INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeib Road, Ann Arbor, MI 48106-1346 USA

UMI®
800-521-0600
RNA and Protein Interactions in the Yeast Spliceosome

by

Deming Xu

A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto

© copyright by Deming Xu, June 1998
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
dedicated to the memory of auntie Juan-Ying
My creative works and my existence are so closely interwoven that if my life flowed as peacefully as a stream through a meadow I believe I would no longer be able to compose anything.

G. Mahler
Acknowledgements

I want to thank my supervisor Jim Friesen for leading me to the wonderful playground of yeast genetics and granting me unlimited freedom with his infectious enthusiasm, endless support and trust. Jim has provided me with the unique opportunity of discovery and self-discovery. He steered my quest from pitfalls. My experience in Jim's lab will benefit me for the rest of my life. I will always be grateful. My deep thanks are due to those who served on my supervisory committee, Barbara E. Funnell, Thomas Yager and Richard Collins, and as well to those who served on my defence committee, John L. Woolford Jr., Paul Sadowski, C. Jim Ingles, Andrew MacMillan, and David E.C. Cole.

The completion of this thesis was only possible with Jim's support and the contributions from people whom I was fortunate to work with, Shou-Jiang Tang, Sherry Nouraini, Arnaud Moris, Brian P. Bobechko, Quoc Huynh and Michael Costanzo. I am particularly grateful to Shou-Jiang's heroic efforts in performing $10^n$ tetrad dissection, $10^n$ mini-preps, $10^n$ transformations and $10^n$ of other experiments. I also wish to thank Dave Jansma and Rita Slaaby for introducing me to the games of yeast genetics and in vitro splicing, respectively. I thank Deborah J. Field, who initiated and provided materials for the genetic screen of synthetic lethal mutations.

I am thankful to the yeast/splicing community for sharing resources and information. In particular, I'd like to thank Jean D. Beggs, Andrew J. Newman, John L. Woolford Jr., Andrew MacMillan and Ben J. Blencowe for their interests in my projects and for their insightful discussion and encouragement. Thanks to those who provided strains and constructs used in this thesis and other experiments.

The completion of this thesis would have been a dull and lonely task without the help and the encouragement of members, present and past, of Jim's lab and my friends. Their
friendships have made my years in Jim's lab enjoyable. The "genetic talks" over beers with Dave and others were always inspirational and rewarding. My collaboration with Sherry not only was fruitful, but also strengthened our friendship and the understanding of each other. Thanks also to Vicki Lay, Ian Donaldson, Jim Hu, Dorian Anglin, Tina Harrington and Andreas Zurlinden for this and that. Of course, when things got off control, Sister Alia Ahmed was always there to make sure that nobody breaks "the Law". My thanks are due to all the "Jacks", Dave Jansma, Mike Kobor, Chris Koth, "Larry" Laurent Brino et al, and to those who participate in the Wednesday evening "ritual" for the wonderful time in the Best Institute. My conversations with Henry Heng always gave me a sense of new dimensions in science. I wish to thank him, Shou-Jiang Tang, Jiang Zhe, Eldad Zacksenhaus, Christine Campbell, Michael Constanzo and other friends who make my life in Toronto exciting and interesting. I also want to thank Donald B. Stoltz for the friendship developed during the years and all sorts of help he gave me when I came to Canada.

My deepest thanks are due to my parents. They always stand behind me, give me strength, and support me whenever needed with their love, respect and understanding. They have done so much for my brother and me during the years, and taught us many of the valuable lessons of life. They have taught us courage, — the courage to take responsibility, the courage to take control and the courage to do the right things. I am eternally grateful. My brother and his family are another source of support and console. I thank them for their understanding.

I dedicate this thesis to the memory of my auntie, Juan-Ying, who, together with my grandparents, among many other things, made my childhood a memorable one.
Abstract

Nuclear pre-mRNA splicing proceeds in a protein-RNA complex, the spliceosome, via a two-step transesterification reaction. Five splicesomal small nuclear RNAs (snRNAs) are brought to the pre-mRNA substrate in a process known as spliceosome assembly. Extensive RNA-RNA interactions and conformational changes occur during spliceosome assembly and maturation. Protein factors exert their functions to facilitate and coordinate these RNA-RNA interactions. In the active spliceosome, interactions between U2 and U6 snRNAs (forming helices Ia, Ib and II) are important for the juxtaposition of the 5' splice site and the branchpoint site and for the formation of RNA structures that are essential for both splicing steps. The highly conserved loop 1 region of U5 snRNA is required for the proper alignment of the two exons for the second splicing step. However, very little is known about how RNA conformational changes are coordinated between the two steps of the splicing reaction, and how recognition of the 3' splice site by other factors is connected to the function of U5 loop 1 prior to and following the first splicing step.

I performed a genetic screen in order to identify factors that are important for the U2/U6 helix II interaction and/or the function of the 5'-end of U2 snRNA, on the basis of synthetic lethality with an 11nt nucleotide substitution in the 5'-end region of U2 snRNA that could also perturb the U2/U6 helix II interaction. Six Slt (synthetic lethal with U2 snRNA) factors were isolated in this screen. Slt11p and Slt22p are new splicing factors, while the remainder correspond to previously identified splicing factors, Slt15p/Prp17p, Slt16p/Smd3p, Slt17p/Slu7p and Slt21p/Prp8p. I have characterized the roles of three of these factors, Slt11p, Slt21p/Prp8p and Slt22p, in the splicing pathway. The genetic interactions among these slt, and selected prp and slu mutations and mutations in U2, U5 and U6 snRNAs were also analyzed. The results suggest that the 5'-end of U2 snRNA is not restricted to interaction with the 3'-end of U6 snRNA (i.e., forming helix II interaction), and that this region of U2 snRNA, in association with three second-step Slt factors identified, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8, may play a role in the second splicing step, which is related to the function of loop 1 of U5 snRNA, particularly the 3' splice site selection.

Slt22p is a large RNA-dependent ATPase, whose activity is preferentially stimulated by pre-annealed U2/U6 snRNAs. Biochemical and genetic analyses indicated that its function is associated with U2/U6 snRNA interaction and that it may unwind intermolecular helix II prior to the formation of the active spliceosome. In the mutant slt22-1 extract, a new complex was formed at the expense of normal splicing complexes that contains U2 and U6 snRNAs, but not
U5 snRNA. This suggests that the helix II region of either U2 or U6 snRNA, after resolved likely by Slt22p, is important for anchoring U5 snRNP to the rest of the spliceosome prior to the first splicing step.

Slt21p/Prp8p has been shown to be required for both steps of the splicing reaction. The slt21/prp8-21 mutation has been mapped to a domain in Prp8p that is important for the recognition of the polypyrimidine tract preceding the 3' splice site. It is synthetically lethal with mutations in only the U2 part of U2/U6 helix II. Consistent with the notion that the 5'-end of U2 snRNA plays a role in the second splicing step, I found that U2 mutations in this region are synthetically lethal with several second-step mutations, including slt15/prp17-100, slu4/prp17-2, slt17/slu7-100, and slu7-1. Furthermore, slt21/prp8-21 is synthetically lethal specifically with slt17/slu7-100 and slu7-1, the corresponding protein of which is involved in the selection of the 3' splice site. However, the combinatorial defect of slt21/prp8-21 and a particular U2 mutation (G21C/C14G) blocks the splicing reaction prior to the first step. Although no apparent splicing defect of slt21/prp8-21 mutation was observed, it is nevertheless defective in spliceosome assembly with a pre-mRNA substrate that lacks the 3' splice site. These results suggest that the 3' splice site is recognized by Prp8p and the 5'-end of U2 snRNA prior to the first step and that the interactions of Prp8p with other second-step factors may coordinate the recognition of the 3' splice site by different factors in spliceosome assembly and between the two steps of the splicing reaction. Consistently, mutations in the loop 1 region of U5 snRNA are synthetically lethal with slt mutations that affect either splicing step. These U5 mutations are synthetically lethal or suppressed by specific mutations in the 5'-end of U2 snRNA.

Slt11p is a putative RNA-binding protein containing two Zn-finger motifs. Although SLT11 is essential for viability only at elevated temperatures, its product is required for the efficiency of the splicing reaction. In the absence of Slt11p, slt17/slu7-100 and mutations in specific regions of U2, U5 and U6 snRNAs become lethal. The N-terminal Zn-finger motifs and the central Rpl25p-homologous region are important for the functions of Slt11p. My genetic results suggest that Slt11p is likely involved in mediating and coordinating RNA-RNA, RNA-protein and protein-protein interactions in the spliceosome that are important for the two splicing steps.
Table of Contents

Acknowledgments .................................................................................................................. i
Abstract ................................................................................................................................ iii
Table of contents .................................................................................................................... v
List of figures ........................................................................................................................ xi
List of tables .......................................................................................................................... xiv
List of abbreviations ............................................................................................................. xv

Chapter 1. Cooperativity of RNA and protein interactions in pre-mRNA splicing -- A review .................................................................................................................. 1

§1.1. Autocatalytic and spliceosomal RNA splicing: why proteins? ........................................ 3
  1.1.1. Properties of RNA folding .......................................................................................... 3
  1.1.2. Protein factors involved in RNA-based catalysis ....................................................... 6
  1.1.3. Cooperativity demands proteins in the spliceosome ................................................ 7

§ 1.2. Spliceosome assembly and establishment of the network of RNA-RNA interactions ........ 11
  1.2.1. Commitment complexes/recognition of 5' splicing site, branchpoint, and cross-intron bridging .............................................................................................. 18
  1.2.2. Pre-spliceosome/recognition of branchpoint site by U2 snRNA ................................. 21
  1.2.3. Holo-spliceosome/recruitment of U4/U6.U5 tri-snRNP .............................................. 24
  1.2.4. Core spliceosome/rearrangement of RNA interactions ............................................ 27
  1.2.5. Activation of the core spliceosome ........................................................................... 29
    1) First catalytic step ........................................................................................................ 29
    2) Second catalytic step .................................................................................................... 30
  1.2.6. Post-splicing recycling of spliceosomal components ................................................ 34

§1.3. Dynamic RNA-RNA interactions in the spliceosome ................................................... 35
  1.3.1. RNA-RNA interactions involved in recognition of splice sites ............................... 35
    1) Recognition of the 5' splice site .................................................................................. 35
    2) Recognition of the branchpoint site .......................................................................... 38
    3) Recognition of the 3' splice site ................................................................................ 38

v
1.3.2. RNA-RNA interactions important for the catalysis of splicing reaction ........................................... 40
  1) RNA conformational rearrangements prior to catalysis ................................................................. 40
     a. Displacement of U1/5' splice site interaction by U6 ............................................................... 40
     b. Disruption of U4/U6 duplex and formation of U2/U6 helix II ................................................. 41
     c. U5/5' splice site interaction ........................................................................................................... 43
  2) Chemistry of catalysis ......................................................................................................................... 43
  3) RNA interactions involved in the catalytic events .............................................................................. 44

§1.4. Rationale and outline of the thesis .................................................................................................. 50

Chapter 2. Identification of yeast SLT genes required for pre-mRNA splicing by means of synthetic lethality with a mutation in U2 snRNA ...................................................... 52

Abstract ...................................................................................................................................................... 53

§ 2.1. Introduction ........................................................................................................................................ 54

§ 2.2. Results .................................................................................................................................................. 57
  2.2.1. Genetic screen ............................................................................................................................... 57
  2.2.2. Genetic characterization ............................................................................................................... 60
  2.2.3. Splicing defects ............................................................................................................................. 64
  2.2.4. Molecular cloning of SLT11 ....................................................................................................... 67
  2.2.5. Molecular cloning of other SLT genes ....................................................................................... 69
  2.2.6. Brief description of Slt factors and their roles in pre-mRNA splicing ........................................ 73

§2.3. Discussion .............................................................................................................................................. 77

§2.4. Material and Methods ....................................................................................................................... 80
  2.4.1. Yeast strains and constructs ......................................................................................................... 80
  2.4.2. Genetic manipulation of yeast ...................................................................................................... 80
  2.4.3. EMS mutagenesis and genetic screen ......................................................................................... 80
  2.4.4. RNA isolation and primer extension ............................................................................................ 81
  2.4.5. Preparation of whole cell splicing extracts and in vitro splicing assay ..................................... 81
  2.4.6. Cloning by complementation ..................................................................................................... 82

vi
Chapter 3. Synthetic lethality of yeast slt mutations with U2 snRNA mutations suggests functional interactions between U2 and U5 snRNPs that are important for both steps of pre-mRNA splicing.  

Abstract  

§3.1. Introduction  

§3.2. Results  

3.2.1. Genetic interactions of slt, slu and prp mutations with U2 snRNA  
3.2.2. Genetic interactions between U2/U6 helix II and slt mutations  
3.2.3. Genetic interactions between U2 and U5 snRNAs  
3.2.4. Genetic interactions among slt and U5 snRNA mutations  
3.2.5. Genetic interactions among slt and slu mutations  

§3.3. Discussion  

3.3.1. Functions of U2/U6 helix II and the 5'-end of U2 snRNA  
3.3.2. Potential interactions between U2 and U5 snRNPs  
3.3.3. Interactions among Slt factors: coordination of the two steps of splicing  

§3.4. Materials and Methods  

3.4.1. Yeast strains and plasmids  
3.4.2. Analysis of synthetic lethality  

Chapter 4. The RNA-dependent ATPase activity associated with Slt22p is involved in U2/U6 snRNA interactions in the formation of the core-spliceosome.  

Abstract  

§4.1. Introduction  

§4.2. Results  

4.2.1. slt22-1 is synthetically lethal with mutations in U2 snRNA.  
4.2.2. SLT22 encodes a large RNA-dependent ATPase/RNA helicase.  
4.2.3. Slt22p contains two separable functional domains.
4.2.4. The ATPase activity of Slt22p is stimulated U2/U6 snRNAs. 131
4.2.5. slt22-1 extract accumulates new splicing complexes. 136

§4.3. Discussion ................................................................. 141
4.3.1. Slt22p is involved in U2/U6 snRNA interaction. 141
4.3.2. Function of U2/U6 helix II ................................. 147
4.3.3. Potential anchoring function of U2 snRNA in tethering U5 snRNP in the spliceosome 149

§4.4. Materials and Methods ............................................ 153
4.4.1. Yeast strains, genetic manipulation .......................... 153
4.4.2. Preparation of yeast splicing extract, in vitro splicing assays and native gel electrophoresis 153
4.4.3. HA-Tagging of Slt22p ........................................ 154
4.4.4. Immunoprecipitation and ATPase assays .................. 154

Chapter 5. Role of Prp8p/Slr21p and U2 snRNA in the formation of the active spliceosome and recognition of the 3' splice site 156

Abstract ........................................................................ 157

§ 5.1. Introduction ............................................................. 158

§ 5.2. Results ................................................................... 161
5.2.1 slt21 is a new allele of prp8. ............................... 161
5.2.2. Allele-specificity and U2 suppressors of synthetic lethality 164
5.2.3. prp8-21 mutation is located in the polypyrimidine tract recognition domain in Prp8p. 168
5.2.4. In vitro splicing defects ........................................ 171
5.2.5. prp8-21 is defective in spliceosome assembly in the absence of the polypyrimidine tract. 177
5.2.6. prp8-21 is synthetically lethal with slu7-1, slt17/slu7-100. 179

§5.3. Discussion .............................................................. 182
5.3.1. Interaction between Prp8p and 5'-end of U2 snRNA 182
5.3.2. Prp8p/U2 snRNA interaction and selection of the 3' splice site 184
5.3.3. Recognition of the PyT and coordination of the splicing steps 186
Materials and Methods ................................................................. 191
  5.4.1. Yeast strains, plasmids, and genetic manipulation .................. 191
  5.4.2. Preparation of yeast splicing extract, in vitro splicing assays and
        native gel electrophoresis .................................................. 191

Chapter 6. Genetic evidence that Slt11p is involved in the snRNA
interactions for the coordination of the two steps of the
splicing reaction ................................................................. 192

Abstract .......................................................................................... 193

§6.1. Introduction .................................................................................. 194

§6.2. Results ......................................................................................... 198
  6.2.1. SLT11 encodes a new splicing factor containing zinc-binding motif. .... 198
  6.2.2. SLT11 is essential for viability at elevated temperatures. .................. 201
  6.2.3. Deletion of SLT11 blocks pre-mRNA splicing prior to the first step. .... 201
  6.2.4. Genetic interactions with U2, U6 and U5 snRNAs ......................... 206
        a) Genetic interactions with U2 snRNA .................................. 206
        b) Genetic interaction with U6 snRNA .................................. 209
        c) Genetic interactions with U5 snRNA ............................... 209
  6.2.5. Genetic interaction with Slt17p/Slu7p ...................................... 215
  6.2.6. Deletional analyses of Slt11p ............................................... 217
        a) Complementation of ΔSLT11 ........................................ 219
        b) Complementation of synthetic lethality of ΔSLT11 with mutations in U2,
           U5 and slt17/slu7-100 .................................................. 219
  6.2.7. Mutational analysis of N-terminal Zn-fingers .......................... 221
        a) Complementation of ΔSLT11 ........................................ 221
        b) Complementation of synthetic lethality of ΔSLT11 with U2, U5 mutations
           and slt17/slu7-100 .................................................. 223
  6.2.8. Expression and purification of His-Slt11p in E. coli, and production of rabbit
        anti-His-Slt11p antibody .................................................. 225
  6.2.9. Detection of Slt11p in total protein extracts and splicing extract .... 228

§6.3. Discussion .................................................................................... 231
  6.3.1. Slt11p as an RNA binding protein/RNA chaperone .................... 231
  6.3.2. Zinc-fingers vs. zinc-ribbon .................................................. 235
6.3.3. Functional duality of Slt11p: coupling and coordination of the two steps of splicing ........................................................................................................ 236

§6.4. Materials and Methods .................................................................................................................. 238
6.4.1. Yeast strains and plasmids ........................................................................................................ 238
6.4.2. Chromosomal deletion of SLT11 ................................................................................................. 238
6.4.3. Preparation of splicing extract and *in vitro* splicing assays ..................................................... 239
6.4.4. Genetic analysis .......................................................................................................................... 239
6.4.5. Construction of Truncations ......................................................................................................... 240
6.4.6. PCR-based site directed mutagenesis ......................................................................................... 240
6.4.7. Expression and Purification of His-Slt11p .................................................................................. 241
6.4.8. Detection of Slt11p in total protein extract and splicing extract of yeast cells .......................... 242

Chapter 7. Summary and future directions ......................................................................................... 244

§7.1. Summary ........................................................................................................................................ 245

§7.2. Future directions ............................................................................................................................. 249
7.2.1. A potential role for the 5' end of U2 snRNA in the second step of splicing ......................... 249
7.2.2. Slt22p: coupling of unwinding of U2/U6 helix II with disruption of U4/U6 duplex in spliceosome assembly ........................................................................ 252
7.2.3. Slt21p/Prp8p ............................................................................................................................... 256
7.2.4. Slt11p .......................................................................................................................................... 260
7.2.5. A possible connection between pre-mRNA splicing and nuclear transport ......................... 262

References ........................................................................................................................................... 265
List of Figures

Chapter 1

Fig. 1.1. Group II intron and nuclear pre-mRNA splicing ........................................ 4
Fig. 1.2. Five yeast spliceosomal snRNAs ................................................................. 9
Fig. 1.3. An outline of spliceosome assembly ............................................................. 12
Fig. 1.4. Comparison of networks of protein-protein and protein-RNA interactions in the formation of early splicing complexes in mammalian and yeast .......................... 20
Fig. 1.5. Genetic interaction among yeast splicing factors and loop 1 of U5 snRNA involved in the second step ................................................................. 33
Fig. 1.6. Rearrangements of RNA-RNA interactions during maturation of the spliceosome and RNA interactions important for the catalytic steps .................................... 37

Chapter 2

Fig. 2.1. Yeast U2 snRNA and genetic screen for slt mutations ...................................... 58
Fig. 2.2. Flow chart of genetic characterization of slt mutations .................................... 62
Fig. 2.3. Growth defects of yeast strains carrying slt mutations .................................. 63
Fig. 2.4. Primer extension analysis of splicing defects associated with slt15 and slt16 mutations ........................................................................................................ 65
Fig. 2.5. In vitro splicing assay of wt, slt11, slt22 and slt17 extracts .............................. 66
Fig. 2.6. Molecular cloning of SLT11 ........................................................................... 68
Fig. 2.7. Molecular cloning of SLT15 and SLT16 ....................................................... 70
Fig. 2.8. SLT17 is identical to SLU7. ........................................................................... 72

Chapter 3

Fig. 3.1. A. Stem I region of yeast U2 snRNA and U2/U6 interactions
B. Growth phenotypes of U2 mutations ...................................................................... 88
Fig. 3.2. Genetic interaction between slt mutations and U2/U6 helix II ........................ 91
Fig. 3.3. Genetic interactions (synthetic lethality and suppression) between substitutions at the G21 position in U2 snRNA and loop 1 mutations of U5 snRNA .... 94
Fig. 3.4. Synthetic lethality of slt mutants with loop 1 mutations of U5 snRNA .... 98
Fig. 3.5. Genetic interactions among slt11-1, slt17/slu7-100 and slt22-1 .................. 100
Fig. 3.6. Summary of genetic interactions among factors involved in either or both step(s) of the splicing reaction ................................................................................ 109

Chapter 4

Fig. 4.1. Isolation and characterization of slt22-1 mutation ......................................... 120
Fig. 4.2. Molecular cloning and characterization of SLT22 ........................................... 122
Fig. 4.3. Chromosomal deletion of SLT22 and mapping of slt22-1 mutation .......... 127
Fig. 4.4. Functional analysis of two domains of Slt22p .................................................. 129
Fig. 4.5. HA-tagged Slt22p and ATPase assays ............................................................. 132
Fig. 4.6. Characterization of ATPase activities of Slt22p and slt22-1p .................. 135
Fig. 4.7. Formation of a new splicing complex in slt22-1 extract ......................... 137
Fig. 4.8. Formation of dead-end complexes in slt22-1 extract ............................... 139
Fig. 4.9. A. U2/U6 helix II interaction and formation of aberrant structures with
mutant U2 snRNAs.
B. Suppression of synthetic lethality of slt22-1 with U2-11nt by rss1-1 .... 144
Fig. 4.10. A role of Slt22p in spliceosome assembly ................................................. 148

Chapter 5

Fig. 5.1. slt21 is a new allele of prp8 ................................................................. 162
Fig. 5.2. U2 suppressors of synthetic lethality of prp8-21 with U2-G21C ............ 166
Fig. 5.3. prp8-21 mutation (Q1902) is located in the polypyrimidine tract recognition
domain in Prp8p. .......................................................... 169
Fig. 5.4. Splicing defects associated with prp8-21 and prp8-21 + U2-G21C/C14G
extracts ........................................................................ 172
Fig. 5.5. Recovery of splicing activity in the prp8-21* extract .............................. 176
Fig. 5.6. prp8-21 is defective in spliceosome assembly with a pre-actin substrate that
lacks the polypyrimidine tract and the 3' splice site. ........................................ 178
Fig. 5.7. Synthetic lethality of prp8-21 with slt17/slu7-100 and slu7-1 ............ 181
Fig. 5.8. Recognition of the polypyrimidine tract (PyT) and the 3' splice site (3' SS) by
mutiple protein and RNA interactions ........................................... 188

Chapter 6

Fig. 6.1. Structure and homology of Slt11p .......................................................... 199
Fig. 6.2. SLT11 is essential for viability at ≥33°C. .............................................. 202
Fig. 6.3. In vitro splicing defects associated with ΔSLT11 ................................. 204
Fig. 6.4. Synthetic lethality of ΔSLT11 with mutations in U2 and U6 snRNAs .... 207
Fig. 6.5. Genetic interactions between ΔSLT11 and mutations in U5 snRNA ..... 210
Fig. 6.6. Summary of mutations in U2, U5 and U6 snRNAs that are lethal in the absence
of Slt11p ........................................................................ 213
Fig. 6.7. slt17/slu7-100 is synthetically lethal with ΔSLT11. .............................. 216
Fig. 6.8. Deletional analysis of Slt11p ................................................................. 218
Fig. 6.9. Zn-finger mutations of Slt11p .............................................................. 222
Fig. 6.10. Expression and purification of His-Slt11p .......................................... 226
Fig. 6.11. Western/protein blot detection of Slt11p ........................................... 229
Fig. 6.12. Role of Slt11p in activation of the spliceosome and its interactions with other components in the spliceosome ................................................................. 233

Chapter 7

Fig. 7.1. A summary of functions of Slt factors in the splicing pathway .................. 246
Fig. 7.2. Genetic experiments designed to test the potential interactions between the intron region of the 3' splice site and the 5' end region of U2 snRNA ......................... 250
Fig. 7.3. Genetic experiments designed to test the potential role for Slt22p in coupling of the unwinding of U2/U6 helix II and the disruption of U4/U6 duplex ......................... 254
Fig. 7.4. A. prp8 mutations and 3' splice site competition. 
B. Potential genetic interaction between ΔMUD2 and prp8 mutations ...... 257
Fig. 7.5. A potential role for Slt11p in U2/U6 helix II interaction .......................... 261
Fig. 7.6. Suppression of synthetic lethality of ΔSLT11 with U2-11nt substitution by rat7 mutations ................................................................. 264
List of Tables

Chapter 1

Table 1.1. Comparison of RNA and protein structural features ....................................... 5
Table 1.2. Yeast splicing factors and their mammalian homologues .................................. 13

Chapter 2

Table 2.1. Growth phenotype of slt strains in the background of SNR20 .............................. 59
Table 2.2. Results of complementation tests ......................................................................... 61
Table 2.3. Summary of SLT genes and proteins ................................................................. 74

Chapter 3

Table 3.1. Allele specificity of synthetic lethality of slt, slu and prp mutations with U2 snRNA mutations .............................................................................................................. 89
Table 3.2. Growth phenotype of U2 and U5 double mutations ............................................ 96
Table 3.3. Summary of tetrad dissection analysis ............................................................... 102
Table 3.4. Summary of synthetic lethality among slt, slu and prp mutations ..................... 104

Chapter 5

Table 5.1. U2 suppressors of the synthetic lethality of slt with U2-G21C ............................ 167

Chapter 6

Table 6.1. RNA-binding proteins involved in pre-mRNA splicing in yeast ....................... 195
Table 6.2. Functional analyses of truncations of Slt11p — complementation of synthetic lethality of ΔSLT11 with U2, U5 and slt17/slu7-100 ......................................................... 220
Table 6.3. Functional analyses of Zn-finger mutations in Slt11p — complementation of synthetic lethality of ΔSLT11 with U2, U5 and slt17/slu7-100 mutations .................................. 224

xiv
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>deoxyribounucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>3'</td>
<td>3 prime</td>
</tr>
<tr>
<td>5'</td>
<td>5 prime</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>pre-mRNA</td>
<td>precursor messenger RNA</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
</tr>
<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoprotein particle</td>
</tr>
<tr>
<td>5' SS</td>
<td>5' splice site</td>
</tr>
<tr>
<td>3' SS</td>
<td>3' splice site</td>
</tr>
<tr>
<td>BPS</td>
<td>branchpoint site</td>
</tr>
<tr>
<td>PyT</td>
<td>polypyrimidine tract</td>
</tr>
<tr>
<td>PRP</td>
<td>pre-RNA processing</td>
</tr>
<tr>
<td>SLU</td>
<td>synthetic lethal with U5</td>
</tr>
<tr>
<td>MUD</td>
<td>mutant U1 die</td>
</tr>
<tr>
<td>SLT</td>
<td>synthetic lethal with U2</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>ts</td>
<td>temperature (thermal)-sensitive</td>
</tr>
<tr>
<td>cs</td>
<td>cold-sensitive</td>
</tr>
<tr>
<td>sg</td>
<td>slow growth</td>
</tr>
<tr>
<td>sl</td>
<td>synthetic lethal</td>
</tr>
<tr>
<td>5-FOA</td>
<td>5-fluoro-orotic acid</td>
</tr>
<tr>
<td>5-FOAr</td>
<td>5-FOA resistant</td>
</tr>
<tr>
<td>5-FOAs</td>
<td>5-FOA sensitive</td>
</tr>
<tr>
<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>mg</td>
<td>miligram</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
</tbody>
</table>
Chapter 1.

Cooperativity of RNA and Protein Interactions in pre-mRNA splicing  
-- A Review
One of the prominent features that differentiates eukaryotic cells from prokaryotes is the compartmentation of RNA biogenesis and DNA replication in the nucleus, separated from the cytoplasmic machinery of protein synthesis. RNA biogenesis commences with synthesis of RNA from dsDNA templates (transcription). Before the messenger RNA (mRNA) is ready for export to the cytoplasm, it must be modified in processes that involve the addition of a 5' cap (m$^7$GpppG) structure and a 3' polyadenosine tail, and removal of non-coding intervening sequences (introns) from precursor, a process known as pre-mRNA splicing (which occurs in a large ribonucleoprotein complex, called the spliceosome). The entire biogenesis of mRNA can be viewed as a set of highly coordinated processes (McCrackon et al. 1997; Steinmetz 1997). Pre-mRNA splicing is also the same. The scope of this chapter shall focus on how a number of RNA-RNA interactions in the spliceosome are coordinated during spliceosome assembly, mediated by a series of protein-protein interactions, and how these interactions contribute to the establishment of catalytically active structures.

Although only a small portion (~210 genes) of the budding yeast, *Saccharomyces cerevisiae*, genome (6,218 ORFs) encode intron-containing genes (Long et al. 1997), the biochemical process of pre-mRNA splicing is highly conserved, if not identical, in all the eukaryotes. Many of the splicing factors and RNA-RNA interactions identified in yeast, by genetic means, are also found and verified in humans and other eukaryotes. The versatility of yeast genetics has proven to be a powerful tool to study the problem of pre-mRNA splicing. Since results of genetic analyses will be presented in the chapters following, particular attention will be given to genetic interactions of the yeast splicing machinery in this review, with a focus on the cooperativity of various RNA-RNA interactions mediated by protein-protein interactions. Biochemical experiments that validate these interactions will also be reviewed, where available.

- 2 -
§ 1.1. Autocatalytic and spliceosomal RNA splicing: why proteins?

The removal of non-coding intervening sequence from a primary transcript of RNA polymerase II (pre-mRNA splicing) proceeds via two consecutive transesterifications, chemically similar to that of the autocatalytic group II intron (Fig. 1.1A). In the first step, the 2' OH of the branchpoint adenosine attacks the 5' OH of the first nucleotide of the intron, resulting in the formation of a covalent 2'-5' phosphodiester bond between these two nucleotides (and thus a lariat intron-exon 2 intermediate) and the release of exon 1. The second step consists of cleavage at the 3' splice site with concomitant ligation of the two exons and the intron is released in a lariat form. In the case of group II introns, these two steps can be achieved by an RNA-based catalysis. Conserved structural elements in the intron are involved directly in the chemistry of the catalysis (for reviews, see Cech 1993; Michel and Ferat 1995). However, pre-mRNA splicing occurs in an elaborate ribonucleoprotein complex, the spliceosome. The protein and RNA components of the spliceosome are essential both in vivo and in vitro (for reviews, see Rymond and Rosbash 1992, Moore et al. 1993; Krämer 1996), even though the RNA components are believed to be the catalytic moieties of the spliceosome (Guthrie 1991; Madhani and Guthrie 1994a; Weiner 1993; Newman 1994; Nilsen 1994). Why are protein factors necessary?

1.1.1. Properties of RNA folding.

The intrinsic properties of the RNA folding process may account for the requirement of proteins in RNA-based catalysis. In proteins, information in the primary amino acid sequence is converted to a well-defined tertiary structure with a defined biological activity (i.e., native state). The folding process is driven by an ensemble of various submolecular interactions which dictates the specificity, and the free energy ($\Delta G$) of folding rarely exceeds 15 kcal/mol.
Fig. 1.1. A. The two steps of group II and nuclear pre-mRNA splicing. Upper. Schematic representation of the two-step reaction. The 5' and 3' exons are shown in filled and open boxes respectively. The intron is shown by a thick line with conserved nucleotides indicated. Lower. The chemistry of the two steps. The 5' and 3' splice sites are indicated by conserved nucleotides shown in the upper panel. B. Conserved elements of a typical yeast pre-mRNA with branchpoint adenosine shown in filled cricle.
RNA folding, however, is profoundly different and problematic (Table 1.1, Herschlag 1995). For a given RNA sequence, it tends to fold into and become kinetically trapped in alternative conformations (i.e., non-native states) which are thermodynamically competitive with the folded native form. The free energy of folding an RNA can be high, and the energy barriers between alternatively folded forms and the native state are huge. [Examples of stably folded inactive RNAs are given in Uhlenbeck (1995).] RNA-binding proteins can facilitate RNA folding in vivo and in vitro by acting as RNA chaperones, which, just as with protein chaperones, prevent RNA misfolding and resolve misfolded RNAs thereby ensuring that RNA is accessible in a biologically functional native conformation.

Table 1.1. Comparison of RNA and protein structural features

<table>
<thead>
<tr>
<th>Feature</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>side chains</td>
<td>4 (similar)</td>
<td>20 (diverse)</td>
</tr>
<tr>
<td>Secondary structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>side chain face</td>
<td>in$^b$</td>
<td>out</td>
</tr>
<tr>
<td>stability</td>
<td>high$^c$</td>
<td>low$^c$</td>
</tr>
<tr>
<td>Tertiary structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>packing</td>
<td>loose, multiple possibility</td>
<td>tight, precise</td>
</tr>
<tr>
<td>cooperativity$^d$</td>
<td>low</td>
<td>high</td>
</tr>
</tbody>
</table>

Notes:

b. In helical structures.
c. As measured by the change in free energy ($\Delta G$). The more negative $\Delta G$, the more favorable (i.e., more stable) the given conformation.
d. Mutual promotion of proper folding by local secondary structures.
Divalent metal ions (Mg$$^{++}$$) can bind to "pockets" in RNA (formed by single-stranded backbone phosphates), and in combination with other counterions (such as Na$$^{+}$$ or a polyamine), can promote RNA folding (for a review, see Pan et al. 1993). Conditions employed for most RNA-based catalysis demand concentrations of ions much higher than under physiological conditions. On the contrary, it has become increasingly evident that protein factors are required for RNA-based reactions under physiological conditions.

1.1.2. Protein factors involved in RNA-based catalysis.

There are several well-documented examples of the influence of RNA-binding proteins on RNA-based catalysis. In all these cases, the functions of protein factors have been implicated in the physical steps of the reactions, which include, for example, association and dissociation of substrate and product with the ribozyme, or adaptation of the functional tertiary structure.

In the reaction cycle of the hammerhead ribozyme, physical steps limit the turnover and specificity of the overall reaction, while the chemical step (catalysis) is a fast one (Hertel et al. 1994). RNA-binding proteins, such as retroviral nucleocapsid protein (Tsuchihashi et al. 1993; Herschlag et al. 1994), snRNP A1 protein (Herschlag et al. 1994) and ribosomal protein S12 (Coetzee et al. 1994) can overcome these limitations by accelerating strand annealing and dissociation and resolving the misfolded ribozyme.

The self-splicing of group I introns of *Tetrahymena* is 50-fold less efficient *in vitro* than *in vivo* (Brehm and Cech 1983). Protein factors involved in the splicing of fungal mitochondrial introns (group I) have been identified by genetic criteria (for reviews, see Burke 1988; Lambowitz and Perlman 1990). In particular, the CYT-18 protein (tyrosyl-tRNA synthetase) of *Neurospora* can promote splicing *in vivo* and *in vitro* (Guo and Lambowitz 1992; Mohr et al. 1992) by inducing the correct tertiary folding of the catalytic core (Caprara et al. 1996).
Similarly, protein factor CBP2 is required to facilitate the assembly of the catalytic core (and other domains) of a yeast mitochondrial group I intron at low salt concentrations (Gampel and Cech 1991; Shaw and Lewin 1995; Weeks and Cech 1995). Ribosomal proteins, including S12, can facilitate the in vitro splicing of group I introns of bacteriophage T4, likely by resolving misfolded RNAs (Coetzee et al. 1994).

Several yeast nuclear-encoded protein factors are required for splicing of mitochondrial group II introns (Wiesengerger et al. 1992; Waldherr et al. 1993). One of them, Mrs2p, contains an RNA binding (KH) motif near the N-terminus and two transmembrane domains at the C-terminus. Both regions are essential for the splicing of group II introns in vivo. Most recent data indicate that this protein may anchor group II intron RNA to the mitochondrial membrane in order to facilitate splicing and translation of intronic ORFs (Bui et al. 1997). A mitochondrial lysate and ATP are required for splicing of the first intron (group II) of cytochrome b precursor under physiological conditions in vitro. Overexpression of a DEAD box protein (pMSS116, an RNA-dependent ATPase) is able to increase the splicing activity in an ATP-dependent manner, suggesting that active unwinding of misfolded RNA is required to promote splicing (Niemer et al. 1995).

1.1.3. Cooperativity demands proteins in the spliceosome.

Pre-mRNA splicing involves both RNA folding and unfolding (see Madhani and Guthrie 1994a; Staley and Guthrie 1998 for reviews on RNA conformational changes in the spliceosome). Unlike self-splicing introns or other ribozymes, pre-mRNAs lack defined primary and secondary structures with the exception of a limited set of conserved sequences at each intron boundary and the branchpoint site (Fig. 1.1B). These sites must be recognized, brought to close proximity, and juxtaposed in order for splicing to occur. It is clear that these events are achieved by individual interactions with spliceosomal small nuclear RNAs (snRNAs) (for a
review, see Madhani and Guthrie 1994a; also see Fig. 1.2 for the structures of five yeast spliceosomal snRNAs). The RNA-RNA interactions in the core of the active spliceosome are assembled from alternatively folded structures which must undergo extensive rearrangement during the splicing cycle. Following the completion of the splicing reactions, products and individual snRNAs must unfold and dissociate in order to allow recycling of snRNAs and other factors.

The splice sites of a pre-mRNA substrate are selected and recognized sequentially by snRNAs in association with a number of protein factors. These selection/recognition events must be coordinated spatially (i.e., the splice sites must be brought into close proximity and juxtaposition with spatial precision) and temporally (i.e., the participants of the first and second transesterification reactions must be brought together with precise temporal order). Furthermore, the two steps of transesterification are likely catalyzed by two active sites in the spliceosome by two distinct mechanisms (Moore and Sharp 1993; Sontheimer et al. 1997). If so, the switch between the two active sites demands another level of coordination between readjustments of the catalytic moiety and substrate in the spliceosome. Cooperativity of individual RNA-RNA interactions, i.e., mutual promotion of proper RNA-RNA interactions, is essential to achieve high level of efficiency and fidelity of splicing. Multiple protein-protein interactions provide the scaffold in which individual snRNA-substrate interactions are connected (for an example, see Abovich and Rosbash 1997), snRNA-snRNA interactions are assembled, and these two sets of RNA interactions are integrated to form the catalytic core of the spliceosome.

Although, the two-step transesterification reaction is intrinsically energy neutral, hydrolysis of ATP by a group of RNA-dependent ATPases (RNA helicases) is essential for the pre-mRNA splicing pathway, suggesting that active unfolding (unwinding) of RNA (helical) structures occurs in the process. The consumption of ATP also indicates that RNA structures required for splicing are not necessarily thermodynamically strongly favored. Protein factors
Fig. 1.2. Five yeast spliceosomal snRNAs. The proposed secondary structures are based on phylogenetic conservation (see Patterson and Guthrie 1988). A. U1 snRNA. The roman numbers indicate stems, some of which are conserved. B. U5 snRNA. C. U2 snRNA. The stem II region can form two alternative stem/loop structures. The central non-essential region is not shown. D. U4/U6 snRNA duplex and free U6 snRNA. U4 and U6 snRNAs form extensive base-pairing interactions (stems I and II). However, a free U6 snRNA can adapt a different configuration.
may provide proofreading (for a review, see Burgess and Guthrie 1993b) and other stabilizing functions.

§ 1.2. Spliceosome assembly and establishment of the network of RNA-RNA interactions.

In eukaryotic cells, pre-mRNA splicing is a nuclear process. It is increasingly clear that in mammalian cells pre-mRNA processing can occur cotranscriptionally (for a review, see Steiznmetz 1997). Preliminary studies indicate that pre-mRNA splicing is also cotranscriptional in yeast (Elliot and Rosbash 1996). However, this may not be the case for all transcripts, as suggested by the existence of a class of inefficiently spliced pre-mRNAs with different susceptibility to UPF1 decay, a mechanism responsible for degradation of frame-shifted cytoplasmic transcripts (He et al. 1993; Long et al. 1995). One early study, however, seemed to indicate that pre-mRNAs are transported into the cytoplasm by default if splicing is blocked by mutations in either intron or the splicing machinery (Legrain and Rosbash 1989).

Regardless of this, the mechanism and components of pre-mRNA splicing are fundamentally conserved (Table 1.2), as an increasing number of homologues of yeast splicing factors (identified mostly by genetic means as Prps, for Pre-RNA Processing) have been identified in mammalian systems (Fig. 1.4). The entire pre-mRNA splicing process can be divided into several stages (Fig. 1.3) based on complexes identified by native gel electrophoresis (Pikielny and Rosbash 1986; Pikielny et al. 1986; Cheng and Abelson 1987). Splice sites in the pre-mRNA are recognized through a series of interactions with snRNAs at different stages in the spliceosome assembly. Concomitantly, snRNAs undergo extensive conformational rearrangement in order to bring the splice sites into close proximity and to form structures which are important for the catalysis. Briefly, pre-mRNA splicing initiates with the recognition by U1 snRNA of the 5' splice site, and is immediately followed by U2 snRNA basepairing with the
Fig. 1.3. An outline of spliceosome assembly, showing RNA/RNA interactions in each complex. Only conserved structural elements in snRNAs are shown. The snRNA/pre-mRNA interaction established during the formation of each complex is circled, and shown on the left. See text for detail.
Table 1.2. Yeast splicing factors and their mammalian homologues
part 1 of Table 1.2

<table>
<thead>
<tr>
<th>snRNP /Events</th>
<th>Protein</th>
<th>Structural motif(s)</th>
<th>Functions / snRNP association</th>
<th>Interaction with</th>
<th>Mammalian homologues / related proteins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>U1 snRNP and formation of commitment complexes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snp1p</td>
<td>RRM</td>
<td>U1 snRNP</td>
<td>Prp8p</td>
<td>U1 70K</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mud1</td>
<td>RRM</td>
<td>U1 snRNP</td>
<td>--</td>
<td>U1 A</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>YU1C</td>
<td>RRM</td>
<td>U1 snRNP</td>
<td>--</td>
<td>U1 C</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Prp40p</td>
<td>WW</td>
<td>U1 snRNP, SL with mud2</td>
<td>Mud2p, Prp8p</td>
<td>--</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Prp39p</td>
<td>tetra tricopeptide</td>
<td>U1 snRNP</td>
<td>--</td>
<td>--</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Prp42p</td>
<td>tetra tricopeptide</td>
<td>U1 snRNP</td>
<td>--</td>
<td>--</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>for other U1 snRNP proteins, see reference 6a.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mud2p</td>
<td>RRM</td>
<td>5' SS/BP/PyT bridging SL with U1 snRNA mutation binds to BP, SL with mud2</td>
<td>BBP, Prp11p</td>
<td>U2AF65</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>BBP/Msl5p</td>
<td>KH, Zn knuckle, pro.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **U2 snRNP and formation of pre-splicosome:** | | | | | | |
| Msl1p | RRM | U2 snRNP, SL with mud2 | -- | U2 B+ | 9 |
| SF3a | Prp9p | Zn-finger | binding of U2 snRNP to BP | Prp21p | SF3a60/SAP61 | 10 |
| SF3a | Prp11p | Zn-finger | binding of U2 snRNP to BP SL with mud2 | Prp21p, Mud2p | SF3a66/SAP62 | 11 |
| SF3a | Prp21p/Spp9p | SURP x2, pro. | binding of U2 snRNP to BP suppressor of prp9 | Prp9p, Prp11p | SF3a120/SAP114 | 12 |
| SF3b | Cus1p | RRM | | | | |
| SF3b | Hsh49p | DEAD | | | | |

Mutations in prp5, 9, 11 and 21 are synthetically lethal with each other and with mutations in the stem II region of U2 snRNA.
**Part 2 of Table 1.2.**

**U4/U6.Us tri-snRNP and formation of holo-spliceosome:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>SnRNP</th>
<th>Protein</th>
<th>SnRNP</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp3p</td>
<td>WD repeats</td>
<td>U4/U6 snRNP</td>
<td>Prp4p</td>
<td>hPrp3p</td>
<td>17</td>
</tr>
<tr>
<td>Prp4p</td>
<td>Zn-finger like, LZP</td>
<td>U4/U6 snRNP</td>
<td>Prp3p</td>
<td>hPrp4p</td>
<td>18</td>
</tr>
<tr>
<td>Prp6p</td>
<td></td>
<td>U4/U6 snRNP</td>
<td></td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>

Prp3p, Prp4p and Prp6p are important for the stability of U6 snRNA.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>SnRNP</th>
<th>Protein</th>
<th>SnRNP</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp24p</td>
<td>RRM</td>
<td>U6 snRNP</td>
<td>Prp8p</td>
<td>pro.</td>
<td>--</td>
</tr>
<tr>
<td>Prp8p</td>
<td>pro.</td>
<td>U5 snRNP, tri-snRNP stability</td>
<td>Prp40p</td>
<td>hPrp8p/U5-p220</td>
<td>21</td>
</tr>
<tr>
<td>Slt22p/Brr2</td>
<td>DEIH x2, LZP</td>
<td>U5 snRNP</td>
<td>Prp31p</td>
<td>recruitment of tri-snRNP</td>
<td>--</td>
</tr>
<tr>
<td>Prp38p</td>
<td>tri-asnRNP, U6 snRNP stability</td>
<td>--</td>
<td>--</td>
<td>U5-p200</td>
<td>22</td>
</tr>
<tr>
<td>Snu114p</td>
<td>EF-2</td>
<td>U5 snRNP</td>
<td>Prp8p</td>
<td>pro.</td>
<td>U5-p116</td>
</tr>
</tbody>
</table>

**Formation of core-spliceosome:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>SnRNP</th>
<th>Protein</th>
<th>SnRNP</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp38p</td>
<td>Dissociation of U4/U6</td>
<td>--</td>
<td>Prp40p</td>
<td>U5-p220</td>
<td>24</td>
</tr>
<tr>
<td>Prp8p</td>
<td>Dissociation of U4/U6</td>
<td>--</td>
<td>Prp24p</td>
<td>Sf3a120/SAP114</td>
<td>26</td>
</tr>
<tr>
<td>Prp21p</td>
<td>2 SURP, pro.</td>
<td>U2/U6 helix II interaction</td>
<td>--</td>
<td>U5-p200</td>
<td>27</td>
</tr>
<tr>
<td>Slt22p/Brr2</td>
<td>DEIH x2, LZP</td>
<td>SL with U2 mutations</td>
<td>--</td>
<td>U5-p100</td>
<td>28, Chapter 4</td>
</tr>
</tbody>
</table>

**Activation of spliceosome:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>SnRNP</th>
<th>Protein</th>
<th>SnRNP</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp8p</td>
<td>pro.</td>
<td>SL with U2 mutations</td>
<td>Prp40p</td>
<td>hPrp8p/U5-p220</td>
<td>32</td>
</tr>
<tr>
<td>Prp2p</td>
<td>DEAH, Zn-finger like</td>
<td>RNA conformational changes</td>
<td>Spp2p</td>
<td>--</td>
<td>33</td>
</tr>
<tr>
<td>Spp2p</td>
<td></td>
<td>activation</td>
<td>Prp2p</td>
<td>--</td>
<td>34</td>
</tr>
<tr>
<td>(HP)*6</td>
<td>unknown</td>
<td>activity required for activation</td>
<td>--</td>
<td>--</td>
<td>35</td>
</tr>
<tr>
<td>Slt11p</td>
<td>Zn-fingers</td>
<td>SL with U2, U5, U6 snRNA</td>
<td>--</td>
<td>--</td>
<td>Chapter 6</td>
</tr>
</tbody>
</table>
### Part 3 of Table 1.2

**Second step:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain</th>
<th>Function</th>
<th>Protein</th>
<th>Domain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp8p</td>
<td>pro.</td>
<td>Py/T recognition, 3' SS selection</td>
<td>Prp40</td>
<td>U5-p220</td>
<td>36, Chapter 5</td>
</tr>
<tr>
<td>Prp16p</td>
<td>DEAH</td>
<td>remodeling, proofreading</td>
<td>hPrp16p</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>Prp22p</td>
<td>DEAH</td>
<td>ATP-independent</td>
<td>HRH1</td>
<td></td>
<td>37a</td>
</tr>
<tr>
<td>Prp17p</td>
<td>WD repeats</td>
<td>SL with U5 loop 1</td>
<td>hPrp17</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>Prp18p</td>
<td>WD repeats</td>
<td>SL with U2 interactions</td>
<td>hPrp18</td>
<td></td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Slu7p</td>
<td>Zn-knuckle</td>
<td>3' SS selection, SL w. U5 loop 1</td>
<td>Prp18p</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SL with U2 mutations</td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutations in <em>prp8, 16, 17, 18</em> and <em>slu7</em> are synthetically lethal with each other</td>
<td></td>
<td></td>
<td>40, Chapter 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>activity required for the 2nd step</td>
<td></td>
<td></td>
<td>41</td>
</tr>
</tbody>
</table>

**Post-splicing:**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain</th>
<th>Function</th>
<th>Protein</th>
<th>Domain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prp22p</td>
<td>DEAH</td>
<td>release of lariat intron</td>
<td>HRH1</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>Prp43p</td>
<td>DEAH</td>
<td>release of lariat intron</td>
<td>mDEAH9</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Prp24p</td>
<td>RRM</td>
<td>U4/U6 annealing</td>
<td>--</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Prp26p</td>
<td>lariat intron turnover</td>
<td>--</td>
<td>--</td>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>

**Notes:**

* The structural motifs are listed according to their order in protein. Abbreviations used: RRM = consensus RNA recognition motif; WW = tryptophan-rich; tetradricopeptide = tetradricopeptide repeat; KH = hnRNP K homology; SURP = motif characteristic for Prp21p and SWAP homologs; pro. = proline rich repeat; DEAD, DEAH, DEIH = motifs characteristic for ATP-dependent ATPases/RNA-helicases; WD repeats = motif characteristic for β-transducin; LZP = leucine-zipper; EF-1 = homology of ribosomal translocase EF-2.

* Abbreviations used: SL = synthetic lethal; 5', 3' SS = 5', 3' splice site; BP = branchpoint; PyT = polypyrimidine tract.

* Determined by 2-hybrid interaction and/or direct protein binding.

* Reference:


e. Biochemical fractions.
branchpoint. The pre-assembled U4/U6.U5 tri-snRNP is recruited into the complex. A series of rearrangements and displacements of RNA interactions take place during the maturation of the spliceosome. They include displacement of U1/5' splice site interaction by U6 snRNA, dissociation of extensive U4/U6 base-pairing and replacement by U2/U6 interactions, which, in turn, bring the 5' splice site and the branchpoint to close proximity. The RNA interaction involved in the recognition of the 3' splice site is complex and is less well understood. U5 snRNA plays an essential role in tethering the two exons via non-canonical basepairing interactions (Fig. 1.3). Protein factors play important roles in 3' splice site selection. The network of RNA-RNA interactions established in assembly of the spliceosome is then rearranged to form a catalytically active structure. RNA interactions are coordinated by protein-protein interactions during spliceosome assembly and in the mature spliceosome.

1.2.1. Commitment complexes/recognition of 5' splicing site, branchpoint, and cross-intron bridging.

The formation of the earliest identified splicing complexes, commitment complexes (CCs), containing U1 snRNP, is the only ATP-independent step in pre-mRNA splicing (Legrain et al. 1988; Séraphin and Rosbash 1989a). The RNA base-pairing interaction between the 5' splice site and the 5' end of U1 snRNA is involved (Siliciano and Guthrie 1988; Séraphin et al. 1988; Séraphin and Rosbash 1989b). Indirect experiments suggest that the highly conserved branchpoint is required for the U1-containing complex (Legrain et al. 1988; Ruby and Abelson 1988, see below). The cap structure, m7G(5')ppp(5')N, seems to be important since cap binding complex (CBC) can quantitatively stabilize CC (Colot et al. 1996) and affect splicing (Izaurralde et al. 1994; Colet et al. 1996; Lewis et al. 1996).

A genetic screen was conducted to isolate factors that are involved in the initial stages of pre-mRNA splicing on the basis of synthetic lethality with a mutation in U1 snRNA
containing a deletion of the yeast-specific region in combination with a point mutation in the conserved A loop (Liao et al. 1993). Several MUD (mutant U1 die) genes were isolated. While some of Mud factors are U1 snRNP associated proteins [Mud1p (U1 A protein, Liao et al. 1993), Mud15p and Mud16p (=Prp42p) (McLean and Rymond 1998; Gottschalk et al. 1998)], Mud2p (Abovich et al. 1994) is the yeast homologue of a well-defined mammalian splicing factor, U2AF65. This factor interacts directly with the polypyrimidine tract in the intron and is important for the formation of early complex (complex E, equivalent of yeast CC) (Singh et al. 1995). Mud13p (Colot et al. 1996), on the other hand, is the small subunit of yeast CBC. The genetic interaction between mud13 and U1 snRNA was consolidated by the observation that in both mammalian and yeast systems, CBC promotes the recognition of 5' splice site and stabilizes early complexes (Colot et al. 1996; Lewis et al. 1996).

Although MUD2 is not essential for viability (Abovich et al. 1994), the protein itself is involved in a set of protein-protein interactions that are responsible for connecting recognition of the 5' splice site by U1 snRNA to the recognition of the branchpoint and the polypyrimidine tract. Early experiments indicated that stable association of U1 snRNP with the 5' splice site is independent of the branchpoint (forming CC1), whereas a second complex with lower mobility (CC2) requires a functional branchpoint site, in the absence of U2 snRNA (Séraphin and Rosbash 1989a; 1991). A functional branchpoint site is indeed recognized during the formation of CC2 by a protein factor (Berglund et al. 1997; Pascolo and Séraphin 1997). This factor, branchpoint bridging protein (BBP), was identified in another genetic screen for mutations that are synthetically lethal with a truncation of Mud2p, containing only the third RNA-binding domain (with strong homology to the corresponding region in U2AF65) (Abovich and Rosbash 1997). Other factors identified included Prp40p (a U1 snRNP protein, Kao and Siciliano 1996) and Mud1p (U1A protein, Liao et al. 1993). The genetic interactions among Mud2p, BBP and Prp40p were confirmed by direct protein-protein interactions of BBP with Mud2p, and BBP with Prp40p (Abovich and Rosbash 1997). Taken together, these data suggest that the recognition of three elements in the intron (5' splice site, via U1 snRNA; branchpoint, via BBP
Fig. 1.4. Comparison of networks of protein-protein and protein-RNA interactions in the formation of early splicing complexes in mammals and yeast. In mammals, the recruitment of U1 snRNP to the 5' splice site by SF2/ASF involves an interaction between the RS domain of both proteins. There are two levels of cross-intron bridging interactions, through RS proteins (shown as RS) interacting with SF2 (at the 5' splice site) and U2AF (35 and 65, bound at the polypyrimidine tract), and through SF1/mBBP interacting with a possible homologue of Prp40 of U1 snRNP and U2AF65. SFb (SAP49 and SAP145) and SFb (SAP49 and SAP145) bind to the anchoring site upstream of the branchpoint and recruit U2 snRNP. U2AF65 also interacts with a DEAD protein (UAP56) and promotes U2 binding. In yeast, cross-intron bridging is mediated through BBP, interacting with Prp40 of U1 snRNP and Mud2 bound at the polypyrimidine tract. SF3a (Prp9p, Prp11p and Prp21p) and SF3b (Cus1p and Hsh49p) interact with the stem II region of U2 snRNA and promote its binding to the pre-mRNA. Prp5p, a DEAD protein, is also involved in this process.
and polypyrimidine tract, via Mud2p) occurs during the initial steps of spliceosome assembly (Fig. 1.4) and that the aforementioned protein-protein interactions provide a framework to coordinate recognition of the branchpoint by U2 snRNA and the polypyrimidine tract by other factors (e.g., Prp8p) in the subsequent events.

This cross-intron bridging via protein-protein interactions seems to be highly conserved (Fig. 1.4), since homologues of both Mud2p (U2AF65) and BBP (mBBP=SF1) have been identified in the mammalian system, where both proteins function in a similar manner (Abovich and Rosbash 1997; Berglund et al. 1997). However, commitment of pre-mRNA to splicing in higher eukaryotes is more complex than in yeast, and is complicated by a class of serine/arginine rich (SR) splicing factors, which bind to pre-mRNA and recruit U1 snRNP and other splicing factors (Fu 1995, Fig. 1.4). Single SR proteins are sufficient to form committed complex(es) with distinct substrate specificity (Fu 1993). [Apparently, multiple RNA recognition motifs (RRMs) in SR proteins act coordinately to achieve substrate specificity (Chandler et al. 1997).] The formation of a stable U1-containing complex appears to involve interaction between SR domains of SF2/ASF and U1-70K (Kohtz et al. 1994, Staknis and Reed 1994a). It has also been proposed that SR protein interactions mediate communication between the 5' and 3' splice sites in the E complex (Fu and Maniatis 1992; Wu and Maniatis 1993; Fig. 1.4). On the other hand, a high concentration of SR proteins can bypass the requirement of U1 snRNP in vitro (Crispino et al. 1994; Tarn and Steitz 1994). A class of naturally occurring pre-mRNAs has been identified that can be spliced in a U1 snRNP-independent manner (Crispino et al. 1996).

1.2.2. Pre-spliceosome/recognition of branchpoint site by U2 snRNA.

The formation of commitment complexes is ATP-independent. In the presence of ATP (or hydrolyzable analogs) and with the addition of U2 snRNP, commitment complexes are
converted quickly to pre-spliceosomes (Liao et al. 1992). Although the branchpoint is recognized by BBP during the formation of CCs (see above), the binding of U2 snRNP has sequence-specificity (Nelson and Green 1989), due to the recognition of the branchpoint by U2 snRNA via basepairing interactions (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989), which results in the branchpoint adenosine residue, the nucleophile of the first step, being bulged out (Query et al. 1994, 1995). It appears that the binding of BBP to the branchpoint site is replaced by another set of protein factors after recognition by U2 snRNA, at least in humans (MacMillan et al. 1994).

In addition to interactions between a U1 snRNP protein (Prp40p) and BBP (Abovich and Rosbash 1997), Mud2p, present in the CC, interacts with Prp11p (Abovich et al. 1994), one component of a splicing factor SF3a required for the binding of U2 snRNP to the pre-mRNA. This protein-protein interaction provides a temporal connection of the two sequential events. Furthermore, a mutation in the gene encoding the yeast U2B" (a U2 snRNP protein) was found to be synthetically lethal with deletion of MUD2 (Tang et al. 1996). In humans, the binding of U2 snRNP to the branchpoint depends on U2AF (Nelson and Green 1989; Valcárcel et al. 1996), one component of which, U2AF65, binds directly to the polypyridine tract (Singh et al. 1995). Mud2p functions in a similar fashion to U2AF65 (see above). Thus, in both yeast and human, the branchpoint site is recognized in two steps: initially by BBP/SF1 (ATP-independent), and then by U2 snRNA. A similar conclusion was also reached in an exhaustive genetic study (Rain and Legrain 1997). Mud2p/U2AF65 provides the connection between these two steps via protein-protein interactions. Although it is not clear whether it interacts with SF3a, U2AF65 recruits a human DEAD box protein (an RNA-dependent ATPase) required for the U2 snRNP-branchpoint interaction (Fleckner et al. 1997). A similar function is performed by Prp5p in yeast (see below).

The binding of U2 snRNP to pre-mRNA substrate also requires snRNP-associated activities: SF3a and SF3b, consisting of three and four polypeptides, respectively. They are
identified as SAPs, spliceosome-associated proteins (for a review, see Krämer 1996). SF3b interacts with isolated 12S U2 snRNP and the subsequent binding of SF3a converts the intermediate into the 17S form, active in pre-spliceosome (complex A in human, complex B in yeast) assembly (Brosi et al. 1993a and 1993b). Crosslinking studies showed that subunits of SF3a and SF3b bind directly to a region (termed anchoring site) upstream of the branchpoint suggesting a function of these two complexes in tethering the U2 snRNP to pre-mRNA (Champion-Arnaud and Reed 1994; Gozani et al. 1996, Fig. 1.4). Direct protein-protein interactions among subunits in each complex have been observed (Bennett and Reed 1993; Champion-Arnaud and Reed 1994; Chiara et al. 1994; Krämer et al. 1994 and 1995; Gozani et al. 1996).

Homologues of subunits of SF3a and SF3b exist in yeast, all of which have been implicated in formation of the pre-spliceosome (Table 1.2). Furthermore, the protein-protein interactions observed in humans are well conserved in yeast (Fig. 1.4). Functional conservation between yeast Prp9p and human SAP61/SF3a60 has been observed (Behrens et al. 1993; Krämer et al 1994). The yeast SF3a consists of Prp9p, Prp11p and Prp21p. The formation of a complex was demonstrated by two-hybrid interaction (Legrain and Chapon 1993; Legrain et al. 1993) and direct protein-protein binding (Wiest et al. 1996). In this complex, both Prp9p and Prp11p interact simultaneously with Prp21p, but not with each other. Consistent with the function in promoting the addition of the U2 snRNP to pre-mRNA (Abovich et al. 1990; Ruby et al. 1993), these proteins were found to influence the structure of U2 snRNA in the branchpoint-pairing region (Wiest et al. 1996). The RNA-dependent ATPase activity of Prp5p seems to be linked to the conformational change in this region of U2 snRNA prior to binding to the branchpoint (O'Day et al. 1996). These four factors (Prp5p, Prp9p, Prp11p and Prp21p) act coherently in vivo with each other and with U2 snRNA. Pair-wise synthetic lethality has been observed among mutations in these four protein factors (Ruby et al. 1993; Wells and Ares 1994) and with mutations in the region adjacent to branchpoint pairing site and stem II of U2 snRNA (Wells and Ares 1994; Yan and Ares 1996). In addition, mutations in prp9, prp11 and
prp21 that reduce or abolish protein-protein interactions are synthetically lethal with each other (Legrain and Chapon 1993; Legrain et al. 1993). Furthermore, prp21 was also isolated as spp91, a suppressor of prp9 (Chapon and Legrain 1992). Several ts mutations of prp21 were found to be associated with defects in the interaction with Prp9p, but not with Prp11p (Rain et al. 1996).

So far, only two subunits of yeast SF3b have been identified, corresponding to SAP145/SF3b145 and SAP49/SF3b50 (Table 1.2). The yeast SAP145 was isolated as a suppressor of a mutation in the stem II region of U2, CUS1 (cold-sensitive U2 suppressor, Wells et al. 1996). A yeast homologue of SAP49 was identified based on sequence similarity (Igel et al. 1998). Both proteins are loosely associated with U2 snRNP, have non-specific RNA binding activities and interact with each other (Wells et al. 1996; Igel et al 1998). Cus1p is required for pre-spliceosome assembly. Consistent with this function, high-level expression of CUS1 suppresses both prp5-1 and prp11-1 (Wells et al. 1996). A model has been proposed in which yeast SF3a, SF3b and Prp5p act collectively to ensure the structural integrity of regions adjacent to the branchpoint-pairing site in the U2 snRNA in order to establish U2/branchpoint interaction during formation of the pre-spliceosome (Yan and Ares 1996, Fig. 1.4).

1.2.3. Holo-spliceosome/recruitment of U4/U6.U5 tri-snRNP.

In the next step of spliceosome assembly, the pre-assembled U4/U6.U5 tri-snRNP is recruited into the pre-spliceosome, forming the so-called holo-spliceosome (containing all five spliceosomal snRNAs). It has been shown that subunits of SF3a become associated with the pre-spliceosome during its formation and are an integral part of the spliceosomal complexes in the subsequent step in both human (Bennett et al. 1992; Staknis and Reed 1994b) and yeast (Arenas and Abelson 1993; Wiest et al 1996). The mutual genetic and biochemical suppression of prp21-2 and prp24-1 (which encodes a U6 snRNP protein, see below) suggests a role for
Prp21p in the formation of the holo spliceosome (Vaidya et al. 1996). The recent identification of several novel proteins associated with the human tri snRNP or U5 snRNP suggests that recruitment of tri snRNP and the subsequent step(s) are regulated by multiple mechanisms.

The integrity of the tri snRNP is one of the determining factors in its recruitment to the pre spliceosome. Several U4/U6 associated protein factors, including, Prp3p (Anthony et al. 1997), Prp4p (Banroques and Abelson 1989; Petersen-Bjørn et al. 1989; Bordonné et al. 1990; Galisson and Legrain 1993; Hu et al. 1994) and Prp6p (Abovich et al. 1990; Galisson and Legrain 1993) are important for stability of the U6 snRNA. Mutations in these proteins lead to a drastic decrease of U6 snRNA, and of the integrity of the U4/U6.U5 tri snRNP. All these mutations block splicing at the formation of the holo spliceosome. Two prp4 mutations, prp4-11 and prp4-21, can be partially suppressed by overexpression of U6 snRNA (Hu et al. 1994). On the other hand, U4 snRNA and its interaction with Prp4p also play an important role in the stability of U4/U6 snRNP (Bordonné et al. 1990; Xu et al. 1990). In addition, Prp38p is required for the stability of U6 snRNA (Blanton et al. 1992). It has been recently shown that this factor is associated with U4/U6.U5 tri snRNP and is involved in the dissociation of U4 from the spliceosome (Xie et al. 1998). The human U4/U6 snRNP also contains homologues of both Prp3p and Prp4p (Horowitz et al. 1997; Lauber et al. 1997; Wang et al. 1997). The integrity of the U4/U6 snRNA base-pairing interaction (Bordonné et al. 1990; Shannon and Guthrie 1991; Hu et al. 1995) is another essential factor in the formation of the holo spliceosome. U4 snRNA mutations in the regions that base-pair with U6 block splicing prior to the formation of the holo spliceosome (Hu et al. 1995). Another yeast splicing factor, Prp31p, is associated with the U4/U6.U5 tri snRNP, but is not essential for the formation/integrity of U4/U6 snRNP or U4/U6.U5 tri snRNP. Mutations in Prp31p can block the assembly of the holo spliceosome, suggesting that this protein is involved in recruiting the tri snRNP (Weidenhammer et al. 1997). Prp8p, a protein associated with U5 snRNP, is also required at this step (Brown and Beggs 1992).
Due to the complex protein composition of the U4/U6.U5 tri-snRNP (Behrens and Lührmann 1991; Fabrizio et al. 1994), protein-protein interactions must play important roles in the formation of the holo-spliceosome. Roscigno and Garcia-Blanco (1995) provided evidence that phosphorylated SR proteins may contribute to the recruitment of the tri-snRNP into the pre-spliceosome in human. It is possible that interactions between SR proteins and snRNP-associated proteins might occur during and after this step. In fact, a U4/U6.U5 tri-snRNP-specific 27 kDa SR protein has been identified in humans (Fetzer et al. 1997); it is phosphorylated by the snRNP-associated protein kinase (Woppmann et al. 1993). There is increasing evidence that reversible protein phosphorylation can positively and negatively regulate pre-mRNA splicing in humans. Phosphorylation of ASF/SF2 enhances significantly the protein-protein interaction with U1-70K and eliminates the non-specific RNA binding to the RS domains of U1-70K (Xiao and Manley 1997; Cao et al. 1997). This is consistent with the early observation that protein dephosphorylation selectively inhibits an early step during spliceosome assembly (Mernoud et al. 1994). Two peptide inhibitors of protein kinases, the calmodulin binding domain (CBD) and glycogen synthase (GS) fragment, have been shown to inhibit pre-mRNA splicing at two distinct events, early during spliceosome assembly and in the subsequent spliceosomal activation (Parker and Steitz 1997). In contrast, inhibition of dephosphorylation blocks the catalytic steps of splicing, but not spliceosome assembly (Mermoud et al. 1992; Tazi et al. 1992; Cao et al. 1997). Dephosphorylation of U1-70K protein seems to be a prerequisite for the first-step of splicing (Tazi et al. 1993). It is interesting to note that yeast Prp4p is also phosphorylated both in vitro and in vivo (A. Zurlinden, J. Hu, J.D. Friesen et al. personal communication).

A 20 kDa protein associated specifically with human U4/U6.U5 tri-snRNP (via strong association with hPrp4p and hPrp3p) is a novel peptidyl-prolyl cis-trans isomerase (cylophilin) (Horowitz et al 1997; Teigelkamp et al. 1998). Several interesting protein factors have also been found to be associated with the human U5 snRNP, including, a GTP-binding protein with homology to ribosomal translocase EF-2 (Fabrizio et al. 1997) and an RNA-dependent ATPase
Chap

(Laggerbauer et al. 1996; Lauber et al. 1996). The yeast homologue of the latter was first identified as SLT22 in my genetic screen (Xu et al. 1996; Chapter 4). More recently, another U5 snRNP protein (U5-100kD) has been identified as an additional RNA-dependent ATPase with an N-terminal RS domain and strong homology to Prp28p (DEAD protein) (Teigelkamp et al. 1997). Interestingly, U5-100kD protein can be phosphorylated by both U1-snRNP-associated kinase (Woppmann et al. 1993) and Ck/Sty kinase (Colwill et al. 1996) in vitro. These results suggest additional mechanisms that regulate protein and RNA conformational rearrangements in the assembly of the spliceosome and pre-mRNA splicing.

1.2.4. Core spliceosome/rearrangement of RNA interactions.

Following the formation of the holo-spliceosome, further extensive RNA conformational rearrangements take place. These processes include the disruption of U4/U6 snRNA base-pairing and U1/5' splice site interaction such that both U1 and U4 snRNAs are loosely associated with the rest of the spliceosome (Cheng and Abelson 1987; Konarska and Sharp 1987; Lamond et al. 1988; Yean an Lin 1991 and 1996), and the formation of U2/U6 snRNA and U6/5' splice site interactions (see §1.3.1 for detail and references). The invariant loop 1 of U5 snRNA is also brought to the 5' splice site (Wyatt et al 1992; Sontheimer and Steitz 1993; Newman et al. 1995). The spliceosome formed at this stage (prior to catalysis of the splicing reaction) can be called the core spliceosome, since it contains all three snRNAs required for the subsequent catalytic steps (Fig. 1.3). Relatively few splicing factors required for this step have been identified. However, recent genetic studies in yeast have revealed several key factors involved in RNA conformational rearrangements at this stage.

Hyperstabilization of the U1/5' splice site interaction results in the accumulation of a complex containing all five snRNAs; i.e., formation of the core spliceosome is blocked. Such a U1 snRNA mutation is exacerbated specifically by a mutation in Prp28p, a putative DEAD
box RNA helicase (Strauss and Guthrie 1991, 1994), but suppressed by mutations in U6 snRNA that strengthen the U6/5' splice site interaction (J. Staley and C. Guthrie, personal communication). These results suggest a functional role for Prp28p in exchanging U1 with U6 at the 5' splice site. A cold-sensitive mutation in U4 snRNA that masks the conserved ACAGAG sequence in U6 (a region important for interaction with the 5' splice site, Fig. 1.2) hinders the U6/5' splice site interaction (Li and Brow 1996; see §1.3.1). This mutation can be suppressed by mutation in Prp8p, suggesting that Prp8p (a highly conserved U5 snRNP protein) plays a role in the regulation of U4/U6 disassembly in the holo-spliceosome. This new allele of PRP8, prp8-201, is synthetically lethal with a mutation in Prp24p (A.N. Kuhn, Z. Li, and D. Brow, personal communication). The function of another U5 protein, Brr2p (Noble and Guthrie 1996)/Slt22p (Xu et al. 1996)/Snu246 (Lauber et al. 1996) (a putative RNA helicase), has been implicated in the disruption of the base-pairing interaction between U4 and U6 snRNAs during the maturation of the spliceosome (P. Raghunathan and C. Guthrie, personal communication). However, the 3' end of human U6 snRNA appears to be sufficient for promoting the dissociation of the U4/U6 duplex and thus a helicase activity may not be necessary for this process (Brow and Vidaver 1995; also see §1.3.2-1). Apparently, these processes are connected in the spliceosome, since a cold-sensitive mutation of PRP28, prp28-1, is suppressed by a prp8 mutation, but is synthetically lethal with prp24 (Strauss and Guthrie 1991). More recently, Ayadi et al. (1997) showed evidence that mutations in Prp4p (a U4/U6 snRNP protein, see above) also affect dissociation of U4 snRNA and Prp4p from the holo-spliceosome, without effect on the integrity of U4/U6 snRNP or U4/U6.U5 tri-snRNP. The action of Prp19p, which forms a stable complex with several other proteins (Tarn et al. 1994; Chen et al. 1998), has also been implicated in the spliceosome maturation concomitant with dissociation of U4 snRNA (Tarn et al. 1993a; 1993b). However, the exact role of the Prp19p complex is not well understood.
Despite the identification of factors associated with the U5 snRNP (see above), virtually nothing is known about how U5 snRNA is brought to the site of action, or its interaction with the rest of the spliceosome prior to the catalysis of the splicing reaction.

1.2.5. Activation of the core spliceosome.

After dissociation of U1 and U4 snRNAs, the spliceosome undergoes extensive RNA conformational changes (see §1.3.) to form a catalytically active structure for the two-step reaction of pre-RNA splicing (Fig. 1.1).

1) First catalytic step.

In yeast, the core spliceosome (complex A1 = functional spliceosome) is a short-lived complex (Cheng and Abelson 1987; Tarn et al. 1993b), thus activation of the core-spliceosome is not a rate-limiting step. Hydrolysis of ATP by Prp2p, an RNA-dependent ATPase (Kim et al. 1992; Plumpton et al. 1994), is required in this step (King and Beggs 1990; Kim and Lin 1993; Plumpton et al. 1994; Teigelkamp et al. 1994), suggesting RNA conformational rearrangements. The ATPase activity of Prp2p is stimulated preferentially by poly (U) oligoribonucleotides (Kim et al. 1992; Plumpton et al. 1994). The importance of ATP-driven RNA conformational changes was revealed by a dominant negative mutation in one of the conserved helicase motifs (SAT). This particular mutant Prp2p can associate with the spliceosome but fails to execute the potential RNA helicase activity, and thus stalls the spliceosome prior to the first step (Plumpton et al. 1994). Prp2p becomes associated with the spliceosome only in the process of activation. However, during its transient association, Prp2p makes direct contact with the pre-mRNA, and upon hydrolysis of ATP (causing necessary RNA conformational changes, presumably) the protein dissociates from the spliceosome and the splicing reaction follows (Teigelkamp et al. 1994). These results suggest that RNA conformational changes facilitated by Prp2p include the pre-mRNA substrate.
Spp2p, isolated initially as a high copy suppressor of *prp2-1* (Last et al. 1987), is another yeast splicing factor that functions at this step. It acts in a similar fashion as Prp2p, and may interact directly with Prp2p in the spliceosome (Roy et al. 1995a). Kim and Lin (1996) purified a biochemical fraction (HP) required for the spliceosomal activation in the event(s) following hydrolysis of ATP by Prp2p. However, the exact composition of HP fraction remains to be determined.

2) **Second catalytic step.**

Immediately following the first step of the splicing reaction, the second step occurs without any obvious delay, suggesting that these two steps are coupled efficiently. However, the spliceosome has to be remodeled in order to initiate the second step (see below) -- particularly since the chemistry of catalysis is different for these two steps (see §1.3.2-2). Several yeast splicing factors are known to be required for the second step (for a review, see Umen and Guthrie 1995a). The action of another RNA dependent ATPase, Prp16p, is likely to be involved in the process of remodeling. The original *prp16-1* mutation was identified as a suppressor of a branchpoint mutation that blocked the second step due to instability of the lariat intermediate (Couto et al. 1987; Burgess et al. 1990). The protein interacts with the spliceosome transiently (Schwer and Guthrie 1991). Its RNA-dependent ATPase activity (Schwer and Guthrie 1991, 1992a) is associated with a conformational change in the spliceosome that results in protection of the 3' splice site from oligonucleotide-directed RNase H cleavage (Schwer and Guthrie 1992b), which seems to be correlated with the crosslinking of two additional protein factors, Prp8p and Slu7p, to this site (see below). Additional *prp16* mutations (all mapped to the conserved RNA-dependent ATPase motifs that render reduced ATPase activities) were able to suppress other branchpoint mutations. These suppressors increased the stability of the aberrant lariat intermediates that carried mutant branchpoint site (Burgess and Guthrie 1993a). These results led to the proposal of a proofreading mechanism in which hydrolysis of ATP by Prp16p controls a discard pathway for the aberrant intermediates.
In this model, ATP hydrolysis by Prp16p is in a kinetic competition with a productive pathway, which leads to conformational changes for the second step. Failure to achieve such changes before ATP hydrolysis would doom the lariat intermediate to degradation. Otherwise, ATP hydrolysis would serve to lock the conformational changes (Burgess and Guthrie 1993a, 1993b). Remodeling of snRNAs is also required for the second step. This was demonstrated by the genetic suppression of prp16 mutations by single nucleotide deletions upstream of the ACAGAG motif of U6 snRNA (Madhani and Guthrie 1994b). Recently, Prp22p, another RNA-dependent ATPase, has been implicated in the second splicing step. However, its role in the second step is ATP-independent. The distance between the branchpoint and the 3' splice site dictates its requirement (Schwer and Gross 1998).

The function of Slu7p in the second step is linked, on the basis of synthetic lethality, to loop 1 of U5 snRNA (Frank et al. 1992). The original slu7-1 mutation is defective in utilization of the 3' splice sites which are further than ~12 nt downstream of the branchpoint (Frank and Guthrie 1992). Slu7p is dispensable for 3' splice sites with short distance from the branchpoint (≤ 7 nt) (Brys and Schwer 1996). It functions after ATP hydrolysis by Prp16p (Ansari and Schwer 1995; Jones et al. 1995), and is crosslinked to the 3' splice site prior to Prp16p; the crosslinking is enhanced following ATP hydrolysis (Umen and Guthrie 1995c).

The requirement of Prp8p in 3' splice selection was first revealed by prp8-101, an allele that is defective in the recognition of the polypyrimidine tract preceding the 3' splice site (Umen and Guthrie 1995b). Subsequently, additional prp8 mutations were isolated that suppress polypyrimidine tract and 3' splice site mutations. These mutations in Prp8p fell into two distinct domains (Umen and Guthrie 1996). Prp8p interacts with pre-mRNA, lariat intermediate and lariat intron (Whittaker and Beggs 1991; Teigelkamp et al. 1995a) and makes direct contact with both exons, which may assist the tethering function of U5 loop 1 (Teigelkamp et al. 1995b). The weak crosslinking of Prp8p to the 3' splice site occurs prior to
the action of Prp16p, but is greatly strengthened afterward (Umen and Guthrie 1995c). In
humans, p220 (Prp8p homologue, hPrp8p) is likely to be involved in the tethering of the two
exons. hPrp8p contacts the exon region at the 5' splice site prior to the first step (Wyatt et al 1992).
 p220, p110, and p116 (all of which are U5 snRNP associated proteins), replacing
U2AF65, are crosslinked to the polypyrimidine tract just prior to the second step (Chiara et al.
1997). In addition, two proteins, AG100 and AG75, are crosslinked to the last nucleotide of
the intron at the 3' splice site, while p220 crosslinks to both intron and exon regions at the 3'
splice site (Chiara et al. 1996). The kinetics of these crosslinkings is consistent with the
proposal of remodeling of the spliceosome for the second step (Schwer and Guthrie 1992b). It
is interesting to note that antibody against U5200, the human homologue of Slt22p/Brr2p,
inhibits the second step of splicing (Lauber et al. 1996). A set of 14 proteins was identified to
be associated with the spliceosome undergoing the first catalytic step. One of these, PSF, is
essential for the second catalytic step (Gozani et al. 1994). However, the exact roles of other
proteins remain to be determined.

Prp17p and Prp18p are two additional yeast factors involved in the second step. Both
were identified by genetic means (Vijayraghavan et al. 1989). A second allele of prp17, prp17-2,
was isolated as slu4 which is synthetically lethal with a mutation in loop 1 of U5 snRNA (Frank
et al. 1992). Neither gene is essential for viability at permissive temperature, and the second
step of in vitro splicing is only partially inhibited in the absence of either protein (Horowitz and
Abelson 1993a; Jones et al. 1995). However, Prp17p acts prior to ATP hydrolysis by Prp16p
(Jones et al. 1995), while Prp18p functions afterward (Horowitz and Abelson 1993b). The
genetic interactions (suppression and synthetic lethality) suggest that the functions of these
factors are interdependent, and are functionally connected to the tethering function of U5 loop
1 (see Fig. 1.5 for detail). In fact, a direct protein-protein interaction has been demonstrated
between Slu7p and Prp18p (Zhang and Schwer 1997).
Fig. 1.5. Genetic interactions among yeast splicing factors and loop 1 of U5 snRNA involved in the second step. Prp16p is an RNA-dependent ATPase. Factors acting prior to and following ATP hydrolysis by Prp16p are shown in open and filled ovals, respectively.
1.2.6. Post-splicing recycling of spliceosomal components

Upon completion of splicing, products must be released from the spliceosome. Prp22p (Company et al. 1991, Schwer and Gross 1998) and Prp43p (Arenas and Abelson 1997) (both RNA-dependent ATPases/DEAH proteins) are involved in the release of the lariat intron from the post-splicing spliceosome. The human homologue of Prp22p, HRH1, has also been implicated in releasing of mature RNA from the spliceosome and nuclear export (Ohno and Shimura 1996). Another factor, Prp26, is required for debranching of the lariat intron (Chapman and Boeke 1991). In the meantime, components of the spliceosome must be recycled for the next round of spliceosome assembly. The activity of Prp24p, an RNA binding protein, has been implicated in the formation and dissociation of the U4/U6 duplex during splicing. It was first isolated as a suppressor of a cold-sensitive U4 snRNA mutation that destabilizes U4/U6 duplex (Shannon and Guthrie 1991). Results of structural probing and binding assays indicate that Prp24p binds to U6 snRNA to promote its association with U4 snRNA and then dissociates to allow complete annealing (Ghetti et al. 1995; Jandrositz and Guthrie 1995). A direct role for Prp24p in annealing and recycling U4 and U6 snRNPs has recently been demonstrated (Raghunathan and Guthrie 1998). Consistent with this, a conformational switch of an intramolecular stem/loop in U6 snRNA is required for the assembly of U4/U6 snRNP in humans (Wolff and Bindereif 1993) and yeast (Fortner et al. 1994).
§1.3. Dynamic RNA-RNA interactions in the spliceosome.

Unlike autocatalytic group II introns, RNA structures important for pre-mRNA splicing are assembled from separate RNA molecules (pre-mRNA and snRNAs). As described above, the RNA-RNA interactions in the formation of the spliceosome are facilitated by protein-protein interactions during assembly and activation. This section will focus on the RNA structural aspects of splicing, emphasizing the conformational rearrangements during and after maturation of the spliceosome.

1.3.1. RNA-RNA interactions involved in recognition of splice sites.

1) Recognition of the 5’ splice site.

Recognition of the 5’ splice site is achieved by two sets of RNA base-pairing interactions: the U1/5’ splice site (during the formation of CC) and the U6/5’ splice site (following formation of the holo-spliceosome). The base-pairing interaction between the intron portion of the 5’ splice site and the 5’ end of U1 (Fig. 1.6) was demonstrated by genetic means in both humans (Zhuang and Weiner 1986; Yuo and Weiner 1989; Cohen et al. 1994; Hwang and Cohen 1996a) and yeast (Siliciano and Guthrie 1988; Séraphin et al. 1988; Séraphin and Rosbash 1990). However, substitutions at the fifth nucleotide of other yeast introns activate the use of adjacent cryptic cleavage sites (Jacquier et al. 1985; Parker and Guthrie 1985; Fouser and Friesen 1987). Compensatory mutations in U1 snRNA fail to suppress the aberrant cleavage, instead, cleavage at both normal and aberrant sites is increased (Siliciano and Guthrie 1988; Séraphin et al. 1988; Séraphin and Rosbash 1990). Subsequent genetic experiments showed that a realignment of the interaction between the 5’ splice site and U6 snRNA determines the cleavage event in the first catalytic step. Cleavage at the aberrant sites was partially suppressed by mutations at C48 nucleotide of U6 (in the highly conserved ACAGAG hexanucleotide motif)
(Kandels-Lewis and Séraphin 1993), or by increasing complementarity between U6 (at nucleotides 45 and 46) and the adjacent nucleotides in the intron (+7 and +8 position) (Lesser and Guthrie 1993a). Similar enhancement was also observed by Kandels-Kewis and Séraphin (1993). Furthermore, strengthening alternative base-pairing between U6 and the 5' splice site resulted in an increase in cleavage at the aberrant site (Lesser and Guthrie 1993a), emphasizing the determining role of the U6/5' splice site interaction in the first-step cleavage.

In humans, U6/5' splice site interaction plays a central role in splice site selection (Crispino and Sharp 1996; Hwang and Cohen 1996b). However, the U1/5' splice site interaction is important in facilitating the subsequent selection by U6 (Hwang and Cohen 1996b), suggesting cooperation between U1 and U6 snRNAs despite their mutual exclusion (Konforti et al. 1993). Base-pairing with U1 also contributes to the fidelity of the 5' splice site selection (Tarn and Steitz 1994). Although the requirement for U1 can be bypassed by a high concentration of RS proteins (Crispino et al. 1994; Tarn and Steitz 1994) and the 5' end of U1 can be replaced by spliced leader sequences (of C. elegans and L. collosoma) (Bruzik and Steitz 1990), the U6/5' SS interaction becomes rate-limiting in the absence of U1 function (Crispino and Sharp 1995). These results suggest cooperativity between U1 and U6 snRNAs in recognizing the 5' splice site. Preliminary results indicated that this may be the case for yeast as well (Goguel et al. 1991).

In a HeLa extract, U1 snRNA is crosslinked to the 5' splice site in an ATP-independent manner and during early stages of spliceosome assembly (Wassarman and Steitz 1992). The close proximity between U6 snRNA and the 5' splice site was demonstrated by crosslinking experiments in human (Sawa and Shimura 1992; Wassarman and Steitz 1992; Sontheimer and Steitz 1993) and yeast (Sawa and Abelson 1992).
Fig. 1.6. Rearrangements of RNA-RNA interactions during maturation of spliceosome (upper) and RNA interactions important for the catalytic steps (lower). See text for detail.
2) **Recognition of the branchpoint site.**

The branchpoint sequence UACUAAC is recognized initially by BBP in the commitment complex (Berglund et al. 1997) and by U2 snRNA subsequently (see §1.2.2). The branchpoint sequence in yeast is highly conserved, but it is considerably more degenerate in mammals. The U2/branchpoint base-pairing interaction was demonstrated by compensatory base-change experiments in yeast (Parker et al. 1987) and human (Wu and Manley 1989; Zhuang and Weiner 1989). A delicately balanced stem/loop structure of stem II (3' of the branchpoint interaction site) in yeast U2 snRNA (see Fig. 1.2C), in association with SF3a and SF3b (Fig. 1.4), is important in promoting the binding of U2 to the pre-mRNA (Ares and Igel 1990; Zavanelli and Ares 1991; Zavanelli et al. 1994).

The base-pairing interaction between U2 snRNA and the branchpoint is important for the subsequent catalytic step, since the nucleophile of the first step transesterification, the branchpoint adenosine, is bulged out in the duplex (Query et al. 1994; 1995). However, several protein factors, including hPrp8p, are in close contact with the branchpoint, and may play essential roles in the spliceosome (MacMillan et al. 1994). This may account for the lack of conservation of the mammalian branchpoint sequence (except the bulged A residue). The kinetics of crosslinking of p80 to the branchpoint; i.e. ATP-independent and early in spliceosome assembly (MacMillan et al. 1994), suggests that this protein may be SF1/mBBP (>70 kD on SDS-PAGE) (see Abovich and Rosbash 1997). Its crosslinking is displaced by p14 afterward (MacMillan et al. 1994). The temporal correlation between binding of p14 and U2 snRNP indicates that p14 is likely to be involved in the U2 snRNA/branchpoint interaction (Query et al. 1995).

3) **Recognition of the 3' splice site.**

It was originally proposed that the conserved AG dinucleotide at the 3' end of the intron basepairs with the invariant CU sequence adjacent to the recognition site of the 5' splice site in
U1 snRNA (Lerner et al. 1980; Rogers and Wall 1980). This was demonstrated experimentally in fission yeast *Schizosaccharomyces pombe* (Reich et al. 1992). In humans, the AG dinucleotide at the 3' splice site is required for the first catalytic step (presumably due to interaction with U1 snRNP via a protein-protein interaction), depending on the size of the polypyrimidine tract (Reed 1989). The interaction between U1 and the 3' splice site (containing the branchpoint and polypyrimidine tract) is ATP-dependent and requires U2 snRNP and RS proteins (Fu and Maniatis 1992). However, recognition of the 3' splice site does not require a base-pair interaction with U1 snRNA in *S. cerevisiae* (Séraphin and Kandels-Lewis 1993). But a mutant U1 snRNA that lacks the yeast-specific sequence (see Fig. 1.2 for yeast U1 snRNA) is defective in selection of a proximal site when the 3' splice sites are duplicated (Goguel et al. 1991), a defect similar to that caused by *prp8-101* (Umen and Guthrie 1995b, see §1.2.5-2), suggesting an indirect role for U1 snRNP in recognition of the 3' splice site. Following the first catalytic step, the exon region of the 3' splice site is tethered to the 5' exon by loop 1 of U5 snRNA through non-canonical base-pairing interaction (Newman and Norman 1991; Newman et al 1995; O'Keefe et al 1996; O'Keefe and Newman 1998). It has been suggested that protein factors (e.g., Prp8p) are required to stabilize these interactions (Teigelkamp et al. 1995).

An important cis element that influences the efficient recognition of the 3' splice site is the polypyrimidine (uridine-rich, in yeast) tract located between the 3' splice site and the branchpoint (Patterson and Guthrie 1991). Multiple interactions of this element with protein factors have recently been reported (Chiara et al. 1997, see §1.2.5-2), which reflect a requirement for this sequence in the early steps of spliceosome assembly and the second-step reaction (Reed and Maniatis 1985; Ruskin and Green 1985; Lamond et al. 1987; Reed 1989; Zhuang and Weiner 1990). Thus, recognition of the 3' splice site is achieved by collective interactions with snRNAs (U5 and U1) and proteins in several steps of assembly. The spacing between the branchpoint and the 3' splice site is another factor that influences the second step (Chiara et al. 1997; Luukonan and Séraphin 1997), suggesting that components that recognize the 3' splice site are constrained spatially by their interactions with the branchpoint. Consistent
with this, insertion of hairpin structures between the branchpoint and the 3' splice site effectively blocked the second step of the splicing reaction (Liu et al. 1997)

1.3.2. RNA-RNA interactions important for the catalysis of splicing reaction.

1) RNA conformational rearrangements prior to catalysis.

Following the formation of the holo-spliceosome, extensive RNA conformational rearrangements occur in order to form the active center, composed of RNA structures important for the catalytic steps. They include displacement of the U1/5' splice site interaction by U6, disruption of U4/U6 base-pairing, formation of U2/U6 interactions (Fig. 1.6), and other events, less well understood, such as positioning of U5 snRNA and its interaction with rest of the spliceosome. The precise temporal order of most events is ambiguous. However, a tentative order is emerging from recent findings.

a. Displacement of U1/5' splice site interaction by U6. Recent results of Staley and Guthrie (personal communication) suggest that displacement of the U1/5' splice site interaction, facilitated by Prp28p (an RNA-dependent ATPase/RNA helicase, also see §1.2.4), triggers a series of conformational changes. Hyperstabilization of U1/5' splice site interaction or mutation in prp28 results in accumulation of a splicing complex containing all five snRNAs with U4/U6.U5 tri-snRNP loosely attached. This new complex is a functional intermediate in the assembly pathway, since it can be chased into the active spliceosome. The highly conserved ACAGAG motif in U6 snRNA is involved in the recognition of 5' splice site (§1.3.1-1). This U6 motif is not base-paired with U4 (Fig. 1.6). Substitutions in the conserved GAAA sequence of U4 snRNA that extend U4/U6 stem I (including the ACAGAG motif in U6) confer lethal or cold-sensitive growth defects without any significant effect on the stability of the U4/U6 duplex (Li and Brow 1996). Cis-acting suppressors consist of substitutions and deletions in U4 snRNA that destabilize or eliminate the extended U4/U6 base-pairing. Similar trans-acting
suppressors of U6 snRNA were also obtained in the same study. The most intriguing U6 suppressor contains an insertion of ACAGA sequence in the upstream region of the extended base-pair (Li and Brow 1996). Genetic experiments suggested that the conserved ACAGAG motif in U6 serves at least two functions: 1) in recognition of the 5' splice site which leads to dissociation of U4/U6 duplex; 2) in subsequent catalytic events. The insertion of such an element in the extended U4/U6 duplex dissected the early role from late functions, and provided compelling in vivo evidence that U6 and 5' splice site interaction precedes the disruption of its association with U4 snRNA (Li and Brow 1996). In other systems, the importance of the GAAA motif in U4 snRNA was also demonstrated (Vankan et al. 1992; Weisg and Bindereif 1992). In one case, substitutions in this motif abolish splicing activity and result in an aberrant complex containing only U1, U2 and U4 (Vankan et al. 1992).

b. Disruption of U4/U6 duplex and formation of U2/U6 helix II. The dissociation of U4/U6 duplex is a prerequisite for the formation of the active spliceosome. This is accompanied by the formation of an intramolecular stem/loop structure (known as the Brow stem, Fig. 1.6) in U6 (required in the subsequent steps, see below), and the intermolecular interactions between U2 and U6 snRNAs. The Brow stem in both human (Wolff and Bindereif 1993) and yeast (Fortner et al. 1994) U6 is intricately balanced. Strengthening the stem inhibits the formation of the U4/U6 duplex, which can suppressed by other cis-acting destabilizing mutations (Fortner et al. 1994). However, weakening the stem structure leads to significant reduction in splicing activity (Wolff and Bindereif 1993; Sun and Manley 1997). The 3' end of human U6 snRNA, which encompasses the sequences that interact with U2 snRNA (forming helix II, Fig. 1.6), plays an important role in the dissociation of U4/U6 duplex (Brow and Vidaver 1995). In the deproteinized HeLa nuclear extract, native U4/U6 duplex is surprisingly unstable. However, binding of oligonucleotides complementary to the 3' end of U6 (mimicking the formation of helix II) or a region upstream of U4/U6 stem I stabilizes the formation of duplex. Furthermore, the removal of the entire 3' end of U6 drastically stabilizes the duplex by increasing the melting temperature by ~20°C (Brow and Vidaver 1995). It was suggested that
the interaction between U2/U6 snRNAs may serve to stabilize U4/U6 in order to prevent premature formation of U2/U6 helix I and Brow stem, both of which are important for catalytic events. Hence, energy released from the switch between alternative conformations, rather than the direct action of RNA-dependent ATPase/ RNA helicase, may be the driving force for the dissociation of U4/U6 duplex (Brow and Vidaver 1995). Results presented in Chapter 4 (Xu et al. 1996) and those of P. Raghunathan and C. Guthrie (see §1.2.4) are also consistent with a role of U2/U6 helix II in U4/U6 dissociation (Fig. 4.11) and in the subsequent events (see respective sections for detail).

Structural probing studies suggest that binding of U2 to the branchpoint increases the accessibility to RNase H of the 5' end of U2 snRNA (Lamond et al. 1989; Barabino et al. 1989). On the other hand, strengthening the bottom part of the stem structure in this region of U2 can effectively block splicing (Wu and Manley 1992). Interaction between the 3' end of U6 and the 5' end of U2 (i.e., helix II, Fig. 1.6) was first identified by psoralen crosslinking (Hausner et al. 1990) and then by genetic suppression in splice-site competition assays (Datta and Weiner 1991; Wu and Manley 1991). Subsequently, an additional trimolecular U2/U4/U6 crosslink, present in much higher abundance than the U2/U6 crosslink, was identified by Wasarman and Steitz (1992). These results are consistent with the notion that U2/U6 helix I is formed following helix II, which might be unwound. It is perhaps no surprise that the helix II interaction in yeast is not essential (Fabrizio et al. 1989; Fabrizio and Abelson 1990; Madhani et al. 1990; Miraglia et al. 1991; McPheeters and Abelson 1992; Field and Friesen 1996), considering that the structure of free yeast U6 snRNA is grossly different from that of human (see Brow and Vidaver 1995 for comparison). However, alternatively, this difference in struture may reflect the different requirement of helix II in two systems, or could be due to functional redundancy (Field and Friesen 1996). Consistent with a regulatory role of helix II, residues in both U2 and U6 parts of the helix I, and Brow stem are important for the catalytic event(s) (Fig. 1.6).
c. **U5/5' splice site interaction.** Loop 1 of U5 snRNA is in the vicinity of the exon region of the 5' splice site prior to the first catalytic step (Wyat et al. 1992; Newman et al. 1995). Indirect experiments suggest that conformational rearrangements that expose the conserved loop 1 are modulated by disruption of the bottom portion of stem 2 of human U5 (Ast and Wiener 1997a). An antisense oligonucleotide (complementary to the U5 portion mentioned above) causes such conformational changes (Ast and Wiener 1997a) and induces the formation of a complex containing U1, U4 and U5 snRNAs (Ast and Weiner 1996), in which loop 1 of U5 is crosslinked to the 5' end of U1 snRNA. These findings indicate the proximity of both snRNAs at the 5' splice site (Ast and Weiner 1997b). The formation of this complex may represent a transient step in spliceosome assembly in which U1 and U4 snRNPs are in the process of dissociating from the spliceosome. If U5 snRNA, following induced conformational changes, fails to contact the rest of the spliceosome, it may preferentially associate with leaving U1 and U4 snRNPs. If this is the case, an anchoring function must be provided by other components of the active spliceosome in order to position U5 snRNA to its site of action. Results presented in Chapter 4 suggest that the U2 snRNA and/or the U6 snRNA part(s) of the helix II interaction, in association with several other splicing factors identified in this study (i.e., Prp8p), may serve such an anchoring function during the formation of the active spliceosome.

2) **Chemistry of catalysis.**

Comparison has been made between important elements of autocatalytic RNA and structural elements in the catalytic center of the spliceosome (for reviews, see Weiner 1993; Madhani and Guthrie 1994a; Staley and Guthrie 1998). However, it is difficult to distinguish if these similarities reflect a common evolutionary origin or are merely products of chemical determinism (i.e., the structural similarities are a result of mechanistic restriction imposed by a particular chemical reaction which is catalyzed by RNA) (Weiner 1993). A two-metal-ion mechanism for catalytic RNA, including spliceosomes, has been proposed by Steitz and Steitz
Chapter 1

(1993). In their model, the first metal ion activates the attacking water or sugar hydroxyl (in the case of pre-mRNA splicing, the 2' OH of branch adenosine for the first step and the 3' OH of last nucleotide of the cut-off 5' exon for the second step), while the second ion coordinates and stabilizes the oxyanion-leaving group (the 3' OH of the last nucleotide of 5' exon for the first step and 3' OH of the last nucleotide of the intron for the second step). This has been shown to be the case for the leaving group of the first catalytic step of pre-mRNA splicing, but not for the metal ion-leaving group interaction in the second catalytic step (Sontheimer et al. 1997), suggesting that the two steps of pre-mRNA splicing proceed by different catalytic mechanisms and thus have distinct active sites. Stereochemistry of the two catalytic steps in the spliceosome are consistent with the proposal of two distinct catalytic centers for the two steps (Moore and Sharp 1993). However, the constituents of these centers may overlap and conformational rearrangements are required between the two steps. Experiments of substitution of 2' OH at the 5' and 3' splice sites also reached the same conclusion (Moore and Sharp 1992).

Although metal binding sites have not been identified in spliceosomal RNAs, chemical modification experiments (Fabrizio and Abelson 1992; Yu et al. 1995; Kim et al 1997) indicate several candidate residues in U6 snRNA. Some of these residues are important for splicing activities shown by mutational analyses (Fig. 1.6).

3) RNA interactions involved in the catalytic events.

Based on the premise that pre-mRNA splicing is an RNA-catalyzed reaction, RNA interactions important for the two catalytic steps are defined as those, which when mutated, block the first or second step reaction without any effect on assembly.

The tentative temporal order of RNA conformational rearrangements (see above) places the formation of U2/U6 helix I (Madhani and Guthrie 1992, 1994c; Sun and Manley 1995; Fig. 1.6) concomitant with spliceosomal activation. In yeast, deletions in the U2 part of helix I
result in accumulation of complex A1 (ΔA27 and ΔU28) or an unstable active spliceosome (ΔA25 and ΔG26) and drastic decrease in overall splicing activity (McPheeters and Abelson 1992). Similarly, substitution G60C in U6 also destabilizes the formation of the active spliceosome (Fabrizio and Abelson 1990). It is no coincidence that nucleotides important for catalytic events are clustered around or part of helix I. Furthermore, the formation of helix I brings (and likely juxtaposes) the two principal participants of the first catalytic reaction to close vicinity: the branchpoint (recognized by U2) and the 5’ splice site (recognized by U6) (Fig. 1.6).

Recognition of the 5’ splice site by the 5’ portion of the ACAAGG motif plays dual functions during maturation of the spliceosome (Li and Brow 1996; see above) and in later catalytic event(s). Mutations in these three nucleotides inhibit or reduce drastically the overall splicing activity (Fabrizio and Abelson 1990) and most of them confer lethal or severe growth defects (Madhani et al. 1990). Although the G52 nucleotide is not involved in a base-pairing interaction with the 5’ splice site, recent experiments showed that it interacts with the first nucleotide of the intron before the first step and that some combinations of 5’ splice site and U6 mutations produce a synthetic block of the first splicing step (Luukkonen and Séraphin 1998). Loop 1 of U5 snRNA is dispensable for the first catalytic step (O'Keefe et al. 1996), but its interaction with the 5’ splice site can influence the precision of the catalytic event, as shown by activation of a cryptic cleavage site(s) by mutation(s) in loop 1 when the mutant 5’ splice is present (Newman and Norman 1991; Newman and Norman 1992). Similar, although more complex, results were obtained in mammal cells (Cortes et al 1993).

Dissociation of the U4/U6 duplex allows the formation of the intramolecular U6 Brow stem (Wolff and Bindereif 1993; Fortner et al. 1994). Destabilization of the Brow stem structure in human U6 snRNA reduces the splicing activity without affecting early step(s) (Wolff and Bindereif 1993). The structural features, rather than identities, of the Brow stem are important (Sun and Manley 1997). Several mutations in this stem of yeast U6 confer a lethal
phenotype (Madhani et al 1990), and one of them, U80G, strongly inhibits splicing activity (Fabrizio and Abelson 1992). Chemical modifications of nucleotides also demonstrated the importance of this structure in the catalytic event (Fabrizio and Abelson 1992; Kim et al. 1997, also see Fig. 1.6).

The ACAGAG motif in yeast U6 snRNA is also important for the second catalytic step. This region is in close contact with the 5' splice site even prior to Prp2p action. The interaction persists following the first step in the lariat intermediate (Kim and Abelson 1996). A functional role of this interaction in the second catalytic step is suggested by the interaction between first and last nucleotides of the intron (Parker and Siliciano 1993; Chanfreau et al. 1994; Luukkonen and Séraphin 1997). It has also been suggested that the proofreading function of Prp16p is exerted on such an intermediate (Burge and Guthrie 1993a). In an attempt to identify potential targets for Prp16p, U6 snRNA suppressors of a cold-sensitive prp16-302 mutation were isolated, all of which contain single deletions in the 6 nucleotides immediately 5' of ACAGAG motif (Madhani and Guthrie 1994b). These results are consistent with the idea that interaction between U6 and the 5' intron-branchpoint region in the lariat intermediate, also identified by crosslinking studies (Sawa and Abelson 1992; Sawa and Shimura 1992), may constitute part of RNA structures important for the second step (e.g., recognition of the 3' splice site, see below). Luukkonen and Séraphin (1998) recently showed that some combination of mutations in the GAG trinucleotide in U6 and the 5' splice site can block the second step, suggesting a role for the corresponding nucleotides in the proofreading step prior to the second catalytic step (also see below). The use of a non-adenosine branchpoint can effectively block the second step (Query et al 1994, 1995; McPheeters 1996). However, mutations in the bottom part of Brow stem, or at position 57 (part of helix I) of yeast U6 suppress non-adenosine branchpoint (McPheeters 1996). These results reiterate the importance of several regions of U6 snRNA in the second step reaction.
The 3' two nucleotides of the ACAGAG motif play crucial roles in the second step. Substitutions at these residues in both human (Wolff et al. 1994), nematode (Yu et al. 1993) and yeast (Fabrizio and Abelson 1990) U6 snRNAs inhibit strongly only the second catalytic step (Fig. 1.6). U6 residues C58 and A59 (of yeast, and corresponding residues in human) opposite the bulge region of helix I and their pairing partners U23 and G26 of U2 snRNA are involved in the second step (Fabrizio and Abelson 1990; McPheeters and Abelson 1992; Wolff et al. 1994). Although base-pairing is necessary and sufficient for the Ia portion of the yeast helix I (Madhani and Guthrie 1994c), there is a phenotypic asymmetry resulting from mutations at A59 position in U6 and its U2 partner U23 in the Ib portion of helix I (Madhani and Guthrie 1992, 1994c). While substitution at 59, 60 and 61 positions confer deleterious growth defect in vivo (Madhani and Guthrie 1992) and inhibit splicing in vitro (Fabrizio and Abelson 1990), their U2 partners are more tolerant to mutations (with the exception of G21C, Madhani and Guthrie 1992). Only two U6 mutations in Ib region, A59C and A59G, are suppressed by compensatory mutations at 23 position of U2 (Madhani and Guthrie 1992; Luukkonen and Séraphin, personal communication). This asymmetry suggests that A59 residue of U6 plays an additional role in the second step, and Ib portion of U2/U6 helix I is not rate-limiting, or forms transiently (thus its function is secondary). Although the identities of the two bulged nucleotides of U2 are not critical (McPheeters and Abelson 1992; Madhani and Guthrie 1994c), altering the spacing of the bulge is deleterious (Madhani and Guthrie 1994c; Wolff et al. 1994).

One nucleotide in the bulge region, A25 (of U2 snRNA), influences the selection of the 3' splice site. Alterations of the AG dinucleotide at the 3' splice, which block the second catalytic step, are suppressed by A25G, although the suppression is not allele-specific (Madhani and Guthrie 1994c). Similar non-specific suppression of the 3' splice site alterations by mutation G52U of U6 snRNA (the last nucleotide in the ACAGAG motif) was also observed (Lesser and Guthrie 1993a). Moreover, these two residues in U2 and U6 are implicated in a possible tertiary interaction (Madhani and Guthrie 1994c). Taken together, these results imply that such a tertiary interaction is part of the structure that controls the selectivity of the second catalytic
step (Madhani and Guthrie 1994c). The crosslinking between U23 residue of U2 snRNA and the first nucleotide of the second exon (Newman et al. 1995) lends support for the close proximity of this region of U2, via such a tertiary interaction, to the 3' splice site.

The most conserved loop 1 region of U5 snRNA (Frank et al. 1994) is involved in interaction with the exon region of the 5' splice site (see above). Although this interaction is not essential for the first catalytic step (O'Keefe et al. 1996), it persists through both steps (Sontheimer and Steitz 1993; Newman et al. 1995). Alteration of the AG dinucleotide of the 3' splice site can be suppressed partially when complementarity between the first and second nucleotides of exon 2 and loop 1 is created (Newman and Norman 1992). A tethering function of U5 loop 1 is further suggested by crosslinking to exon 2 after the first catalytic step (Sontheimer and Steitz 1993; Newman et al. 1995; O'Keefe et al 1996). In yeast, the size, rather than the sequence, of U5 loop 1 is important for the proper alignment of the two exons for the second step in vitro (O'Keefe and Newman 1998).

Stem II of U2 snRNA, important for the binding of U2 to pre-mRNA (see §1.3.2-2), may be required for the catalytic step. A biotinylated 2'-O-allyl oligoribonucleotide complementary to the stem IIa of human U2 specifically inhibits the second step splicing reaction without any noticeable effect on the first step (Barabino et al. 1992). More recently, crosslinking between stem IIa and the exon region of the 5' splice site (present in a oligoribonucleotide) in HeLa extract has also been reported (Ast and Wiener, personal communication). This interaction is suggested to tether the "cut-off" exon 1 for ligation in the second step.

In summary, the nucleophile of the first catalytic reaction, an adenosine at the branchpoint, is bulged out in the U2/branchpoint site duplex. The intron region of the 5' splice site is recognized by the 5' portion of ACAGAG motif in U6 snRNA. This is aided by interaction between the exon region of the 5' splice site and U5 loop 1, although this additional interaction is not necessary for first catalytic step. The branchpoint and the 5' splice sites are juxtaposed through U2/U6 helix I interactions. These interactions and the intramolecular U6
stem form a highly ordered structure (catalytic center) that might be responsible for the catalysis of the first transesterification reaction. Following the first step, the catalytic center undergoes conformational rearrangements to accommodate the lariat branch site, which may form part of the structure that recognizes the 3' splice site. [The chemical requirement of the branch adenosine is different in three recognition events: during assembly, and the first and second catalytic steps (Query et al. 1995).] The free "cut-off" exon is tethered to the second exon by loop 1 of U5. Protein factors and additional tethering by other RNA component (i.e., stem IIa of U2) are likely to be involved in this process. The rearranged catalytic center can then perform the second transesterification reaction. These two catalytic steps are efficiently coupled.
§1.4. Rationale and outline of the thesis.

In order to understand the functions of the 5'-end of U2 snRNA and U2/U6 helix II, a genetic screen was performed. As described in Chapter 2, six slt mutant strains were isolated based on synthetic lethality with a U2 mutation in the 5'-end of U2 snRNA that also perturbs the helix II interaction. They were characterized and the five corresponding SLT genes were cloned by complementation. In Chapter 3, genetic interactions among slt, slu, prp, U2, U5 and U6 mutations will be described in detail. The results will demonstrate a network of synthetic lethality among factors involved in both steps of the splicing reaction. Evidence will show that this network of interactions is related to the genetic interactions between the 5'-end of U2 snRNA and loop 1 of U5 snRNA, a region that is involved in the alignment of the two exons for the second splicing step. These results suggest a potential role for U2 snRNA in the second splicing step. Several Slt factors and Prp16p may act as two overlapping functional units to regulate and coordinate the two steps of the splicing reaction.

In the following chapter, the characterization of a new splicing factor, Slt22p, will be presented. The results will show that the RNA-dependent ATPase activity of Slt22p is related to the U2/U6 helix II interaction (i.e., it is likely that helix II is the target for the potential RNA helicase activity of Slt22p), and that the ATPase function of Slt22p in association with the U2/U6 interaction is important for retention of U5 snRNP in the formation of the mature spliceosome.

Chapter 5 will be concerned with the role of Slt21p/Prp8p (a protein associated with U5 snRNP) and its interaction with U2 snRNA in spliceosome assembly prior to the first step. Results of genetic and biochemical experiments indicate that the interaction between the the 5'-end of U2 snRNA and Slt21p/Prp8p is required for the selection of the 3' splice site in spliceosome assembly. A bridging function of Prp8p and U2 snRNA will also be discussed in this chapter.
Chapter 6 will present results of genetic and biochemical characterization of another new splicing factor, Slt11p, and its interactions with U2, U5 and U6 snRNAs, and Slt17p/Slu7p (a factor involved in the 3' splice site selection). The results will show that Slt11p may act to mediate RNA conformational changes in the active spliceosome for both steps of the splicing reaction.

A general summary and future directions will be provided in the Chapter 7.
Chapter 2.

Identification of Yeast SLT Genes Required for Pre-mRNA Splicing by Means of Synthetic Lethality with a Mutation in U2 snRNA


D.J.F. initiated the genetic screen and provided the original yeast strain of ΔSNR20 and mutant U2 and U6 constructs. S.-J. T. contributed to molecular cloning of SLT11 and SLT22, and genetic analysis of *slt, prp* and *slu* mutations, A.M., SLT15 and SLT16, B.P.B, SLT17.
Abstract

A genetic screen was conducted in an effort to identify yeast splicing factors that are important for the function of U2 snRNA. Six temperature-sensitive slt (synthetic lethal with U2 snRNA) mutations were isolated on the basis of synthetic lethality in combination with an 11 nucleotide substitution in the 5'-end (i.e., nucleotide 3 through 13) of U2 snRNA. All of them have been characterized and five corresponding wild-type genes were cloned by complementation. While four slt mutations were new alleles of previously identified splicing genes, slt15/prp17-100, slt16/smd3-1, slt17/slu7-100 and slt21/prp8-21, two were new splicing genes, SLT11 and SLT22. Four mutations, slt11-1, slt16/smd3-1, slt21/prp8-21 and slt22-1, blocked pre-mRNA splicing prior to the first catalytic step. The remaining two mutations, slt15/prp17-100 and slt17/slu7-100, are defective in the second step. These results suggest that the interactions of U2 snRNA with these Slt factors are important for both steps of the splicing reaction.
§2.1. Introduction

Pre-mRNA splicing proceeds via a two-step transesterification reaction, the chemistry of which is shared by group II introns that are able to catalyze the reaction \textit{in vitro} in the absence of protein factors. However, nuclear pre-mRNA splicing occurs only in the spliceosome, a dynamic ribonucleoprotein complex, in which both the RNA and protein components contribute equally to the establishment of a network of RNA-RNA interactions important for the two-step reaction (Rymond and Rosbash 1992; More et al. 1993; Madhani and Guthrie 1994a). Genetic approaches in the yeast \textit{Saccharomyces cerevisiae} have allowed the identification of as many as 50 protein factors required for pre-mRNA splicing: 29 genes were identified by screening temperature-sensitive (ts) mutants that fail to efficiently process pre-mRNA \[PRP2\] through \[9\], and \[PRP10=PRP11\] (Hartwell et al. 1970), \[PRP17\] through \[27\] (Vijayraghavan et al. 1989), \[PRP29\] through \[34\] (Maddock et al. 1996), \[PRP38\] (Blanton et al. 1992), \[PRP39\] (Lockhart and Rymond 1994), and \[PRP42\] (McLean and Rymond 1998)], 6 by screening cold-sensitive (cs) mutations \[PRP28\] (Strauss and Guthrie 1991), and \[BRR1\] through \[5\] (Noble and Guthrie 1996)]. Other splicing genes have been identified by means of genetic suppression \[PRP16=PRP23\] (Couto et al. 1987), \[SPP2\] (Roy et al. 1995a), \[PRP40\] (Kao and Siliciano 1996), \[CUS1\] and \[CUS2\] (Wells et al. 1996; M. Ares, personal communication)] and synthetic lethality \([SLU\] genes (Frank et al. 1992), \[MUD\] genes (Liao et al. 1993), \[MSL\] genes (Tang et al. 1996; Abovich and Rosbash 1997)].

Although new splicing factors have been identified by screens of ts and cs mutants, this approach is diminished by the appearance of genes identified previously and the number of new genes identified in later screens has decreased (Maddock et al. 1996; Noble and Guthrie 1996). On the other hand, alternative genetic approaches, such as suppression or synthetic lethality, have revealed certain functional interactions among different factors identified in the screen, regardless new or known. For example, the cross-intron bridging interactions were first suggested by pair-wise synthetic lethality of \[prp40\] with \[mud2\], and of \[msl5\] with \[mud2\].
mutations. PRP40 was initially identified as a suppressor of a mutation in U1 snRNA (Kao and Siliciano 1996) and then as a synthetic lethal mutant of mud2 (Abovich and Rosbash 1997). MSL5 was isolated as a synthetic lethal mutant of mud2 (Abovich and Rosbash 1997). Prp40p is a U1 snRNP protein, MSL5 encodes a protein that binds to the branchpoint site in intron (thus known as BBP, branchpoint binding protein). Their interactions with Mud2p define a cross-intron bridging between the 5' splice site and the branchpoint in the formation of commitment complexes (Abovich and Rosbash 1997; and references therein).

The yeast U2 snRNA contains all the elements that are conserved in U2 snRNAs of other eukaryotes, despite its unusually large size (1,175 nt) (Fig. 2.1A). The stem II region is important for recognition of the branchpoint site and association of the U2 snRNP with the pre-mRNA. Protein factors, SF3a (Prp9p, Prp11p, and Prp21p), SF3b (Cus1 and Hsh49p), and Prp5p (a DEAD box protein) have been implicated in this function (Ruby et al. 1993; Well and Ares 1994; Yan and Ares 1996; Igel et al. 1998 and references therein). This aspect of the splicing pathway is highly conserved in both yeast and humans (for a review, see Krämer 1996). However, very little is known about the factors involved in subsequent steps in spliceosome assembly in which the stem I region of U2 snRNA undergoes extensive conformational rearrangements to form intermolecular U2/U6 snRNA interactions. A synthetic lethality strategy was suggested by Dr. D. Field to identify factors that are important for the function of yeast U2 snRNA, particularly that of the 5' end region. Before the commencement of the genetic screen for mutations that were synthetically lethal with a mutant yeast U2 snRNA carrying an 11nt substitution in stem I region (Fig. 2.1A), it had been demonstrated that, in human, the 5' end of U2 snRNA and the 3' end of U6 snRNA form base-pair interaction, later known as helix II (Hausner et al. 1990; Data and Weiner 1991; Wu and Manley 1991). However, the aforementioned U2 mutation, disrupting the proposed yeast U2/U6 helix II interaction (Hausner et al. 1990), confers only mild growth defect at 16°C and 37°C. It was believed that this interaction was functionally redundant, which was demonstrated subsequently to be so (Field and Friesen 1996), and/or that additional factor(s) are involved. Thus, it was
Chapter 2

anticipated that this genetic screen might yield factors that are important for the U2/U6 interaction.

The genetic screen described in this chapter identified six yeast splicing factors. Two of them are new: Slt11p and Slt22p; the remainder are known factors, Slt15p/Prp17p, Slt16p/Smd3p, Slt17p/Slu7p and Slt21p/Prp8p. This is the first time that proteins have been implicated in the function of the 5' end of yeast U2 snRNA. Characterization of Slt22p revealed that this protein, an RNA-dependent ATPase, is involved in U2/U6 interaction (Xu et al. 1996 and Chapter 4). Results of subsequent characterization of other Slt factors suggested new roles of U2 snRNA, in association with these proteins, in events prior to and following the first step of the pre-mRNA splicing reaction.
§2.2. Results

2.2.1. Genetic screen

During the process of the synthetic lethal screen, additional interactions between U2 and U6 were demonstrated in both yeast and human (Madhani and Guthrie 1992; Sun and Manley 1995; Field and Friesen 1996). Two intermolecular helices were identified and named as I and II (helix I is further divided into Ia and Ib with a 2-nt bulge in the middle, see Fig. 2.1A). For the purpose of clarity and consistence, these terms will be used to describe of U2/U6 interactions. The starting U2 mutation used in the genetic screen contains an 11nt substitution at the 5' end (nucleotides 3 through 13). It disrupts part of the stem I structure, and the proposed U2/U6 helix II interaction (Fig. 2.1A). The yeast strain used in the genetic screen contains a chromosomal deletion of U2 snRNA gene, SNR20, with wt and mutant U2 genes carried on URA3 and TRP1 plasmids, respectively (Field and Friesen 1996). After treatment of EMS to a surviving rate of ~10%, the surviving cells were then screened individually for sensitivity to 5-FOA at 30°C, an indication that mutation(s) introduced by EMS might cause lethality in combination with the mutant U2 snRNA (Fig. 2.1B). Twelve 5-FOA-sensitive strains were isolated from a pool of ~8,000 surviving cells, named YDX11 through YDX22. In the presence of wt U2 snRNA, all twelve strains display a temperature sensitive phenotype (Table 2.1).

In order to eliminate artifacts (e.g., mutations that affect the uracil synthesis pathway), the original mutant U2 plasmid (TRP1-marked) of the candidate mutant strains was replaced with a wt U2 gene carried on the TRP1 plasmid (pDF87) to test for 5-FOA resistance. Three of them, YDX14, YDX18 and YDX20, remained sensitive, and were then discarded.
Fig. 2.1. Yeast U2 snRNA and genetic screen for slt mutations. A. The 5' end region of yeast U2 snRNA (top) and RNA-RNA interactions in the spliceosome (bottom). The underlined branchpoint interaction (BP int.) region in U2 snRNA is essential for the recognition of branchpoint site in pre-mRNA via base-pairing to bulge out the adenosine residue, the nucleophile of the first transesterification reaction. Nucleotides in stem I of U2 snRNA are involved in U2/U6 interaction forming three helices: Ia, Ib and II. Nucleotides shown in black boxes correspond to U2 part of the helix II interaction. The 11nt substitution (snr20-11nt) in this region of U2 snRNA, which perturbs the U2/U6 helix II interaction, was used in the genetic screen. B. Genetic screen for slt mutations. A yeast strain of chromosomal deletion of U2 snRNA gene (SNR20) (with wt and mutant U2 snRNA genes carried on URA3 and TRP1 plasmids respectively) was subjected to EMS mutagenesis. Surviving cells were screened for sensitivity to 5-FOA, an indication that an extragenic mutation (slt) is lethal in combination with snr20-11nt and thus relies on the SNR20-URA3 plasmid for viability.
Table 2.1. Growth Phenotype of slt Strains in the Presence of SNR20.

<table>
<thead>
<tr>
<th>Strains</th>
<th>16°C</th>
<th>25°C</th>
<th>30°C</th>
<th>33°C</th>
<th>35°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>YDF16 (wt)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>YDX11&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>YDX12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YDX13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>YDX14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YDX15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>YDX16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>YDX17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YDX18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>YDX19&lt;sup&gt;e&lt;/sup&gt;</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>YDX20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>YDX21</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>YDX22&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes:

a. Wild-type genes were cloned by complementation.
b. Additional mutation was present in the original strain.
c. Artifacts, likely due to mutation(s) in the uracil synthesis pathway.
d. Synthetic lethality is not linked to ts phenotype.
e. At least two mutations are collectively responsible for synthetic lethality.
2.2.2. Genetic characterization

The nine remaining strains were crossed to prp mutant strains in our collection for complementation tests. While YDX21 failed to complement the ts phenotype of prp8-1 at 37°C (see Fig. 5.1A), other strains fully complemented all ts prp mutations tested (Table 2.2). The result of YDX16/prp17-1 complementation was ambiguous, since only one diploid was recovered at 37°C. However, cloning confirmed that slt16 is a new allele of prp17 (see below). The characterization of YDX21 will be described in Chapter 5. The other strains were subject to a set of genetic analyses to determine if synthetic lethality had arisen from mutation at single locus which also conferred a ts phenotype.

The scheme for genetic analyses is outlined in Fig. 2.2. Briefly, each original ts and 5-FOA⁵ mutant strain was first crossed to the wt W303-1A strain to generate a heterozygous diploid (a). In all cases, such heterozygous diploids displayed a wt growth phenotype suggesting that all the mutations are phenotypically recessive. After loss of both URA3 and TRP1 plasmids, the resulting heterozygous diploid was sporulated, and tetrad dissection was performed in order to recover ts haploids in the wt U2 background (b). The ts strains were then crossed to wt cells (c) in order to determine, after sporulation and tetrad dissection, if such a ts phenotype was due to mutation at single locus (d). The progeny ts cells were then back-crossed to wt strain at least three times (e) to confirm that such is the case and to segregate additional mutation(s), if any. Additional mutations were found for YDX11 and YDX22 strains. The resulting haploid ts strain was then crossed to a newly constructed SNR20Δ::HIS3 strain (see 2.4.1) (f) to generate a ts haploid in the background of chromosomal deletion of SNR20 (g) so that synthetic lethality could be tested after transformation of the mutant U2 plasmid (h). During the process of genetic characterization, some the ts strains were also crossed to each other to test for complementation. In all cases tested, full complementation was observed (data not shown).
Table 2.2. Results of complementation tests.

<table>
<thead>
<tr>
<th>YDX</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>19</th>
<th>21</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>prp2-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp3-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp4-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp5-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp6-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp7-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp8-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp9-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp10-1c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp11-1c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp16-1d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp17-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp18-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp19-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp20-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp21-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp22-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp23-1d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp24-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp25-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp26-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp27-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp33-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp34-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Notes:

a. Diploid failed to complement at 37°C; i.e. slow-growth.

b. Only one diploid was obtained at 37°C. *slt15* is a new allele of *PRP17*, *prp17-100* (see §2.2.5-i).

c. *prp10-1* and *prp11-1* are allelic.

d. *prp16-1* and *prp23-1* are allelic.
Fig. 2.2. Flow chart of genetic characterization of slt mutation. Each candidate slt mutation was characterized in order to determine if the ts phenotype was associated with synthetic lethality and was due to mutation at single locus. See text for detail.
Fig. 2.3. Growth defects of yeast strains carrying slt mutations. The ts phenotype of six slt mutations segregated genetically to the background of wt SNR20 (i.e., after step e in Fig. 2.2). Shown is two-day growth on YPD medium. Note that slt15, slt16 and slt22 confer slow growth at 30°C, and that slt21 confers slow growth at 37°C. None of these mutations confer cold-sensitive growth.
Five mutations, representing five different complementing groups (also see below), were found to satisfy the criteria that the synthetic lethality has arisen from mutation at single locus which also confers a ts growth defect. They were named as slt11, slt15, slt16, slt17, and slt22 (according to the original YDX strain number), for synthetic lethal with U2 (Fig. 2.3). The synthetic lethality observed in the other three strains, YDX12, YDX13 and YDX19, failed to fit the above criteria. In the YDX19 strain, the original synthetic lethality is likely due to a combination of at least two mutations in addition to the mutant U2 snRNA.

2.2.3. Splicing defects

Prior to molecular cloning of SLT genes, five satisfactory mutant strains, slt11, slt15, slt16, slt17 and slt22, were subject to primer extension and/or an in vitro splicing assay to determine whether splicing defects were associated with these mutations.

To determine whether the ts growth defect of slt mutations is associated with failure to process pre-mRNA in vivo, total yeast RNA was isolated from slt cells (containing the wt U2 gene, obtained after step e in Fig. 2.2) prior to and following a shift to non-permissive temperature (37°C) from 25°C. The level of spliced and unspliced actin and U3 RNAs were measured by primer extension using labeled oligonucleotides complementary to the second exons of each transcript (Fig. 2.4). Inhibition of pre-mRNA splicing was noted in slt16 (Fig. 2.4, lanes 12 to 14) and slt22 (data not shown) cells at both temperatures, whereas accumulation of unspliced precursor was detected in slt11 cells only following the shift (data not shown). The slt15 mutation exhibited a decrease of mature RNA at the permissive temperature and following the temperature shift (Fig. 2.4, lanes 2, 6-8). These data indicate that these four slt mutations affect the processing of pre-mRNA in vivo.
Fig. 2.4. Primer extension analysis of splicing defects associated with *slt15* and *slt16* mutations. Total yeast RNA was extracted from wt, *slt15* and *slt16* cells grown at 25°C and following a shift to 37°C for the time indicated. Lanes 1 - 8: 32P-labeled primer complementary to the second exon of pre-actin transcript was used to determine level of spliced and unspliced RNAs in wt and *slt15* cells under the condition indicated. Lanes 9 - 14: 32P-labeled primer complementary to the second exon of pre-U3 transcript was used to determine splicing defect associated with *slt16* mutation. Bands labeled as A and B are precursor transcripts of U3A and U3B genes. Note that splicing defect was observed in both *slt15* and *slt16* cells grown at permissive temperature (25°C). Both mutations confer a slow growth defect at permissive temperatures (Fig. 2.3).
Fig. 2.5. *In vitro* splicing assay of wt, *slt11*, *slt22* and *slt17* extracts. A. Mutations *slt11* and *slt22* block splicing prior to the first catalytic step. *In vitro* splicing reactions of wt, *slt11* and *slt22* extract were performed with 32P-labeled pre-actin substrate at 25°C and 33°C for a 20 min incubation. B. Mutation *slt17* impairs the efficiency of the second catalytic step. At the 25°C (lanes 5 and 6) and 33°C (lanes 7 and 8), the *slt17* extract accumulated products of the first step reaction (free exon 1, lariat intron-exon 2, as indicated by arrows).
Whole-cell splicing extracts were prepared from slt11, slt17 and slt22 cells that contain the wt U2 gene on the chromosome II (obtained after step e in Fig. 2.2) so that any observed splicing defect could be attributed solely to the slt mutations. When untreated extracts were assayed at 25°C, the splicing activity of slt11 extract is relatively lower than that of the wt extract (lane 2, Fig. 2.5A), while that of slt22 was extremely low (lane 3). At the 33°C, the activity of both mutant extracts were completely abolished; no mature products were detected (lanes 5 and 6) while the wt extract was still functional, although less efficient (lane 4). In both mutant extracts, pre-mRNA splicing was blocked at the step before/at the first splicing step, in agreement with the in vivo splicing defect observed (see above). On the other hand, the slt17 extract was active at both 25°C and 33°C (lanes 5-8, Fig. 2.5B). However, partial inhibition of the second step, as indicated by the preferential accumulation of lariat-intron-exon 2 intermediate, was noted (arrows in Fig. 2.5B), suggesting that the slt17 mutation affects primarily the efficiency of the second step of the splicing reaction. This is in agreement with of molecular cloning result that showed that slt17 corresponds to SLU7, which encodes a factor involved in the second step of splicing (see below).

2.2.4. Molecular cloning of SLT11

Molecular cloning of SLT genes was facilitated by the growth defect conferred by all five slt mutations (Fig. 2.3). Since the cloning procedure was essentially the same in all cases, the identification of SLT11 gene will be described in detail to provide an example.

The slt11-1 mutation confers a ts growth defect at $\geq$ 33°C (Fig. 2.3). A yeast genomic library, carried on a low-copy plasmid (YCP50, URA3, CEN-ARS, Rose et al. 1987), was introduced into slt11-1 cells, and the resulting transformants were selected at 37°C for full complementation of the ts phenotype. Twelve ts-complementing clones were isolated, which fell into three types but all shared common restriction fragments. The nucleotide sequences
Fig. 2.6. Molecular cloning of SLT11. A. Restriction map of the SLT11-containing region on Chromosome II. All slt11-complementing clones are located on Chromosome II and the overlapping region contains several ORFs. Note that U2 gene, SNR20, and PRP5 are also located on the right arm of Chromosome II. B. Minimal slt11-compelmenting genomic fragments. These clones were isolated by a mini-library method (see text). Since all of them contained YBR065c, it was identified as SLT11. C. YBR065c complements the synthetic lethality of slt11 with U2 snRNA mutations. A 2.2 Kb BglII fragment containing only YBR065c, on pRS315 (LEU2, CEN-ARS) was introduced into the mutant slt11 strain with a chromosomal deletion of SNR20 (left). The original 11nt sub. and a single substitution, G21C, of U2 snRNA were tested. YBR065c was able to complement the synthetic lethality (at 30°C on 5-FOA containing medium, right), and ts phenotype of slt11 (at 37°C in the presence of wt U2).
(-150 nts) at the ends of the three representative clones were determined and used to search Genbank. The results indicated that all three clones were located on the right arm of Chromosome II (Feldamm et al. 1994, Fig. 2.6A). Within the ~10 Kb overlapping fragment, six ORFs were present.

A mini-library method was devised to identify the complementing ORF. Clone #2 (Fig. 2.6A) was first digested with HindIII, PstI, SacI, BglII and XhoI to completion. The resulting restriction fragments were then ligated to pRS315 (LEU2, CEN-ARS) at respective sites, and transformed into E. coli. Transformants from an individual ligation were pooled, and total plasmid DNA was prepared ("mini-library", containing all the fragments of the original clone). The total plasmid DNA was then transformed into the slt11-1 cells, and transformants were selected for full complementation of the ts growth defect at 37°C. Positive clones were isolated from five "mini-libraries" (Fig. 2.6B). In the cases of HindIII, PstI and SacI libraries, the minimum complementing clones contained two restriction fragments religated in the correct orientation. By restriction analysis, the slt11-complementing ORF was determined to be YBR065c (Fig. 2.6B). When this ORF, on a ~2.0 Kb BglII fragment, was introduced into a yeast strain that contains slt11-1 and chromosomal disruption of U2 gene (Fig. 2.6C), it was able to complement fully the synthetic lethality of slt11-1 with U2-11nt and U2-G21C, and the ts growth defect of slt11-1 in the presence of wt U2. It was concluded that YBR065c is SLT11 (see Chapter 6 for additional data). Thus, SLT11 is a new splicing gene, and, when mutated, it blocks the first step of splicing reaction both in vivo (data not shown) and in vitro (Fig. 2.5A).

2.2.5. Molecular cloning of other SLT genes

i). SLT15 Eight slt15-1 complementing genomic clones were isolated, which fell into two types. Upon sequencing, both clones were localized on Chromosome IV in a region that contains PRP17 (kindly verified by Dr. U. Vijayraghavan) (Fig. 2.7A). It was evident that
Fig. 2.7.  

A. **SLT15** is identical to **PRP17**.  
(Upper) Restriction map of slt15-complementing clones. The two clones obtained (shown in gray lines on top) both contain **PRP17** gene in the overlapped region.  
(Lower) Amino acid sequence of Prp17p. The underlined amino acids indicate putative WD repeats.  

B. **SLT16** is identical to **SMD3**.  
(Upper) Restriction map of slt16-complementing genomic fragment. The minimal complementing clone, 0.9Kb EcoRI fragment (containing SMD3), is shown at bottom.  
(Lower) Amino acid sequence of Smd3p.
SLT15 is identical to PRP17, since the region shared by both complementing clones encompasses only PRP17. Furthermore, when slt15-1 strain was crossed to slu4-1 (prp17-2, Frank et al. 1992) strain, it failed to complement the ts growth defect (data not shown). In the original complementation test (Table 2.2), slt15 strain (YDX15) was crossed to prp strains and complementing diploids were selected at 37°C (assuming slt15 complements all prp mutants tested). However, only one diploid was obtained at 37°C, likely due to a suppressor(s). The new allele is named as prp17-100.

ii). **SLT16** Two identical slt16-1 complementing clones were isolated. Sequence analysis revealed that these two clones contain SMD3, in addition to two unknown ORFs, on Chromosome III (Fig. 2.7B). A 0.9Kb EcoRI fragment, containing only SMD3, was able to complement fully slt16-1. When slt16-1 strain was crossed to a SMD3 disruption strain (Roy et al. 1995b, courtesy of Dr. J. Woolford), the diploid remained ts in the absence of maintenance plasmid (data not shown). Thus, SLT16 is identical to SMD3. This mutation is the first ts allele of smd3, and is named as smd3-1.

iii). **SLT17** Four slt17-1 complementing clones were isolated, and all shared a large region, but with minor differences. A minimum complementing fragment was identified by the "mini-library" method. It contains two HindIII fragments with total size of ~6.4 Kb, and was found to contain SLU7 (Frank and Guthrie 1993) and two additional ORFs, upon sequencing (Fig. 2.8A). The complementing activity was further narrowed down to an 1.8Kb XbaI-EcoRI fragment containing only SLU7. When crossed to slu7-1 strains (Frank et al. 1992), slt17 failed to complement the ts phenotype at 37°C (Fig. 2.8C). Thus, SLT17 is identical to SLU7. The new allele is named as slu7-100.

iv). **SLT22** Only one complementing genomic clone was isolated. It contained YER172c and RAD3. SLT22 is identical to YER172c (Fig. 4.2A and §4.2.2). The same gene was
Fig. 2.8. **SLT17** is identical to **SLU7**. A. Restriction map of the region shared by slt17-complementing clones. Shown at bottom are results of complementation of slt17 with three subclones, +: full complementation, -: no complementation. B. Amino acid sequence of Slu7p. The underlined C and H residues indicate a zinc-knuckle motif that is also present in retroviral nucleocapsid protein implicated in RNA binding. C. Growth of slt17-1/slu7-1 diploid, two parental haploid and wt cells at 37°C on YPD. Note that slt17-1 and slu7-1 failed to complement each other.
also identified independently as BRR2 (Noble and Guthrie 1996), SNU246 (Lauber et al. 1996) and RSS1 (Lin and Rossi 1996).

The results of molecular cloning/characterization of six SLT genes, including, SLT21/PRP8, are summarized in Table 2.3.

2.2.6. Brief description of Slt factors and their roles in pre-mRNA splicing

Among six SLT genes identified in our genetic screen, two encode new splicing factors, SLT11 (Chapter 6) and SLT22 (Chapter 4), and both were characterized genetically and biochemically. In addition, the interaction between Slt21p/Prp8p and U2 snRNA and the role Slt21p/Prp8p in the formation of the active spliceosome were also investigated (Chapter 5). Genetic interactions among slt, slu, prp, U2, U5, and U6 mutations were analyzed and are described in Chapter 3. The following is a brief summary of functions of Slt factors in yeast pre-mRNA splicing.

i). Slt22p  
SLT22 encodes a large RNA dependent ATPase of 2,164 amino acids, or 246 kD. It is an essential gene. Slt22p contains RNA dependent ATPase motifs that are distantly related to previously identified DEAD or DEAH proteins involved in pre-mRNA splicing. The ATPase activity of Slt22p is associated with the U2/U6 intermolecular interactions, particularly helix II. This activity is required for the formation of the core-spliceosome (Chapter 4, and Xu et al. 1996).

ii). Slt21p/Prp8p  
Prp8p is the largest splicing factor identified so far; its function has been implicated in spliceosome assembly, recognition of both 5' and 3' splice sites, and the second step of splicing (see §5.1 for detail). The slt21 mutation (Q1902K) has been mapped to a region that has been implicated in recognition of the uridine tract which is important for 3' splice site selection (Umen and Guthrie 1996). The interaction between slt21/prp8-21 mutation
Table 2.3. Summary of SLT genes and proteins

<table>
<thead>
<tr>
<th>SLT</th>
<th>genes</th>
<th>proteins</th>
<th>splicing step(s)</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sequence</td>
<td>sizes (a.a.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>other names</td>
<td>motifs, homology and/or function</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>names</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLT11</td>
<td>YBR065c</td>
<td>364 Zn++-fingers, Rbl25 homology</td>
<td>1st step</td>
<td>Chapters 3, 6</td>
</tr>
<tr>
<td>SLT15</td>
<td>YDR364c PRP17/SLU4/CDC40</td>
<td>455 WD repeats</td>
<td>2nd step</td>
<td>1, 2, Chapter 3</td>
</tr>
<tr>
<td>SLT16</td>
<td>YLR147c SMD3</td>
<td>101 Sm core protein</td>
<td>snRNP biogenesis</td>
<td>3, Chapter 3</td>
</tr>
<tr>
<td>SLT17</td>
<td>YDR088c SLU7</td>
<td>382 Zn++-knuckle</td>
<td>2nd step</td>
<td>1, 2, 4, Chapter 3</td>
</tr>
<tr>
<td>SLT21</td>
<td>YHR165c PRP8/DBF3</td>
<td>2,413 proline-rich repeats</td>
<td>1st and 2nd steps</td>
<td>5, 6, 7, Chapter 5</td>
</tr>
<tr>
<td>SLT22</td>
<td>YER172c BRR2/SNU246/RSS1</td>
<td>2,163 RNA-dependent ATPase</td>
<td>1st step</td>
<td>8, 9, 10, 11, Chapter 4</td>
</tr>
</tbody>
</table>

References:
1. Jones et al. 1995;
7. Umen and Guthrie. 1995a, b.
9. Xu et al. 1996.
10. Lauber et al. 1996.
and U2 snRNA was characterized by genetic means. This interaction, related to 3' splice site selection, is important for activation of the spliceosome (Chapter 5).

iii). **Slt11p**  
**SLT11** encodes a protein of 364 amino acids or 41 kD. The gene is not essential for viability at ≤ 30°C, however, disruption of **SLT11** confers a ts growth defect at ≥ 33°C. Slt11p contains a zinc-binding motif in the N-terminal region and a central region with homology to yeast ribosomal protein L25, and is a putative RNA binding protein. It is required for the first step prior to catalysis. Genetic evidence indicates that Slt11p may act as an RNA chaperone to modulate and coordinate RNA and protein interactions in the active spliceosome that are required for both steps of the splicing reaction (Chapter 6).

The remaining three Slt factors have been characterized previously and their roles in splicing are summarized as following.

iv). **Slt15p/Prp17p**  
**PRP17** was first identified in a genetic screen for ts mutations that also affect pre-mRNA splicing (Vijayraghavan et al. 1989). Subsequently, it was identified as **SLU4** in another genetic screen for mutations that are synthetically lethal with U5 mutants (Frank et al. 1992). Prp17p contains WD repeats (Fig. 2.7A), which are also present in another splicing factor, Prp4p, known to be involved in spliceosome assembly (Hu et al. 1994; Ayadi et al. 1997). This factor is involved exclusively in the second step of splicing (Jones et al. 1995).

v). **Slt17p/Slu7p**  
Slu7p (Fig. 2.8B), containing a so-called zinc-knuckle, is also a second-step splicing factor (Frank and Guthrie 1992; Jones et al. 1995; Brys and Schwer 1996). Both slu4-1/prp17-2 and slu7-1 were isolated in a genetic screen for splicing mutants that are synthetically lethal with mutations in the invariant loop 1 of U5 snRNA (Frank et al. 1992), a region that has been implicated in the alignment of two exons important for the second step of splicing (O'Keefe et al. 1996). However, Slu7p and Prp17p exert their functions at different steps with respect to hydrolysis of ATP by Prp16p (Jones et al. 1995), a putative RNA helicase,
whose function has been implicated in remodeling of the spliceosome (Schwer and Guthrie 1992b) and the control of fidelity of the first step reaction (Burgess and Guthrie 1993a), both of which are required for the second step. The function of Slu7p has been implicated in the 3' splice site selection (Frank and Guthrie 1992; Brys and Schwer 1996). The protein is crosslinked to the 3' splice site (Umen and Guthrie 1995b).

vi). Slt16p/Smd3p The slt16 is the first ts allele of SMD3, which encodes a yeast core Sm protein (Fig. 2.8B) with homology to human Sm D3 (Roy et al. 1995b). The protein is associated with all five spliceosomal snRNAs and is thought to be involved in the biogenesis of snRNPs in yeast (Roy et al 1995b). In an extract depleted of Smd3, splicing is blocked prior to or at the first splicing step (Roy et al. 1995b). A similar in vivo defect was observed (Fig. 2.4, lanes 12 - 14). The human SmD protein has been shown to be cross-linked to the 5' splice site in a site-specific manner (Wyatt et al. 1992; MacMillan et al. 1995). To my knowledge, this is the first time that an Sm protein has been shown genetically to be important for function of a particular snRNA in splicing. It remains to be determined if the genetic interaction between Smd3p and U2 snRNA is direct (i.e., Smd3p affects the function of U2 snRNA) or indirect (i.e., it affects the stability of snRNAs).
§2.3. Discussion

The genetic screen described here has identified six yeast splicing factors which, when mutated, become synthetically lethal with mutations in stem I region of U2 snRNA. A similar approach was used to identify slu (Frank et al. 1992), mud (Liao et al. 1993) and msl (Tang et al. 1996; Abovich and Rosbash 1997) mutations that are related to the function of U5 snRNA, U1 snRNA, and Mud2p, respectively. The Mud and Msl factors are involved in early events of spliceosome formation (i.e., commitment complex) (Liao et al. 1993; Abovich et al. 1994; Colot et al. 1996; Abovich and Rosbash 1997; Berglund et al. 1997), while Slu4p/Prp17p and Slu7p are required for the second step (Frank and Guthrie 1992; Jones et al. 1995; Umen and Guthrie 1995b; Brys and Schwer 1996). My genetic screen of synthetic lethal mutations was designed to isolate yeast splicing factors important for the function of U2 stem I region. It was anticipated that it might yield factor(s) involved in the intermolecular U2/U6 interactions (also see §2.1). Indeed, one of the Slt factor, Slt22p, an RNA-dependent ATPase, has been demonstrated to be so (Xu et al. 1996 and Chapter 4). However, other factors identified are involved in events prior to the first step (Slt11p), or in the second step (Slt15p/Prp17p and Slt17p/Slu7p), or in both steps (Slt21p/Prp8p) (Tables 2.3). These findings indicate that the 5' end of U2 snRNA and its interactions with Slt factors and other components of the splicing machinery are important for both steps of the splicing reaction.

Yeast U2 snRNA is exceptionally large (1,175 nucleotides) due to the presence of a non-essential internal region (945 nt, Ares 1986; Igel and Ares 1988; Shuster and Guthrie 1988). However, it contains all the phylogenetically conserved secondary elements (Fig. 2.1A). During spliceosome assembly, the branchpoint site in pre-mRNA substrate is recognized by the branch interaction region (BP int.) of U2 snRNA (Parker, et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989). The adjacent stem II region can form two alternative structures, however, only one of them is active for binding of U2 snRNA to the pre-mRNA (Zavanelli and Ares 1991;
Zavanelli et al. 1994). Results of structural probing (Lamond et al. 1989) suggest that the 5' end (i.e., stem I) of human U2 snRNA undergoes conformational change (becoming more accessible) after it binds to the branchpoint in the pre-mRNA. Strengthening the bottom part of stem I by mutations can block effectively the splicing reaction in HeLa cell extract (Wu and Manley 1992). Apparently, the accessibility of stem I region is necessary for the subsequent steps in the spliceosome assembly; i.e., binding of U4/U6.U5 tri-snRNP (Lamond et al. 1989; Barabino et al. 1989). This is consistent with the idea that formation of U2/U6 helix II (between the 5' end of U2 and 3' end of U6) precedes other U2/U6 interactions and disruption of U4/U6 duplex (§1.3.2-1-b, and see §4.3.2).

The stem I region of U2 snRNA is involved in at least two alternative RNA-RNA interactions. The relative importance of the stem structure of the top part of stem I has been demonstrated experimentally in yeast (McPheeters and Abelson 1992). However, base-pairing of the bottom part of stem I is not essential in either yeast (McPheeters and Abelson 1992) or humans (Wu and Manley 1992). In human, the 5' half of stem I is involved in base-pairing interaction with the 3' end of U6 snRNA, forming helix II (Hausner et al. 1990; Datta and Weiner 1991; Wu and Manley 1991). On the contrary, the corresponding interaction in yeast is not essential (Fabrizio et al. 1989; Fabrizio and Abelson 1990; Madhani et al. 1990; Miraglia et al. 1991; McPheeters and Abelson 1992, Field and Friesen 1996), due to functional redundancy with another U2/U6 interaction, helix Ib (Field and Friesen 1996). The function of helix II interaction is largely unknown. It is not clear whether the formation of helix II is transient. Preliminary results suggest that the formation of this helix may serve to facilitate other RNA-RNA interactions (Brow and Vidaver 1995; also see §1.3.2-1-b and Fig. 1.5). The complex results of mutational analyses of stem I of both human (Wu and Manley 1992) and yeast (Miraglia et al. 1991; McPheeters and Abelson 1992) U2 snRNAs are consistent with the idea that this region may interact with different components of the spliceosome in the splicing cycle; i.e., the function of the 5' end of U2 snRNA is NOT solely restricted to interaction with U6 snRNA. The identification of six Slt factors is the first step to dissecting function of U2/U6
helix interaction and the 5' end of U2 snRNA in pre-mRNA splicing. Genetic studies presented in the following chapter indicate that the function of this region of U2 snRNA is linked to a network of RNA-RNA, RNA-protein and protein-protein interactions important for both steps of the splicing reaction.
S2.4. Material and Methods

2.4.1. Yeast strains and constructs

All the yeast strains used in this study were derived from W303-1A or 1B (Mata or \(M\alpha\) ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100). The original strain used in the genetic screen, containing a chromosomal deletion of \(SNR20\) (the U2 snRNA gene), was described in Field and Friesen (1996). A new \(\Delta SNR20\) disruption was made by deleting the \(Clal-HpaI\) region of \(~810\) nt containing the 5' end half of the U2 gene (\(~750\) nt), replaced with the yeast HIS3 gene. The wt \(SNR20\) gene was carried on pRS316 (URA3 CENARS). Two resultant haploids, YDX2299A (Mata) and YDX22100A (Mata), were used in the subsequent genetic experiments. Plasmids (TRP1 CEN-ARE) carrying \(SNR20\) and \(snr20-11nt\), pDF87 and pDF205, were provided by D. Field.

2.4.2. Genetic manipulation of yeast

Procedures for growth of yeast culture, mating, complementation test, sporulation, tetrad dissection and construction of chromosomal disruption are described in Guthrie and Fink (1990).

2.4.3. EMS mutagenesis and genetic screen

In the initial genetic screen, a yeast strain with a chromosomal deletion of \(SNR20\) (Field and Friesen, 1996) containing \(SNR20\) and \(snr20-11nt\) carried on URA3 and TRP1 plasmids, respectively, was subject to ethyl methanesulfonate (EMS) mutagenesis (Lawrence 1991) at the survival rate of 10-20%. The surviving cells were screened for sensitivity to 5-fluoroorotic acid.
(5-FOA) at 30°C, an indication of dependence of viability on SNR20 (on a URA3 plasmid), which reflects the lethality generated by snr20-11nt in combination with extragenic mutations (i.e., synthetic lethality) (Fig. 2.1B). In order to eliminate artifacts (e.g., mutations affecting uracil pathway), the snr20-11nt/TRP1 plasmids of 5-FOA sensitive cells were replaced by SNR20. Those of the resultant cells that were sensitive to 5-FOA were discarded. A scheme was designed for genetic characterization to ensure that the slt mutants were phenotypically and genetically satisfactory (see Fig. 2.2 and §2.2.2). Briefly, the ts phenotype of each original slt strain was first segregated into chromosomal SNR20 background by crossing to the wild-type strain (W303-1A). The resulting ts haploid was then back-crossed to the wt strain at least three times to determine if the ts phenotype was due to mutation at single locus. The final ts haploid was crossed to the newly constructed SNR20 deletion strain (YDX2299A or YDX22100A). Synthetic lethality was then tested in strains containing both ts and ΔSNR20 derived from the above cross.

2.4.4. RNA isolation and primer extension.

All slt mutant strains used for RNA analysis contained SNR20 on the chromosome, which were obtained following the genetic characterization (in after step e in Fig. 2.2). Total yeast RNA isolation and primer extension were performed as described by Hu et al. (1994).

2.4.5. Preparation of whole cell splicing extracts and in vitro splicing assay

Yeast whole cell extracts (WCEs) were prepared from wt and slt cells according to Lin et al. (1985) with modifications. Substrate, 32P-labeled yeast pre-actin RNA, was synthesized by runoff transcription with T7 RNA polymerase and α-32P-UTP as described (Lin et al. 1985). For in vitro splicing assays, equal volumes (10μl) of WCE and buffer component, containing
labeled substrate, were mixed and incubated at the temperatures indicated. Splicing intermediates and products are resolved in 5% polyacrylamide gels.

2.4.6. Cloning by complementation

To clone wild-type SLT genes, a Ycp50-born yeast genomic DNA library, CENBANK A (Rose et al. 1987), was introduced to slt cells and the transformants were selected for temperature resistance at 37°C (Rose and Broach 1991). Plasmids containing complementing genomic DNA fragments were recovered from the positives. A "mini-library" approach was devised to narrow down the minimal complementing regions: The original complementing plasmid was first digested with restriction enzymes to completion, and the resulting fragments were ligated to another vector(s) with a different selective marker(s). Total plasmid DNA prepared from these mini-libraries was transformed into the slt strains and selected for full complementation. The overlapping region in plasmids rescued from different mini-libraries should contain slt-complementing ORF(s). Nucleotide sequences of both ends of these fragments were determined and then used to search Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/) for a match. DNA sequences of complementing regions were retrieved and, fragments containing only single ORFs were tested for complementation of both growth defect(s) and synthetic lethality.
Chapter 3.

Synthetic Lethality of Yeast *slt* Mutations with U2 snRNA Mutations Suggests Functional Interactions between U2 and U5 snRNPs that Are Important for Both Steps of Pre-mRNA Splicing.


D.J.F. initiated the genetic screen and provided the original yeast strain of ΔSNR20 and mutant U2 and U6 constructs. S.-J. T. contributed to molecular cloning of *SLT11* and *SLT22*, and genetic analysis of *slt*, *prp* and *slu* mutations, A.M., *SLT15* and *SLT16*, B.P.B., *SLT17*. 
Abstract

Interactions among slt and selected prp and slu mutations, U2, U5 and U6 snRNAs were analyzed by genetic means in order to determine the function of the 5'-end of U2 snRNA and its interactions with other factors. While slt11-1 and slt22-1 are synthetically lethal with mutations in the 3'-end of U6 snRNA that affect U2/U6 helix II interaction, slt17/slu7-100 and slt21/prp8-21 showed no synthetic lethality with these U6 mutations, suggesting that the 5'-end of U2 snRNA may interact specifically with other splicing factors (e.g., Slt17p/Slu7p, Slt21p/Prp8p) in addition to U6 snRNA. Mutations in loop 1 of U5 snRNA, a region implicated in the tethering of the two exons for the second step of splicing, are synthetically lethal with certain mutations in U2 snRNA. Specific substitutions at position 21 of U2 snRNA suppress U98A and U97C/U99C of U5 snRNA. These results suggest a possible role for the 5' end of U2 snRNA in the second step of splicing. Four slt mutations, slt11-1, slt15/prp17-100, slt17/slu7-100, and slt21/prp8-21, are also synthetically lethal with mutations in loop 1 of U5 snRNA. Furthermore, synthetic lethality analyses reveal that Slt11p, Slt15p/Prp17p, Slt17p/Slu7p, Slt21p/Prp8p and Prp16p may act as two overlapping functional units. These results, taken together, suggest that interactions among U2 snRNA, U5 snRNA and Slt factors may be responsible for the coupling and coordination of the two-step reaction of pre-mRNA splicing.
§3.1. Introduction

During the process of spliceosome assembly, spliceosomal small nuclear RNAs (snRNAs) and the pre-mRNA substrate, in association with protein factors, undergo extensive conformational changes to establish a network of RNA-RNA interactions that are important for both steps of the splicing reaction (§1.3). Protein factors are essential for the formation and maintenance of these RNA-RNA interactions in the spliceosome (§1.2). With few exceptions (Parker and Siliciano 1993; Madhani and Guthrie 1994b; McPheeters 1996), Watson-Crick RNA base-pairing is important for recognition of the splice sites and other RNA-RNA interactions in the spliceosome (for a review, see Madhani and Guthrie 1994a). During maturation of the spliceosome, intermolecular base-pairing interactions between U2 and U6 snRNAs, forming two helices, I (divided into Ia and Ib) and II (Fig. 1.6), play central roles in the formation of RNA structures important for the catalytic events. In particular, formation of U2/U6 helix I (Madhani and Guthrie 1992; 1994b) brings the 5' splice site (through U6/5' splice site interaction) and the branchpoint site (through U2/branchpoint interaction) into close proximity and perhaps juxtaposes the two sites for the first-step reaction (see §1.3.2.3 for detail). On the other hand, U2/U6 helix II, demonstrated in the mammalian spliceosome, is not essential in the yeast (see §2.1). The helix II part of human U6 snRNA has been shown to influence dissociation of the U4/U6 duplex in vitro (Brow and Vidaver 1995). Indirect experiments suggested that formation of U2/U6 helix II may stabilize the U4/U6 duplex (Brow and Vidaver 1995). If so, the formation (and the subsequent disruption) of U2/U6 helix II interaction may represent a crucial regulatory event in the initiation of the splicing reaction. With the identification of six Slt factors (Chapter 2), it became possible to explore the functions of U2/U6 helix II and the 5'-end of U2 snRNA in the splicing pathway.

Three of the factors identified in the genetic screen, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8p are functionally linked to the highly conserved loop 1 region of U5 snRNA (Frank et al. 1992; Teigelkamp et al. 1995), which is important for the juxtaposition of the two exons
for the second step of splicing (O'Keefe et al. 1996). Two of them, Slt17p/Slu7p (Frank and Guthrie 1992; Brys and Schwer 1996) and Slt21p/Prp8p (Umen and Guthrie 1995b, 1995c, 1996), are required for 3'-splice site selection/recognition. Since canonical base-pairing is not obviously involved in the U5/exon interaction, these protein factors may also act to establish and maintain the fragile interaction between U5 loop 1 and the two exons (Teigelkamp et al. 1995). These results suggest a potential role for U2/U6 helix II and/or the 5'-end of U2 snRNA in the second step of the splicing reaction. In fact, a functional role for U2 snRNA in the alignment of the two exons has been suggested by site-specific crosslinking between the first nucleotide of the 3'-exon and the U23, A30 nucleotides of U2 snRNA (Newman et al. 1995).

In an attempt to determine and differentiate the functions of U2/U6 helix II and the 5' end of U2 snRNA, a series of genetic analyses were performed with six slt mutation isolated in this screen and a few selected prp, slu mutations. A number of mutations in U2, U5 and U6 snRNAs were also tested for genetic interactions with each other and with slt mutations. The results presented in this chapter suggest that Slt22p, an RNA-dependent ATPase, is involved in U2/U6 helix II interaction (also see Chapter 4), and that the functions of the 5'-end of U2 snRNA and some Slt factors may overlap that of loop 1 of U5 snRNA in the alignment of the two exons required for the second step. Furthermore, the interactions among U2, U5 and Slt factors may be responsible for the coupling and coordination of the two-step reaction of pre-mRNA splicing.
§3.2. Results

3.2.1. Genetic interactions of slt, slu and prp mutations with U2 snRNA.

Functions of the six Slt factors identified in the synthetic lethal genetic screen are summarized in Table 2.3. It is worth noting that the 5'-end of U2 snRNA is related genetically to several components/functions of the splicing mechanism, particularly, those involved in the second step. As one approach to understanding how these Slt factors are involved in the function of U2 snRNA, their genetic interactions with mutations in U2 snRNA were examined. Three sets of U2 snRNA mutations were included in this genetic analysis. They lie in regions that are involved in U2/U6 snRNA interactions: helix Ia (G26A and A27C), helix Ib (substitutions at G21) and helix II (11nt and 9nt substitutions) (Fig. 3.1A and B). In addition to the six slt mutations identified in this study, four slu (synthetic lethal with U5, Frank et al. 1992) and four prp mutations were also included (Table 3.1).

A spectrum of allele-specificity was observed with six slt mutations. While slt21/prp8-21 and slt22-1 showed allele-specific synthetic lethality with only three U2 mutations, 11nt and 9nt substitutions, and a single substitution of G21C, the other four slt mutants were synthetically lethal with mutations in three regions of U2 snRNA (see Table 3.1 for detail). Three slu mutants, slu4, slu5 and slu7, were also synthetically lethal with mutations in U2 snRNA. However, other slu and prp mutants (except prp16-1) showed no genetic interaction with U2 mutations tested. Thus, the synthetic lethal interactions with mutations in U2 snRNA are specific to slt and some slu mutants. The lack of allele specificity with respect to mutations in U2 snRNA for most of slts and slus may indicate that a large portion of the 5'-end of U2 snRNA (in combination with U6 snRNA, in some cases, see below), rather than individual structural elements, is involved in interactions with these Slt and Slu factors. In contrast, among the four prp mutations tested, only the prp16-1 showed allele-specific synthetic lethality with one single substitution, G26A, in the U2 part of U2/U6 helix Ia interaction (Table 3.1). Another allele-specific genetic interaction between prp16-203 and helix Ia mutations in U6.
Fig. 3.1. A. Stem I region of yeast U2 snRNA (left) and U2/U6 interactions (right). Dots indicate nucleotides in the stem I region which are involved in intermolecular U2/U6 interactions, forming helices Ia, Ib and II. The 11nt substitution (11nt sub.) used in the genetic screen and other U2 mutations used to test allele-specificity are shown under U2 snRNA in the context of U2/U6 interactions. B. Growth phenotypes of U2 mutations shown in A. These U2 snRNA mutations were tested in YDX2299A.
### Table 3.1. Allele specificity of synthetic lethality of slt, slu and prp mutants with U2 snRNA mutations.

<table>
<thead>
<tr>
<th>U2 snRNA mutations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>helix Ia</th>
<th>helix Ib</th>
<th>helix II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A27C</td>
<td>G26A</td>
<td>G21A</td>
</tr>
<tr>
<td>slt mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>slt11-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>--</td>
<td>-</td>
</tr>
<tr>
<td>slt15/prp17-100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>slt16/smd3-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>slt17/slu7-100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>slt21/prp8-21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slt22-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>slu mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>slu2-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slu3-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slu4/prp17-2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>slu5-1</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>slu7-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>prp mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prp2-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp16-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>prp28-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prp28-102&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Notes:**

- Helices Ia, Ib and II indicate the U2 snRNA parts of the intramolecular interactions between U2 and U6 snRNAs. Also see Fig. 3.1A for locations of these mutation.
- Yeast strains carrying slt, slu or prp mutation and SNR20Δ::HIS3 were transformed with plasmids carrying wt or mutant U2 snRNA genes, and the resultant transformants were then grown on medium containing 5-FOA at 30°C (if the slu or prp mutant in question is viable) and/or 25°C, to test for viability of cells containing both mutations. --: no growth of double-mutant strains, i.e., synthetic-lethal at 25°C and 30°C. -: no growth of double-mutant strains at 30°C, but poor growth at 25°C (≥4 days). +: no additive growth defects of double-mutations.
- Mutations block splicing prior to the first step.
- Required for the second step.
- Required for both steps of the splicing.
snRNA (i.e., U57C and U57A, synthetic lethality and weak suppression, respectively) has been documented (McPheeters 1996). Likely, these results underscore the specific interaction between Prp16p and U2/U6 helix Ia.

The four prp mutations correspond to three RNA-dependent ATPases involved in events which occur during the formation of the holo-spliceosome (Prp28p), concomitantly with spliceosomal activation (Prp2p), or in the second step (Prp16p) (see Table 1.2). With the exception of only one prp16-1 interaction, none of these mutations showed synthetic lethality. These results provided genetic evidence that the RNA conformational rearrangements that are affected by the original U2 mutation (11nt substitution) is specific to Slt22p (also see below).

3.2.2. Genetic interactions between U2/U6 helix II and slt mutations.

Since the original 11nt substitution of U2 snRNA used in the genetic screen could perturb the proposed U2/U6 helix II interaction, two related questions arise: 1) whether slt mutations interact functionally with helix II; and 2) whether Slt factors play redundant roles with helix II, as it has been shown to be so with U2/U6 helix Ib (Field and Friesen 1996). Consequently, yeast strains containing slt mutations in combination of double deletions of SNR20 and SNR6 were constructed (Fig. 3.2A). They were used to determine whether mutations in the U6 part of helix II (Field and Friesen 1996; also see Fig. 3.2B) were synthetically lethal with slts, and whether partial restoration of mutationally disrupted helix II could suppress synthetic lethality that is observed with the original slt mutations with U2-11nt or -9nt substitution. Since a corresponding 9nt substitution in U6 confers lethality by itself (Field and Friesen 1996), four 3nt substitutions (U6-a, -b, -c and -d, Fig. 3.2B) were selected. Four slt mutations were tested in these experiments. Two mutations, slt11-1 and slt22-1, represent new splicing factors (both are required for the first step), while the other two (Slt17p/Slu7p and Slt21p/Prp8p) are involved in the 3’ splice site selection (Frank and Guthrie 1992; Jones et al. 1995; Brys and Schwer 1996; Teigelkamp et al. 1995; Umen and Guthrie 1995b, 1995c).
Fig. 3.2. Genetic interaction between *slt* mutations and U2/U6 helix II. A. A representative yeast strain used in the genetic test for synthetic lethality. B. U2 and U6 mutations used in the genetic test. U6 mutations and their growth phenotypes in the *SLT* and *SNR20* background are described in Field and Friesen (1996). C. Results of two-day growth at 30°C of four *slt* strains containing various combinations of U2 and U6 mutations. Mutant U2 (*TRP1*-marked) and U6 (*LEU2*-marked) plasmids were transformed into yeast strains shown in A. The resultant transformants were grown on 5-FOA-containing selective medium. Note that *slt22-1* mutation confers slow-growth at 30°C.
Consistent with the results that the RNA-dependent ATPase activity of Slt22p is related to U2/U6 helix II (Xu et al. 1996, Chapter 4), slt22-1 showed synthetic lethality with three U6 mutations (b, c and d) that partially disrupt helix II interaction (Fig. 3.2C), suggesting that defects of slt22-1 are exacerbated in vivo by a disrupted helix II caused by mutation in U6. 

slt11-1, corresponding to a putative RNA binding protein (Chapter 6), showed same synthetic lethal interactions with these three U6 mutations (Fig. 3.2C, see §6.3.1 for an explanation). None of these synthetic-lethal interactions was suppressed by partial restoration of helix II through inclusion of the U2-11nt or -9nt substitution (Fig. 3.2C). For slt22-1, this suggests that the helix structures formed by the U6-b, -c or -d mutation with U2-11nt or -9nt substitution are not efficient substrates for the mutant protein.

Neither slt17/slu7-100 or slt21/prp8-21 was synthetically lethal with any of the U6 mutations tested (Fig. 3.2C). Furthermore, synthetic lethality of either slt mutation with 11nt or 9nt substitution was not suppressed by any of the four U6 mutations, suggesting that these two factors are not involved in direct interactions with U2/U6 helix II. These results also indicate that none of the Slt factors tested plays a redundant role with U2/U6 helix II. The asymmetric synthetic lethality associated with slt17/slu7-100 and slt21/prp8-21 provides evidence that the functions of two corresponding proteins are linked specifically to the 5'-end of U2 snRNA, which may interact with other components, in addition to the 3'-end of U6 snRNA, in the spliceosome.

3.2.3. Genetic interactions between U2 and U5 snRNAs.

An intriguing possibility was suggested by three Slt factors identified in the screen. Since Slt15p/Prp17p and Slt17p/Slu7p are linked genetically to loop 1 of U5 snRNA (Frank et al. 1992), and Slt21p/Prp8p is involved in stabilization of exon alignment by U5 snRNA (Teigelkamp et al. 1995) and the 3' splice site selection (Umen and Guthrie 1995b, 1995c), it is possible that the 5'-end of U2 snRNA is related to the function of loop 1 of U5 snRNA. I
explored this possibility by genetic means. It was reasoned that if the 5'-end of U2 snRNA is important for the function of U5, one might observe genetic interactions between these two regions of U2 and U5 snRNAs. The two U5 snRNA mutations used in the genetic screen for slu mutants (Frank et al. 1992), U98A and U97C/U99C, and a series of U2 snRNA mutations were tested for genetic interaction, using a yeast strain shown in Fig. 3.3A.

The original 11nt or 9nt substitutions of U2 snRNA showed no synthetic lethality with either U5 mutation (Table 3.2). However, all mutations at C22 and U23 positions of U2 snRNA were synthetically lethal or debilitating with either of the U5 mutations tested, even though these U2 mutations themselves conferred little or no growth defect at these temperatures (Table 3.2). It is worth noting that U23 of U2 snRNA is in close contact with the first nucleotide of the 3'-exon (Fig. 3.3C), which is tethered to the 5'-exon through interactions with U5 loop 1 after the first step (Newman et al. 1995). Mutations at the adjacent G21 position showed mixed genetic interactions: at 25°C, all three substitutions of G21 in U2 snRNA suppressed the slow growth conferred by U5-U98A mutation. At 30°C, two G21 substitutions (G21A, G21U) had little effect while G21C was synthetically lethal with this U5 mutation. On the other hand, only U2-G21A substitution suppressed U5-U97C/U99C double mutations at 25°C and 30°C, while G21C was synthetically lethal at both temperatures (Fig. 3.3B). G26A and A27C, the only two viable substitutions in the U2 part of U2/U6 helix Ia, a region that is important for both steps of splicing (Fabrizio et al. 1990; McPheeters and Abelson 1992), were synthetically lethal with both U5 mutations (Table 3.2). No synthetic lethality was observed with mutations at other positions in U2 snRNA (e.g. substitutions G20C, U19G, C14G and C14A, data not shown).

These results demonstrate allele-specific genetic suppression of two U5 loop 1 mutations by substitution(s) at the G21 position of U2 snRNA and synthetic lethality of both U5 mutations with a broad range of mutations in a particular region of U2 snRNA (Fig. 3.3C and Table 3.2). However, neither U5 mutation showed synthetic lethality with 11nt and 9nt substitutions at the 5'-end of U2 snRNA, suggesting that the corresponding region in U2 snRNA
Fig. 3.3. Genetic interactions (synthetic lethality and suppression) between substitutions at the G21 position in U2 snRNA and loop 1 mutations of U5 snRNA. A. Yeast strain containing double-deletions of U2 and U5 genes. Mutant U2 (TRP1-marked) and U5 (LEU2-marked) plasmids were transformed into this strain to test for genetic interactions. B. Four-day growth at 25°C and 30°C of U5 snRNA-wt, -U98A, -U97C/U99C in combination with U2 snRNA-wt, -G21A, -G21C, -G21U in the absence of maintenance plasmid. Note that U2-G21C is synthetically lethal with U5-U97C/U99C. C. Summary of genetic interactions between U2/U5 snRNAs and location of U2 and U5 mutations in context of other RNA-RNA interactions (following the first splicing step) in the spliceosome. Positions (97, 98 and 99) in loop 1 that correspond to two U5 mutations are highlighted. Filled circles indicate mutations at these positions of U2 that were synthetically lethal with both U5 mutation (also see Table 3.2 for summary), open circles ones that are not. The highlighted G21 position in U2 indicates that mutations at this position were able to suppress the two U5 mutations. The helix II portion of U2 snRNA is shown by shade (see §3.3.1 and §4.3.1 for unwinding of helix II). Lines between U23, A30 of U2 and exon 2 indicate site-specific crosslinking (Newman et al 1995).
Table 3.2 Growth phenotype of U2 and U5 double mutant strains.

<table>
<thead>
<tr>
<th>U2 region or nucleotide mutation</th>
<th>Growth phenotype of double mutant containing indicated U5 mutationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U2</td>
</tr>
<tr>
<td>WT</td>
<td>+++</td>
</tr>
<tr>
<td>helix II 11nt sub. 9nt sub.</td>
<td>+++c</td>
</tr>
<tr>
<td>helix II 11nt sub. 9nt sub.</td>
<td>+++c</td>
</tr>
<tr>
<td>G21b</td>
<td>+++c</td>
</tr>
<tr>
<td>G21C</td>
<td>+++c</td>
</tr>
<tr>
<td>G21U</td>
<td>+++c</td>
</tr>
<tr>
<td>C22</td>
<td>+++</td>
</tr>
<tr>
<td>C22G</td>
<td>+++</td>
</tr>
<tr>
<td>C22U</td>
<td>+++</td>
</tr>
<tr>
<td>U23</td>
<td>+++</td>
</tr>
<tr>
<td>U23G</td>
<td>+++</td>
</tr>
<tr>
<td>U23C</td>
<td>+++</td>
</tr>
<tr>
<td>G26</td>
<td>++c</td>
</tr>
<tr>
<td>A27</td>
<td>+++c</td>
</tr>
</tbody>
</table>

a. Four-day growth at temperatures indicated. ++++, indistinguishable from the growth of the wt; +/-, pinpoint colonies; -, no growth; ND, not determined.

b. See Fig. 3.3C for growth phenotypes in combination with U5 mutations.

c. See Fig. 3.1B for growth phenotypes at 30 and 33°C.
is not directly involved in interaction with U5 snRNA. Although crosslinking experiments have shown that this region and helix Ia are in close contact with exon 2 (Newman et al. 1995), there is no evidence for a direct RNA-RNA interaction between loop 1 of U5 snRNA and nucleotides 21, 22 and 23 of U2 snRNA. However the genetic results presented here suggest that this region of U2 snRNA, in an unidentified form, may be involved the alignment of the two exons (and thus overlaps the function of loop 1 of U5 snRNA) for the second step of the splicing reaction (see §5.3 for further discussion). This notion is consistent with the finding that two Slt factors, Slt17p/Slu7p and Slt21p/Prp8, are involved in 3' splice site selection (Frank and Guthrie 1992; Jones et al. 1995; Brys and Schwer 1996; Teigelkamp et al. 1995; Umen and Guthrie 1995b, 1995c).

3.2.4. Genetic interactions among slt and U5 snRNA mutations.

I then addressed the question of whether slt mutations were also synthetically lethal with the two U5 mutations mentioned above, since alleles of slt15 and slt17 were identified originally as being synthetically lethal with U5 mutations (Frank et al. 1992). Furthermore, since the tethering function of U5 loop 1 occurs after the first splicing step (O'Keefe et al. 1996), it is interesting to determine the effect of slt mutations on U5 mutations that block first step splicing (i.e., slt11-1, slt21/prp8-21 and slt22-1). Using yeast strains carrying slt mutations in the background of ΔSNR7 (Fig. 3.4A), I tested synthetic lethality of five slt mutations, slt11-1, slt15/prp17-100, slt17/slu7-100, slt21/prp8-21 and slt22-1, with both U5 mutations.

Among the three first-step mutations, slt11-1 showed synthetic lethality with both U5 mutations at 25°C and 30°C (Fig. 3.4B and data not shown). The slt21/prp8-21 mutation, on the other hand, showed lethality with the U5-U97C/U99C mutations at both temperatures (Fig. 3.4B and data not shown). Weak synthetic lethality was observed between slt21/prp8-21 and U5-U98A (Fig. 3.4B). However, slt22-1 showed some growth with the two U5 mutations.
Fig. 3.4. Synthetic lethality of *slt* mutations with loop 1 mutations of U5 snRNA. A. left: Yeast strains carrying *slt* mutations and a chromosomal deletion of the U5 snRNA gene (*SNR7*Δ:*HIS3*), with *SNR7* on a *URA3*, *CEN-ARS* plasmid, were transformed with wt and mutant U5 plasmids (*LEU2*, *CEN-ARS*). right: The structure of yeast U5 snRNA with loop 1 region shown in detail. The highlighted residues are mutated in the two mutations used in original genetic screen for *slu* mutations (Frank et al. 1992) and tested in B for synthetic lethality with *slt* mutants. B. Results of five-day growth at 30°C of the resultant transformants on medium containing 5-FOA. Note that *slt22-1* and U5 snRNA double mutants grew significantly slower than strains carrying either mutation alone.
at both temperatures (Fig. 3.4B and data not shown). Although the original slu4/prp17-2 and slu7-1 were synthetically lethal with substitution U98A in U5 snRNA (Frank et al. 1992), it was found that slt15/prp17-100 was lethal with both U98A and U97C/U99C substitutions, while slt17/slu7-100 was lethal with U97C/U99C only (data not shown).

Thus, four slt mutations identified on the basis of synthetic lethality with U2 mutation were linked genetically to the function of loop 1 of U5 snRNA. Although the tethering function of U5 loop 1 is only required for the second step of the splicing reaction (O'Keefe et al. 1996), my genetic analyses showed that mutations in loop 1 are synthetically lethal with two slt mutations (slt11-1 and slt21/prp8-21) that affect the first step. It is thus possible that these Slt factors are involved in the functions of both U2 and U5 snRNAs, and in the potential interactions between them which are important for both steps of splicing.

3.2.5. Genetic interactions among slt and slu mutations.

The aforementioned possibility prompted me to investigate genetic interactions among slt and other splicing mutations. If Slt and other splicing factors act coherently as a group, mutations in these factors could exacerbate each other. I assessed this possibility by testing pair-wise synthetic lethality of slt, as well as selected slu and prp mutations. This was done by attempting to create double-mutant haploids (Fig. 3.5 and §3.3.2). Failure to obtain such haploids indicates that the two mutations in question are synthetically lethal. Results of these experiments are summarized in Tables 3.3 and 3.4, and Fig. 3.6.

Strong pair-wise synthetic lethality was observed with following mutations, slt11-1 (first step mutation), slt15/prp17-100, slu4/prp17-2, slt17/slu7-100, slu7-1, and prp16-1 (all second step mutations). Two members of this group, slt17/slu7-100 and prp16-1, also showed weak but significant synthetic lethality with slt21/prp8-21 (the corresponding factor is required for both steps, also see Fig. 5.7). Although not identified in our genetic screen, prp16-1 is
Fig. 3.5. Genetic interactions among \textit{slt11-1, slt17/slu7-100} and \textit{slt22-1}. A. Results of tetrad dissection of heterozygous diploids. Top. Tetrads containing spores with double mutations that are viable; i.e., the two mutations are NOT synthetically lethal. Shown is the tetrad dissection result of \textit{slt11-1/SLT11 SLT22/slt22-1} diploid, grown on YPD for 3 days at 30°C (showing slow-growth phenotype of \textit{slt22-1} cells). Note that a large portion of the tetrads contain four viable spores and that all \textit{slt22-1}-containing spores, some of which may also harbor \textit{slt11-1} mutation, give rise to homogenous colonies; i.e. the two mutations show no additive growth defect. Bottom. Tetrads containing spores with double mutations that are inviable. Shown is the tetrad dissection result of \textit{slt11-1/SLT11 SLT17/slt17} diploid, grown on YPD for 2 days at 30°C. Note that only a small portion of tetrads contain four viable spores (parental ditype, DP), whereas most tetrads contain only three or two viable spores (mostly tetratype, T, and non-parental ditype, NPD). B. Schematic representation of three type of tetrads obtained from the \textit{slt11-1/SLT11 SLT17/slt17} diploid. Spores that inherit both mutations, inviable in this case, in T and NPD are shown in dashed circles. Detailed results of these tetrad analyses are summarized in Table 3.3.
Chapter 3

A

\( \text{slt11} \times \text{slt22} \)

\( \text{slt11} \times \text{slt17} \)

B

\( \text{slt11} \times \text{slt17} \)

\( \text{PD} \)

\( \text{T} \)

\( \text{NPD} \)

\( \text{SLT11} \)

\( \text{slt11} \)

\( \text{SLT17} \)

\( \text{slt17} \)
Table 3.3. Summary of tetrad dissection analysis

<table>
<thead>
<tr>
<th>Tetrad types&lt;sup&gt;d&lt;/sup&gt;</th>
<th>slt&lt;sub&gt;11&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;11&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;11&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;11&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;15&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;15&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;17&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;17&lt;/sub&gt; x</th>
<th>slt&lt;sub&gt;17&lt;/sub&gt; x</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>slt&lt;sub&gt;15&lt;/sub&gt;</td>
<td>slu&lt;sub&gt;4&lt;/sub&gt;</td>
<td>slt&lt;sub&gt;17&lt;/sub&gt;</td>
<td>slu&lt;sub&gt;7&lt;/sub&gt;</td>
<td>prp&lt;sub&gt;16&lt;/sub&gt;</td>
<td>slt&lt;sub&gt;17&lt;/sub&gt;</td>
<td>slu&lt;sub&gt;7&lt;/sub&gt;</td>
<td>prp&lt;sub&gt;16&lt;/sub&gt;</td>
<td>slu&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>4 (2ts:2ts)</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3 (2ts:1ts)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (1wt:2ts:1ts)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 (1wt:2ts)</td>
<td>27</td>
<td>25</td>
<td>39</td>
<td>23</td>
<td>17</td>
<td>23</td>
<td>19</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>NPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (2wt:2ts)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 (2wt:1ts)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 (2wt)</td>
<td>5</td>
<td>8</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>total #</td>
<td>38</td>
<td>40</td>
<td>58</td>
<td>35</td>
<td>26</td>
<td>34</td>
<td>29</td>
<td>16</td>
<td>22</td>
</tr>
</tbody>
</table>

Notes:

<sup>a</sup> The three types of tetrads were determined as follows: (i) the PD tetrads contained four or three viable spores, all with the original temperature sensitivity (ts) phenotype; (ii) the T tetrads contained four viable with only one with the wt phenotype (the one with double mutants [ts]) or three viable spores with only one with the wt phenotype and two with the original temperature sensitivity phenotype; and (iii) the NPD tetrads contained four viable spores with two with the wt and two with the temperature sensitivity phenotype (double mutants, ts), three viable spores with two with the wt and one with the temperature sensitivity phenotype (double mutants, ts), or two viable wt spores.

<sup>b</sup> slt15 is prp17-100, slt16 is smd3-1, slt17 is slu7-100, and slu4 is prp17-2. The slt11-1 mutation (underlined) affects the first step of splicing, while the other mutations affect the second step of splicing. Results of tetrad dissection of heterozygous diploids that generate viable double-mutations are not shown but are summarized in Table 3.4.
nevertheless synthetically lethal with one particular mutation, G26A, in U2 snRNA (Table 3.1), and four \textit{slt} mutations (Fig. 3.6). All other combinations tested showed no significant synthetic lethality.

I suggest that Slt11p, Slt15p/Prp17p, Slt17p/Slu7p, Prp16 and Slt21p/Prp8p form two overlapping functional units (Fig. 3.6). The genetic interactions described above also suggest that regulation of RNA/RNA interactions in the spliceosome is achieved collectively through the action of a number of protein factors which act as functional units. The fact that these factors affect both splicing steps might indicate a hitherto-unknown connection between the two steps of the pre-mRNA splicing reaction.
Table 3.4. Summary of synthetic lethality among slt, slu and prp mutants

<table>
<thead>
<tr>
<th>Mutations</th>
<th>slt11&lt;sup&gt;b&lt;/sup&gt;</th>
<th>slt15&lt;sup&gt;c&lt;/sup&gt;</th>
<th>slt16&lt;sup&gt;b&lt;/sup&gt;</th>
<th>slt17&lt;sup&gt;c&lt;/sup&gt;</th>
<th>slt21&lt;sup&gt;d&lt;/sup&gt;</th>
<th>slt22&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>slt11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>slt15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>+/e</td>
<td>+</td>
</tr>
<tr>
<td>slt16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slt17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slt21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slt22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slu4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>slu7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NA</td>
<td>+/e</td>
<td>+</td>
</tr>
<tr>
<td>prp16-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>+/</td>
<td>+/</td>
</tr>
<tr>
<td>prp2-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Notes:

a. slt15 is prp17-100, slt16 is smd3-1, slt17 is slu7-100, slu4 is prp17-2, and slt21 is prp8-21. Symbols: +, the double mutant is viable and without additive defect; -, the double mutant is lethal (i.e., it has synthetic lethality); +/-, the double mutant is viable but confers a severe growth defect (slow growth at 25°C, i.e., it has partial synthetic lethality); and *, also see Frank et al. 1992. NA, not applicable; ND, not determined.

b. Mutations block splicing prior to the first step.

c. Required for the second step.

d. Required for both steps.

e. Also see Fig. 5.7.
§3.3. Discussion

Among the six Slt factors identified in the genetic screen, Slt22p is likely to be involved in U2/U6 helix II interactions (Xu et al. 1996; Chapter 4), and three Slt factors (Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8p) are linked to the tethering function of loop 1 of U5 snRNA (Frank et al. 1992; Teigelkamp et al. 1995). Interactions between mutations in the 5'-end of U2 snRNA and loop 1 of U5 snRNA were observed in the genetic analyses (Fig. 3.2). It is thus evident that the 5'-end of U2 snRNA is not restricted to interaction with the 3'-end of U6 (in forming helix II). This region of U2 snRNA may play a role in tethering of the two exons, a function parallel to that of U5 loop 1. Genetic analyses (Table 3.1 and Fig. 3.4) also indicate that several Slt factors, including the new one, Slt11p, interact functionally with both U2 and U5 snRNAs. Furthermore, these Slt factors (Slt11p, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8p) and Prp16p may function as two overlapping units (Fig. 3.6) to regulate collectively RNA-RNA interactions in the active spliceosome and to coordinate events of both steps of the splicing reaction.

3.3.1. Functions of U2/U6 helix II and the 5'-end of U2 snRNA.

The formation of U2/U6 helix II was first detected in HeLa extract (Hausner et al. 1990; Datta and Weiner 1991; Wu and Manley 1991), but the proposed interaction is not essential in yeast (Fabrizio et al. 1989; Madhani et al. 1990; Miraglia et al. 1990; McPheeters and Abelson 1992; Field and Friesen 1996). However, a mutation in the 3'-end of yeast U6 snRNA that disrupts this interaction confers lethality and can not be suppressed by a compensatory mutation in the corresponding U2 region (Field and Friesen 1996). Similar phenotypic asymmetry was also observed for another U2/U6 interaction, helix Ib; i.e., mutations in the Ib part of U6 snRNA confer deleterious growth defect in vivo (Madhani and Guthrie 1992) and block splicing in vitro (Fabrizio and Abelson 1990), while their partners in U2 snRNA are more
tolerant to mutations (with the exception of G21C, Madhani and Guthrie 1992; 1994b). Nevertheless, the existence of helix II in yeast was demonstrated genetically (Field and Friesen 1996), and was supported by the observation that the RNA-dependent ATPase activity of Slt22p is stimulated by annealed U2/U6 snRNAs (Xu et al. 1996; Chapter 4). The formation of the intermolecular helix II and its function are likely secondary, as compared with U2/U6 helix Ia. As discussed in §4.3.2, one possible function of U2/U6 helix II is to antagonize the premature formation of RNA structures important for the splicing reaction, by indirectly holding the U4/U6 duplex. After helix II is resolved by Slt22p, the U6 part of the U2/U6 interaction is likely to play other roles that are important for the subsequent events. It is equally possible that the U2 part may interact with other components of the spliceosome.

Three factors that are related to the tethering function of loop 1 of U5 snRNA were also identified in the genetic screen. Slt21p/Prp8p, shown to be crosslinked to both the 5' and 3' splice sites in humans (Wyatt et al. 1992; Chiara et al. 1996) and yeast (Teigelkamp et al. 1995; Umen and Guthrie 1995b, 1995c), may act to stabilize the fragile exon/loop 1 interaction (Teigelkamp et al. 1995). Slt21p/ Prp8p is also involved in recognition of the polypyrimidine tract (Umen and Guthrie 1995b, 1996) and 3' splice site selection (Umen and Guthrie 1996). The other two factors, Prp17p and Slu7p, are linked genetically to the function of U5 loop 1 (Frank et al. 1992). Slu7p is required for 3' splice site selection (Frank and Guthrie 1992; Jones et al. 1995; Brys and Schwer 1996) and is in close contact with the 3' splice site after ATP hydrolysis by Prp16p occurs (Ansari and Schwer 1994; Jones et al. 1995; Umen and Guthrie 1995c). Prp17p is required for the second step but acts prior to or concomitant with Prp16p action (Jones et al. 1995). The genetic interactions of Slt17p/Slu7p and Slt21p/Prp8p with U2 snRNA differentiate the function of the 5'-end of U2 from that of U2/U6 helix II, since slt17/slu7-100 and slt21/prp8-21 are synthetically lethal only with mutations in U2, but not U6, portion of helix II (Fig. 3.2). These Slt factors may interact directly with the 5'-end of U2 snRNA. A mutation corresponding to slt21/prp8-21 has been mapped in the polypyrimidine tract recognition domain in Prp8p (§5.2.3). These results suggest a potential role for the 5'-end
of U2 snRNA in efficient selection/ recognition of the 3' splice site for the second step of splicing, which is related to the tethering of the two exons by U5 loop 1 (see below).

3.3.2. Potential interactions between U2 and U5 snRNPs.

Characterization of the slt22-1 mutation (§4.2.5) revealed a potential anchoring function for U2 snRNP in holding U5 snRNP to the spliceosome (see §4.3.3 for detail). Factors (Slt11p, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8p) identified on the basis of synthetic lethality with a U2 mutation were found to be lethal in combination with mutations in loop 1 of U5 snRNA (§3.2.4, Fig. 3.4). These results suggest that the 5'-end of U2 snRNA is related functionally to the tethering function of U5 loop 1.

Although nucleotides 21, 22 and 23 of U2 snRNA are involved in the intermolecular U2/U6 helix Ib interactions (see Fig. 1.6), direct interaction has been demonstrated for only one base-pair; mutations at the other two positions in U6 failed to be suppressed by compensatory mutations in U2 partner (Madhani and Guthrie 1992, 1994b). It is likely that either or both helix Ib region(s) of U2 and/or U6 may play additional role(s) in the spliceosome. The observed genetic interactions between mutations in U2 and U5 snRNAs (§3.2.3) suggest a possible function for this region of U2 snRNA in the second step: (i) the 5'-end region of U2 snRNA may interact with U5 snRNA to assist the tethering function of loop 1 and/or (ii) these nucleotides may interact with either or both splice site(s)/exon(s) to provide additional tethering, a function that is parallel to that of U5 loop 1. Since the U23 nucleotide in this region is in close contact with the exon region of the 3' splice site (Newman et al. 1995), the second possibility is more likely, and is supported by synthetic lethality with mutations in second-step factors Slt15p/Prp17p and Slt17/Slu7p (Table 3.1).

Recently, Chiara et al. (1997) have shown that the crosslinking of U2AF65 to the polypyrimidine tract between the branchpoint site and the 3' splice site is replaced by
crosslinking of three U5 snRNP proteins, p110, p116 and p220 (Slt21p/Prp8p homology) prior to the second step in the mammalian spliceosome. These U5 snRNP proteins also interact with the substrate pre-mRNA in the region spanning from immediately downstream of branchpoint site (U2 snRNP binding site) to just upstream of the 3’ splice site (Chiara et al. 1997). These observations provide evidence that an interaction (direct or indirect) between U2 snRNP, bound to the branchpoint site, and the U5 snRNP is important for positioning the latter on the 3’ splice site, and thus for selection of the 3’ splice site. My genetic analyses lead to a similar conclusion.

3.3.3. Interactions among Slt factors: coordination of the two steps of splicing.

The recognition of the 3’ splice site is achieved collectively by a number of cis- and trans-acting elements/factors (see §5.1 for detail). Two of these protein factors, Slt17p/Slu7p and Slt21p/Prp8p, were isolated in this genetic screen. It has been shown that the crosslinking of these two factors to the 3’ splice is enhanced following the first step of splicing (Umen and Guthrie 1995c) and that the tethering of the two exons by U5 loop 1 occurs only after the first step (Sontheimer and Steitz 1993; Newman et al. 1995; O’Keefe et al. 1996). However, the same region of U5 snRNA is in close contact with the 5’ splice site prior to the first step, even though this interaction is not essential for this step (O’Keefe et al. 1996). It is likely that the 3’ splice is recognized/selected prior to the first step (see §5.1), but little is known about how this is connected temporally to the tethering function of U5 loop 1, prior to and after the first step. The genetic interactions described in this chapter may suggest a mechanism responsible for the coupling and coordination of the two steps of splicing.

Four Slt factors (Slt11p, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8p) as well as Prp16p may act as two units with overlapping functions (Fig. 3.6). Although not identified in my genetic screen, prp16-1 is synthetically lethal with a single mutation in the helix Ia region of U2 (G26A, Table 3.1). The behavior of the mutant protein indicates an important connection between the two steps of the splicing reaction. The RNA-dependent ATPase activity of Prp16p
Fig. 3.6. Summary of genetic interactions among factors involved in either or both step(s). Thick lines indicate synthetic lethality at all temperatures. Thin lines indicate partial synthetic lethality (see Table 3.4). None of the slt16/smd3-1, slt22-1 and prp2-1 mutations show synthetic lethality with other mutations tested. Genetic interaction between second-step factors, including Prp18p, and Prp8p have also been described elsewhere (Frank et al. 1992; Jones et al. 1995; Umen and Guthrie 1995b, also see Fig. 1.5).
is required for proofreading of the first step reaction and remodeling of the spliceosome for the second step (see §1.2.5-2 for detail). In the group II intron self-splicing, the analogous proofreading function has been implicated in a mechanism of kinetic coordination of the two-step reaction. It has been suggested that branchpoint nucleophilic attack (the first step) is the rate-limiting step in group II self-splicing (Jacquier and Jacquesson 1991). This reaction is reversible and may serve as a proof-reading function to control the fidelity of splice-site selection such that the second step may serve as a trap for intermediates that have undergone productive branching, thus driving the full splicing reaction to completion (Chin and Pyle 1995). It has been always observed that the two steps of nuclear pre-mRNA splicing are efficiently coupled without obvious temporal delay, suggesting that recognition of the 3' splice site and tethering of the two exons are highly coordinated.

The five factors mentioned above represent different components/functions of the spliceosome that are important for the two steps of splicing: Slt11p (activation of the spliceosome, see Chapter 6), Prp16p (proofreading of the first step and remodeling of the spliceosome), Slt21p/Prp8p plus Slt17p/Slu7p (recognition/selection of the 3' splicing) and Slt15p/Prp17p (efficiency of the second step). The observed synthetic lethality among mutations in these factors/functions suggests that these events are integrally connected in the active spliceosome. These functions may also overlap with those of U2 snRNA (interacting with U6 forming catalytically important RNA structures and juxtaposing the 5' splice site and the branchpoint, a potential role in the second step, see §5.3.2) and U5 snRNA (tethering of the two exons). In addition, the potential protein-protein interactions among these protein factors may provide the structural bases for efficient remodeling of the spliceosome in order to form RNA structures important for the second step without temporal delay.
§3.4. Materials and Methods

3.4.1 Yeast strains and plasmids.

All the yeast strains used in this study were derived from W303-1A or 1B (*Mata* or *Matα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100). The original strain used in the genetic screen, containing a chromosomal deletion of *SNR20* (the U2 snRNA gene), was described in Field and Friesen (1996). A new Δ*SNR20* disruption was described in §2.4.1. U2 mutations tested for allele-specificity (Table 3.1) were provided by D.J. Field.

Chromosomal deletion of *SNR6* (the U6 snRNA gene) was constructed by deleting the entire coding region and replaced with the yeast *HIS3* gene. Two haploid Δ*SNR6* strains, YXU86 (*Matα*) and YXU87 (*Mata*), were obtained. Strains containing *slt11*, *slt17*, *slt21* and *slt22* in a background of Δ*SNR20* were crossed to either Δ*SNR6* strain to generate haploid strains of double deletions with *slt* mutations (with *SNR20* and *SNR6* on the same maintenance plasmid). These strains were used to test genetic interactions among *slt*, U2 and U6 mutations. U6 snRNA mutations (3 nt substitutions near the 3'-end, a, b, c and d, Fig. 3.2) were described in Field and Friesen (1996).

The Δ*SNR7* (the U5 snRNA gene) disruption was constructed by deleting the entire coding region and replacing it with the yeast *HIS3* gene. Two disruption strains, YXU37A (*Mata*) and YXU37B (*Matα*) were used in subsequent experiments. Strains containing chromosomal deletions of both *SNR20* and *SNR7* were created by crossing both single-deletion haploid strains, and a maintenance plasmid carrying both *SNR20* and *SNR7* genes was then introduced into the resultant heterozygous diploid before sporulation. The double deletion strains (YXU53, YXU54) were obtained from the progeny following sporulation and tetrad dissection. Mutant U5 snRNA constructs (U98A, U98C, U97C/U99C) (Frank et al. 1992) were courtesy of C. Guthrie (UCSF). PCR was used to subclone mutant U5 snRNA fragments into pRS315 (*LEU2, CEN-ARS*). *slt* mutations in the wt chromosomal *SNR20* background
(obtained after successive back crosses to W303, see below) were segregated to the ΔSNR20 or ΔSNR7 background to test synthetic lethality with mutations in U2 or U5 snRNA.

Strains containing slu mutations (Frank et al. 1992) were courtesy of C. Guthrie (UCSF). prp2-1 strain and the PRP8 gene were courtesy of J. Beggs (Univ. Edinburgh). Both prp2-1 and prp28-102 plasmids were obtained from R.-J. Lin (Beckman Research Institute of the City of Hope) and T.-H. Chang (The Ohio State University), respectively. To test potential synthetic-lethal interaction between slu (or prp) and U2 snRNA mutations, the slu (or prp) mutations were segregated genetically into the background of ΔSNR20 through at least two consecutive crosses with YDX2299A or YDX22100A. Following tetrad dissection, progeny haploids containing both slu (or prp) and ΔSNR20 were selected and used to test synthetic lethality with U2 snRNA mutations. To exclude genetic background differences, at least four independent ΔSNR20/slu (or prp) isolates were used in these experiments. Genomic fragments containing prp2-1 and prp28-102 mutations were introduced to W303-1A by two-step gene replacement. Both mutations were then segregated to the ΔSNR20 background as described above.

3.4.2. Analysis of synthetic lethality.

To examine potential synthetic lethality of two mutations, a heterozygous diploid was first generated from two parental slt (slu or prp) haploid strains, and then subjected to sporulation and tetrad dissection. If haploids containing both mutations are viable, three types of tetrads should be obtained: parental ditype (PD, each spore inherits either of the original mutations), non-parental ditype (NPD, two spores inherit both mutations, while the other two neither, i.e., wt) and tetratype (T, two spore inherit either of the original mutations, one both mutations and the remaining one neither; i.e., wt). However, if the combination of both mutations is lethal, only PD tetrads can be obtained from such a diploid. T tetrads should contain only three viable spores (one wt and two with either mutation), and NPD tetrads only two wt spores; i.e., the lack of complete (i.e. four viable spores) NPD and T tetrads would
indicate that the two mutations in question are synthetically lethal. In most cases, at least 20 scoreable tetrads from each heterozygous diploid were analyzed phenotypically to determine synthetic lethality.
Chapter 4.

The RNA-Dependent ATPase Activity Associated with Slt22p Is Involved in U2/U6 snRNA Interactions in the Formation of the Core-Spliceosome.


S.N. contributed to characterization of the RNA-dependent ATPase activity of HA-Slt22p. D.J.F. initiated the genetic screen and provided the original yeast strain of ΔSNR20 and mutant U2 constructs. S.-J. T. contributed to molecular cloning and genetic characterization of SLT22 and slt22-1, and construction of various plasmids.
Abstract

ATP hydrolysis by a group of RNA-dependent ATPases (DExD/H proteins) is required for spliceosome assembly, but not for the subsequent transesterification reactions. Relatively little is known about the function of these ATPases in relation to the RNA conformational changes that occur in formation of active structures, in which U2/U6 small nuclear RNA (snRNA) interactions play essential roles in the splicing reactions. One of the yeast splicing genes isolated in the genetic screen, SLT22, encodes a new RNA-dependent ATPase/RNA helicase, containing motifs that are conserved only in a new class of large DExH proteins. The slt22-I mutation (E909K) has been located C-terminal to one of the conserved motifs, QMxGRAGR, shown to be important for activities of the prototype RNA helicase, eIF-4A. The RNA-dependent ATPase activity associated with HA-tagged Slt22p is stimulated preferentially by annealed U2/U6 snRNAs. Both mutant slt22-1p and U2 snRNA (used in the original genetic screen) cause a reduction in the stimulated activity. At a non-permissive temperature, the slt22-I mutation blocks splicing before the first-step reaction, resulting in the accumulation of an unusual complex, which lacks U5 snRNA, diverged from the normal spliceosome assembly pathway prior to the formation of the core-spliceosome. These data and others presented in the preceding chapter suggest that the Slt22p is involved in U2/U6 snRNA interactions and may act to unwind the U2/U6 helix II. It is likely that the 5' end region of U2 snRNA and/or the 3' end of U6 snRNA may serve as part of the structure required for the anchoring of U5 snRNA in the formation of the core-spliceosome.
§4.1. Introduction

One prominent feature of spliceosome assembly is the requirement of ATP hydrolysis in many steps, even though the subsequent two-step transesterification reaction itself does not need an exogenous source of energy. In yeast, seven DExD/H proteins are involved in the hydrolysis of ATP (Table 1.2). They are members of a family of RNA-dependent ATPases/RNA helicase (Schmid and Linder 1992). Their actions have been implicated in various steps in the splicing pathway. The RNA-dependent ATPase activity of Prp5p is associated with the conformational changes in the 5' end of U2 snRNA (O'Day et al. 1996) which, in association with SF3a and SF3b, are required for the binding of U2 snRNA to the pre-mRNA during formation of the pre-spliceosome (Ruby et al. 1993; Yan and Ares 1996; also see §1.2.2). Prp28p has been implicated in displacement of the U1/5' splice site interaction by U6 snRNA during maturation of the spliceosome (J. Staley and C. Guthrie, personal communication; §1.2.4). Slt22p (Xu et al. 1996) was also identified independently by three other groups as Brr2p (Noble and Guthrie 1996), Snu246 (Lauber et al. 1996) and Rss1p (Lin and Rossi 1996). Results presented in this chapter place the action of Slt22p in events during the formation of the core-spliceosome. The ATPase activity of Prp2p is associated with certain RNA conformational change(s) required for activation of the spliceosome (Kim and Lin 1993, 1996; Teigelkamp et al. 1994; Plumpton et al. 1994; §1.2.5-1). Prp16p, on the other hand, is required for remodeling of the spliceosome essential for the second catalytic step (Schwer and Guthrie 1992; §1.2.5-2). Its ATPase activity is also associated with a mechanism that proofreads the lariat intermediate of the first step (Burgess and Guthrie 1993a and 1993b). Following completion of the splicing reaction, release of the splicing products requires the function of Prp22p (Company et al. 1991) and Prp43p (Arenas and Abelson 1997; §1.2.6).

In the network of spliceosomal RNA-RNA interactions, the intermolecular U2/U6 snRNA interactions (helices I and II) are central to spliceosomal activation. Helix II was first
identified in human. However, the proposed yeast helix II interaction is not essential (see §3.1), and is shown genetically to be redundant with part of helix I (i.e., Ib, Field and Friesen 1996). The results presented in the previous chapter suggest that the helix II region of U2 snRNA is not restricted to the interaction with the 3'-end of U6 and that the 5'-end of U2 snRNA may play additional role(s) in the splicing pathway. If so, the formation of helix II is transient (thus, its function might be secondary).

Indirect experiments suggested that U2/U6 helix II may play a regulatory role in the dissociation of the U4/U6 duplex in spliceosome assembly (Brow and Vidaver 1995). The native U4/U6 duplex, present in the deproteinized HeLa extract, is extremely unstable, and dissociates rapidly at 30°C. However, binding of oligonucleotides complementary to the 3' end of U6, mimicking the formation of U2/U6 helix II, increases drastically the stability of the U4/U6 duplex. The removal of the entire 3' end of U6 resulted in an increase of melting temperature of the U4/U6 duplex by ~20°C (Brow and Vidaver 1995). Since this region can base-pair with the central domain of U6 (upstream of U4/U6 stem I), it has been suggested that this intramolecular interaction is responsible for the dissociation of U4/U6. However, this destabilizing interaction is balanced by the formation of intermolecular U2/U6 helix II. Thus, helix II could potentially stabilize the U4/U6 interaction in the spliceosome to prevent the premature formation of helix I and the intramolecular Brow stem, both of which are important for the catalysis of the splicing reaction (§1.3.2-3). If such is the case, helix II must be resolved for the release of U4 and for the formation of U6 Brow stem and U2/U6 helix I. It has been further suggested that energy released from the switch between alternative conformations (i.e., unwinding of helix II) may be the driving force for the dissociation of the U4/U6 duplex (Brow and Vidaver 1995).

Slt22p was identified on the basis of synthetic lethality with a particular U2 mutation that could perturb U2/U6 helix II interaction. Biochemical and genetic characterization of Slt22p and splicing defects associated with the slt22-1 mutation indicate that the RNA-
dependent ATPase activity of Slt22p is associated with U2/U6 (likely helix II) interaction. The results presented in this chapter also revealed hitherto-unsuspected interactions of the 5' end of U2 and/or helix II with U5 snRNA in the active spliceosome. These results are consistent with the idea that the unwinding of helix II, likely by Slt22p, is a crucial regulatory step in the initiation of splicing.
§4.2. Results

4.2.1. \textit{slt22-1} is synthetically lethal with mutations in U2 snRNA.

The original \textit{slt22-1} mutant was isolated on the basis of synthetic lethality with a mutant U2 snRNA (an 11 nt substitution in the 5' end stem I region that could potentially perturb U2/U6 helix II interaction, see Fig. 2.1). Genetic analyses confirmed that the synthetic lethality phenotype arose from a single-gene mutation that also conferred a temperature-sensitive (ts) (≥33°C) and a slow-growth phenotype (23°C-30°C) (Fig. 2.3 and Fig. 4.1B). In addition to the U2-11nt substitution, \textit{slt22-1} is also synthetically lethal with U2-9nt, U2-G21C (Fig. 4.1A), but is otherwise highly allele-specific with respect to other U2 mutants tested (Table 3.1 and data not shown).

A whole cell splicing extract was prepared from \textit{slt22-1} cells, and \textit{in vitro} splicing assays were first tested at 25°C. Compared with wt extract (Fig. 4.1C, lanes 1-3), relatively low splicing activity was detected in the mutant extract (Fig. 4.1C, lanes 4 -6) in the 20 min incubation, neither exon 1 or lariat intron-exon 2 intermediate was preferentially accumulated. However, when assayed at 33°C, the activity of mutant extract was completely abolished (Fig. 4.1C, lanes 10 - 12). A splicing defect was also observed in \textit{slt22-1} extracts that had been heated at 37°C for 45 min prior to splicing reaction at 25°C (Fig. 3.1C, lane 14); neither intermediates or products were detected. Thus Slt22p is a \textit{bona fide} splicing factor, which in mutated form blocks the splicing reaction before or at the first step.
Fig. 4.1. Isolation and characterization of the slt22-1 mutation. A. U2 snRNA and mutations. Top: Schematic representation of yeast U2 snRNA. BP int: region that interacts with branchpoint site in pre-mRNA, Sm: Sm site. The non-essential internal region is shown in dots. Bottom: Structures of stem I of wt and three mutant U2 snRNAs (also see Fig. 3.1A) that are synthetically lethal with slt22-1. B. Synthetic lethality of slt22-1 and snr20-G21C. Top: Yeast strains (derived from W303-1) in which synthetic lethality was tested. Bottom: Growth of SLT22 and slt22-1 in combination with wt and mutant U2 snRNAs on medium containing 5-fluoroorotic acid (5-FOA). C. In vitro splicing assays of wt and slt22-1 extracts. Heat-treated (37°C for 45 min) wt and slt22-1 extracts (Δwt and Δ22) were also assayed at 25°C (lanes 13 and 14, respectively). The precursor, final products (mature RNA and lariat intron) and lariat intron-exon 2 intermediate are indicated on the left side.
4.2.2. **SLT22 encodes a large RNA-dependent ATPase/RNA helicase.**

The wild-type **SLT22** gene was cloned by complementation (Rose and Broach 1991) (Fig. 4.2A). **SLT22** is identical to **YER172c** (on the right arm of yeast Chromosome V), which encodes a protein of 2,163 amino acids (264 kDa). Its central region (residues 500-900; Fig. 4.2A and D) contains motifs that are conserved in a class of RNA-dependent ATPases/RNA helicases (Schmid and Linder 1992). However, Slt22p is distantly related to DEAD (including Prp5p and Prp28p) and DEAH (including Prp2p, Prp16p, Prp22p, and Prp43p) RNA helicase families in that it contains DEIH in ATPase box B and two additional motifs that are conserved only in a class of relatively large RNA helicase-like proteins shown in Fig. 4.2D: PxKAL and GlGxHHA/GGL motifs. I suggest that Slt22p represents a new family of RNA-dependent ATPase/RNA-helicases. The protein can be divided into three regions: The N-terminal unique region I (a.a. 1-499) shows low homology to several yeast proteins with unknown function and is relatively highly-charged. The central region II (a.a 500 - 1,348) contains all the conserved RNA helicase motifs (see Fig. 4.2D, for alignment). The C-terminal Region III (a.a. 1,349 - 2163) shows homology (20% identity and 40% similarity) to the central RNA-helicase Region II (Fig. 4.2B). This region contains a second GKT (nucleotide binding) and a noncanonical DDAH boxes, separated by approximately 100 amino acids. This GKT/DDAH region is referred to as ATPase-like. Other RNA helicase motifs, however, are not well conserved in this region (Fig. 4.2D). In addition, a putative leucine zipper motif (LX₆LX₆LX₆LX₆LX₆LX₆L) is present near the C-terminus of region II (a.a. 1,073 - 1,115).

To date, only two Slt22p homologues have been identified, one yeast protein, encoded by **YGR172w**, and the other human U5-200 kD protein, identified as a splicing factor associated with human U5 snRNP, although the N-terminus of which has not been identified yet (Lauber et al. 1996). The function of the yeast homologue is unknown (but is not essential for viability, Martegani et al. 1997), however, the unique structural features; i.e., two ATPase/RNA helicase domains, are well conserved in this protein. In addition, the N-terminal
Fig. 4.2. Molecular cloning and characterization of SLT22. A. Schematic representation of SLT22-containing genomic DNA fragment and protein encoded. SLT22 is identical to YER172c (accession #P32639) and encodes a protein of 2,163 amino acids (246 kD) containing motifs conserved in a group of DExH putative RNA dependent ATPases/RNA helicases. Filled boxes indicate conserved RNA helicase motifs the alignment of which is shown in D (next page). Slt22p can be divided into three regions, I, II and III, based on sequence homology between regions II and III. Box near the C-terminus of region II indicates a putative leucine zipper. B. Dot-matrix plot of regions II and III, showing sequence homology (DNA strider 1.2, with window of 30 amino acid, homology of 20%). C. Yeast (Ygr271p, #S64604) and human (U5-200 kD, Lauber et al. 1996, #Z70200) homologues of Slt22p. All three proteins contain two homologous RNA-dependent ATPase regions and new motifs shown in D. The homology between Slt22p and U5-200 kD is described in Lauber et al. (1996), note that the N-terminal (~200 amino acid) portion of U5-200 kD has not been identified yet. LZP: putative leucine zipper. D (p124-125). Amino acid sequence alignment of six motifs that are conserved in the first (Slt22 II, Ygr271 N and U5-200 kD N) and the second (Slt22 III, Ygr271 C and U5-200 kD C) halves of Slt22p, Ygr271p and U5-200 kD, and a class of putative RNA helicases, including: Hfm1p of S. cerevisiae (1,048 a.a., #U22156), a hypothetic protein deduced from a cDNA of human myeloid cell line (1,045 a.a. fragment, #P42285), antiviral protein Ski2p of S. cerevisiae (1,286 a.a., #P35207), Ski2p homolog of human (1,246 a.a., #Z48796, Dangel et al. 1995), Yjl050wp of S. cerevisiae (1,073 a.a., #Z49325), a hypothetic protein (C28H8.3) of C. elegans (1,722 a.a., #Q09475), and a large-helicase-related protein (LHR) of E. coli (1,538 a.a., #P30015). The consensus sequences are shown at the bottom of the alignments. Dots indicate similarity. The position of E909K mutation in slt22-1p is underlined in the alignment of the last motif.
A

SLT22/YER172c

I  unique
RNA-dependent
region
II  ATPase region
III  ATPase-like
region

RAD3

B

Region II

Region III

C

Slp22p

Ygr271w

U5-200 kD
D

1. **GKT motif (ATPase box A)**

<table>
<thead>
<tr>
<th>Motif</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt2 II</td>
<td>502</td>
<td>IYQKVFHANHFGDSNLICAPTGSNGKNIALLTVLAK</td>
<td>539</td>
<td></td>
</tr>
<tr>
<td>Slt2 III</td>
<td>1351</td>
<td>IQSQVFESLYNSDVSFGVSGKTGKTAELALLNH</td>
<td>1388</td>
<td></td>
</tr>
<tr>
<td>Ygr271w N</td>
<td>291</td>
<td>IYQSLVYPAYTNENMLICAPTGSNGKTIALLTVINT</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td>Ygr271w C</td>
<td>1143</td>
<td>IQSQVYFGLTNENAFVGSPTSGKTVVEILAIWHA</td>
<td>1180</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD N</td>
<td>49</td>
<td>IYQSKLYRAALETDENLLLCAPTGSNGKTDIALLT</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD C</td>
<td>896</td>
<td>IQTVQFNTVYSDDNVFVGAPTGSNGKTECAEFAILRML</td>
<td>933</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homolog</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfml</td>
<td>1</td>
<td>MQSEAFPSIYSENCTISSPTSGKTVVFELAILLRI</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Hu ORF</td>
<td>146</td>
<td>AFQREAIQCVDNNQSVLVSHTGATKVCAEYAIALAL</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Ski2</td>
<td>313</td>
<td>TFQKEAVYHLEQDGVSFWAHSGKTVEAYAIAMAH</td>
<td>369</td>
<td></td>
</tr>
<tr>
<td>Ski2 homolog</td>
<td>313</td>
<td>VQKQAILKLEHDGSEVFAHTGSNGKTVVAEYAIHAQSL</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>Yjl050w</td>
<td>152</td>
<td>PFDATAICIDRGESVLSHTGATKVVEYAIALAQ</td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>C. elegans ORF</td>
<td>795</td>
<td>GWQRMLDSVDRGNSALIIAPTSAGKTFVSYYCEKVL</td>
<td>832</td>
<td></td>
</tr>
<tr>
<td>E. coli LHR</td>
<td>32</td>
<td>AVQPQTHVAARESEHLAAPTSGKTLFAAFLYALDRL</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

2. **PxKAL motif**

<table>
<thead>
<tr>
<th>Motif</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt2 II</td>
<td>556</td>
<td>KIVYIAPLKLAVQEQVRE</td>
<td>573</td>
<td></td>
</tr>
<tr>
<td>Slt2 III</td>
<td>1395</td>
<td>AVYINPSGEKIDFLLSDW</td>
<td>1412</td>
<td></td>
</tr>
<tr>
<td>Ygr271w N</td>
<td>348</td>
<td>KIVYVAPLKLAAEIVDK</td>
<td>365</td>
<td></td>
</tr>
<tr>
<td>Ygr271w C</td>
<td>1187</td>
<td>KIVYIAPMKALVRERVDD</td>
<td>1204</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD N</td>
<td>102</td>
<td>KIIYIAPMTRLQEMVG</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD C</td>
<td>941</td>
<td>CVYITMPRLWGEQYVMW</td>
<td>958</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homolog</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfml</td>
<td>49</td>
<td>KIIIYIAPTSKSLCYEMKN</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Hu ORF</td>
<td>188</td>
<td>RVIFTSPIKALSNQKRE</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Ski2</td>
<td>374</td>
<td>KIYIYTSPIKALSNQKFRE</td>
<td>391</td>
<td></td>
</tr>
<tr>
<td>Ski2 homolog</td>
<td>355</td>
<td>RITYIYTSPIKALSNQKFRD</td>
<td>372</td>
<td></td>
</tr>
<tr>
<td>Yjl050w</td>
<td>194</td>
<td>RVYIYTSPIKALSNQKYRE</td>
<td>221</td>
<td></td>
</tr>
<tr>
<td>C. elegans ORF</td>
<td>839</td>
<td>VVVYVAPSKALINQVCGS</td>
<td>856</td>
<td></td>
</tr>
<tr>
<td>E. coli LHR</td>
<td>87</td>
<td>RILYTSPIKALINQVCGS</td>
<td>104</td>
<td></td>
</tr>
</tbody>
</table>

3. **DEI(V)H motif (ATPase box b)**

<table>
<thead>
<tr>
<th>Motif</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt2 II</td>
<td>624</td>
<td>IVELVRLIIIDEIHLHLD</td>
<td>641</td>
<td></td>
</tr>
<tr>
<td>Slt2 III</td>
<td>1464</td>
<td>NIQSLELMYDAAHEISQ</td>
<td>1481</td>
<td></td>
</tr>
<tr>
<td>Ygr721w N</td>
<td>417</td>
<td>LVSKVLIIIDEHHLHLE</td>
<td>435</td>
<td></td>
</tr>
<tr>
<td>Ygr721w C</td>
<td>1245</td>
<td>FVQDSLIIKIMDEIHLAS</td>
<td>1273</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD N</td>
<td>170</td>
<td>YTQLVRLIVLDEIHLHLD</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD C</td>
<td>1009</td>
<td>NQNMLIFVDEVHILG</td>
<td>1026</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homolog</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfml</td>
<td>117</td>
<td>LFELVKLVLVDLEIHTIKE</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Hu ORF</td>
<td>246</td>
<td>VMREVAWFIDEHMYRM</td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>Ski2</td>
<td>434</td>
<td>LIRDVEVIFDEVHYN</td>
<td>451</td>
<td></td>
</tr>
<tr>
<td>Ski2 homolog</td>
<td>413</td>
<td>VIRDLEWIFDEVHYIND</td>
<td>430</td>
<td></td>
</tr>
<tr>
<td>Yjl050w</td>
<td>252</td>
<td>VMREVAWFIDEHMYRM</td>
<td>269</td>
<td></td>
</tr>
<tr>
<td>C. elegans ORF</td>
<td>911</td>
<td>FVSHKIYVFDEVHISGA</td>
<td>928</td>
<td></td>
</tr>
<tr>
<td>E. coli LHR</td>
<td>169</td>
<td>TLRQGEVTIIIDEHVAVG</td>
<td>186</td>
<td></td>
</tr>
</tbody>
</table>

4. **SAT motif**

<table>
<thead>
<tr>
<th>Motif</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt2 II</td>
<td>624</td>
<td>IVELVRLIIIDEIHLHLD</td>
<td>641</td>
<td></td>
</tr>
<tr>
<td>Slt2 III</td>
<td>1464</td>
<td>NIQSLELMYDAAHEISQ</td>
<td>1481</td>
<td></td>
</tr>
<tr>
<td>Ygr721w N</td>
<td>417</td>
<td>LVSKVLIIIDEHHLHLE</td>
<td>435</td>
<td></td>
</tr>
<tr>
<td>Ygr721w C</td>
<td>1245</td>
<td>FVQDSLIIKIMDEIHLAS</td>
<td>1273</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD N</td>
<td>170</td>
<td>YTQLVRLIVLDEIHLHLD</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>U5-200 KD C</td>
<td>1009</td>
<td>NQNMLIFVDEVHILG</td>
<td>1026</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homolog</th>
<th>ORF</th>
<th>Start</th>
<th>End</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfml</td>
<td>117</td>
<td>LFELVKLVLVDLEIHTIKE</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Hu ORF</td>
<td>246</td>
<td>VMREVAWFIDEHMYRM</td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>Ski2</td>
<td>434</td>
<td>LIRDVEVIFDEVHYN</td>
<td>451</td>
<td></td>
</tr>
<tr>
<td>Ski2 homolog</td>
<td>413</td>
<td>VIRDLEWIFDEVHYIND</td>
<td>430</td>
<td></td>
</tr>
<tr>
<td>Yjl050w</td>
<td>252</td>
<td>VMREVAWFIDEHMYRM</td>
<td>269</td>
<td></td>
</tr>
<tr>
<td>C. elegans ORF</td>
<td>911</td>
<td>FVSHKIYVFDEVHISGA</td>
<td>928</td>
<td></td>
</tr>
<tr>
<td>E. coli LHR</td>
<td>169</td>
<td>TLRQGEVTIIIDEHVAVG</td>
<td>186</td>
<td></td>
</tr>
</tbody>
</table>

---

- 124 -
5. GIGxHHA(G)GL motif

- Slt22 II  793  LRKLIESGIGTHHAGLTRSDRSLSEDLFADGLLQVLVCTATLAW
- Slt22 III  1629  LRAPLKHGVGILYKGMASNDERVKRLYEYGAVSLLISKDCSA
- Ygr271w N  587  MKEIFQFGFGIIHAGMARSDRNLTEKMFKDGAIKVLVCTATLAW
- Ygr271w C  1423  LKLSLQFGIGLHLHAGLVRQKDSHSHLFQKNIQILIATATLAW
- U5-200 kD N  339  LKDLLPYGFAIHAGMTRVDRTLVEDLFGDHQVVLVSTATLAW
- U5-200 kD C  1174  LKETLLENGVHEGLSPMERRLVEQLFSSGAIQVVASRSCLW

- Hfm1  272  LNECMQQGIAFHHAGISLEDRTAVKEFLAGSINILCSTSTLAV
- Hu ORF  448  VPLLLKRGIGIHHGGGLPILKETEILFSEGLIKLALFATETFAM
- Ski2  684  TRSLLERGIAVHGGLLLPIVKELIELFSGKFIKLVFATETFAM
- Ski2 homolog  624  MSELLNRGLGVSILPILKEVIMFLPSUNGVLKVFATETFAM
- Yji050w  464  ILPLLRICGIIHSGLLPILKEVIEILFQEGFLKVFATETFAM
- C. elegans ORF  1282  LLKLFERGIGYHHAGLNTVERGAVEVLFRSVNLAVFLSTSTLSL
- E. coli LHR  344  VQSSDVFIARSHHSVSKEQGRAITEQALKSGLQVCVATSSLLE

--- GIG-HH•GL---•E-LF--•G•--•L--T---

6. QMxGRAGR motif

- Slt22 II  867  DVLQMLGRAPRYTDF-GEGIITDQSNSVYLSVLNLQPIESQFV
- Slt22 III  1702  ELLEMVGLAS--GNDSMAGKVLILTHSNMKAYKFLIEPLTESYLQ
- Ygr271w N  661  DVIQIFGRGGRPGFGSANGTGLCTSDRLDHLYVSLTQQHPIESRFG
- Ygr271w C  1496  DILQMMGRAPAYDT-TGTAIVYTKESKKMKYKHFLNVGFPESSLH
- U5-200 kD N  413  DILQMGIRGAPQYDTK-GEGLITSHELQYVGLSLNLQPIESQMV
- U5-200 kD C  1248  DILQMVHHANRDL-DEGRVCIMCQGKSDFKKFKEYPLPVESSLH

- Hfm1  344  DVLQMIGRAMGRPFETH-GCAVIMTDKMKQTYENLHHTVLESSHL
- Hu ORF  520  EYIQMSGRAGRRGMDDR-GIVILMVDEKMSPTIKQLKGSADPLNWA
- Ski2  755  GEFITLAGRRGRGLST-GEVIVMYANPSLIAFTFKIEVFIGNQVTPRQ
- Ski2 homolog  696  EVEVQAGRAGRRGDPT-GEVILLCKGRVPEMALHRMMGKPSQLQ
- Yji050w  536  EYIQMSGRAGRRGDRE-GEVIMGKDEMPEVAKGVMKQADRLDSE
- C. elegans ORF  1347  LVRQMISGRAGRRGFHGS-NVIFMSIPTSKEVRRLTASLSLNQGNPPF
- E. coli LHR  408  SGLQRIGRAHQQGYS-KGLFFPRTRDLVDASAVECOMFAGRLENL
region (290 a.a.) also shows homology (22% identity and 35% similarity) with the corresponding region in Slt22p (Fig. 4.2C). The other two regions show higher homology, 40% identity and 72% similarity for the first ATPase/RNA helicase domain, and 27% identity and 63% similarity for the second domain (Fig. 4.2C).

SLT22 is an essential gene. Deletion of the central BgIII fragment (2.5 Kb, replaced by the HIS3 gene), that encodes these conserved RNA-dependent ATPase motifs, confers lethality (Fig. 4.3A & B). The mutant gene was cloned from the slt22-1 cells using the gapping method with an SLT22 gene bearing large internal deletion (i.e., the 2.5 Kb BgIII fragment). The slt22-1 mutation (E909K, Fig. 4.3C) is located in region II downstream of the H/QxxGRAGR motif, which is important for RNA binding, ATP hydrolysis and RNA unwinding of the prototype RNA dependent ATPase/RNA helicase, eukaryotic translation initiation factor 4A (eIF 4A) (Pause et al. 1993). The corresponding residue is also conserved in region III and the two homologues (Fig. 4.2D). I have confirmed that the E909K mutation is both necessary and sufficient to confer a ts growth phenotype in the presence of SNR20 and synthetic-lethality in combination with three U2 mutations shown Fig. 4.1, by introducing it into the yeast chromosome as the sole copy of SLT22 gene using two-step gene replacement (data not shown). The slt22-1 ts phenotype can be partially suppressed by itself when overexpressed from a low-copy (CEN-ARS) plasmid. However, when overexpressed from a high-copy (2μ) plasmid, the slt22-1 mutation showed a partial dominant negative phenotype (data not shown).
Fig. 4.3. Chromosomal deletion of SLT22 and mapping of slt22-1 mutation. A & B. SLT22 is an essential gene. A. The internal 2.5 Kb BglII fragment encoding the central RNA dependent ATPase region was deleted and replaced with the yeast HIS3 gene to create a chromosomal deletion of SLT22. B. Results of tetrad dissection of SLT22/SLT22Δ::HIS3 diploids in the absence (top) and presence (right) of maintenance SLT22/URA3 plasmid. C. slt22-1 mutation is located the C-terminal region of the GRAGR motif. The asterisk in A indicates the location of slt22-1 mutation. The arrow indicates another mutant allele of slt22, rss1-1 (Lin and Rossi 1996). Shown is the sequence ladders of regions in both wt and slt22-1 genes surrounding the mutation G → A that results in E909K change.
4.2.3. Slt22p contains two separable functional domains.

The functional domain structure of Slt22p was studied with the aid of deletion mutations (Fig. 4.4A): N1, N2 and N3 contain various parts of the N-terminal region, and C1, C2 and C3 the C-terminal region. Carried on a low-copy plasmid, these deletions were tested for their ability to complement three defects: 1) the synthetic lethality of slt22-1 in combination with snr20-G21C, 2) the ts phenotype of slt22-1 (in combination with wild-type U2 snRNA), and 3) the null chromosomal deletion of SLT22 (ΔSLT22). While none of the deletions were able to complement ΔSLT22, deletion N2, which contains the regions I and II portion of the protein plus the two ATPase-like motifs (GKT/DDAH) of region III, complemented partially both the slow-growth and synthetic-lethal phenotypes (Fig. 4.4B). Deletion N3, which lacks the ATPase-like motifs of region III, lost the ability to complement any slt22-1 phenotype (data not shown). Surprisingly, the combination of deletions C2 and N2 was able to supply sufficient Slt22p activity to complement ΔSLT22 at permissive temperatures (≤33°C; Fig. 4.4B). However, trans-complementation could be achieved only when both deletions were expressed from low-copy plasmids; complementation was not observed if either or both were over-expressed from (a) high-copy plasmid(s) (data not shown). Moreover, the C-terminal ATPase-like motifs (GKT and DDAH) must be present in both of the deletions in order that complementation be observed. It is thus evident that the C-terminal region III is essential for viability – even a small deletion (~220 a.a.) at the C-terminus of this domain (N1 in Fig. 4.4A) is sufficient to render Slt22p inert. However, the results of trans complementation suggest that Slt22 comprises two functional domains which need not be covalently linked, provided that both are present in stoichiometric equivalence and neither exceeds wild-type level. None of other combinations (N2-C1, N2-C3 or N3-C3) was able to complement in trans, regardless of whether they were carried on a low- or high-copy plasmid (Fig. 4.4C, and data not shown).

Since the slt22-1 mutation resides in the central RNA helicase domain (Fig. 4.3B), I tested a deletion of slt22-1, N2' (equivalent to N2 of SLT22) for complementation of the ts and
Fig. 4.4. Functional analysis of two domains of Slt22p. A. Two groups of Slt22p deletions. Two filled boxes in region III indicate GKT and DDAH motifs. N group deletions: an EcoRV site, introduced at 3'-end of the gene by site-directed PCR mutagenesis, was used in combination with other convenient restriction sites in the gene to create deletions that retained the intact 3'-end untranslated region of the gene. Yeast vectors pRS315 (LEU2, CENARS) were employed for the construction. C group deletions: C1 was constructed by an in-frame deletion of the internal 2.5 Kb BgIII fragment. The other two were constructed using PCR and convenient restriction sites. In all cases, the native promoter of SLT22 was used. These genes were carried on pRS314 (TRP1, CENARS). C2 was also subcloned into pRS315. B. In vivo functional assay and trans-complementation of deletions N2 and C2. Deletion mutants were assessed for the ability to complement the synthetic lethality of slt22-1 + U2-G21C (left column), a null chromosomal deletion of SLT22 (the central column), and slow-growth (at 30°C) phenotype of slt22-1 (right column). Results obtained with SLT22, slt22-1 and vector(s) were shown in top three rows. ND, not done. C. Summary of in vivo functional assays of SLT22 deletions. -, no complementation; +/− partial complementation.
A

![Diagram showing deletions and synthetic lethality](image)

B

<table>
<thead>
<tr>
<th>synthetic lethality of slt22-1 + U2-G21C</th>
<th>deletion of SLT22</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>ND</td>
</tr>
<tr>
<td>N2</td>
<td>+/-</td>
</tr>
<tr>
<td>N3</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>-</td>
</tr>
<tr>
<td>N1+C1</td>
<td>ND</td>
</tr>
<tr>
<td>N1+C2</td>
<td>ND</td>
</tr>
<tr>
<td>N1+C3</td>
<td>ND</td>
</tr>
<tr>
<td>N2+C1</td>
<td>ND</td>
</tr>
<tr>
<td>N2+C2</td>
<td>+/-</td>
</tr>
<tr>
<td>N2+C3</td>
<td>+/-</td>
</tr>
<tr>
<td>N3+C1</td>
<td>-</td>
</tr>
<tr>
<td>N3+C2</td>
<td>-</td>
</tr>
<tr>
<td>N3+C3</td>
<td>-</td>
</tr>
</tbody>
</table>

- 130 -
synthetic-lethal phenotypes of \textit{slt22-1} and deletion of \textit{SLT22}. It failed, both by itself and in combination with deletion C2 (data not shown), reiterating the functional importance of E909K mutation in \textit{slt22-1p}.

4.2.4. The ATPase activity of Slt22p is stimulated U2/U6 snRNAs.

A 9 amino acid hemagglutinin (HA)-epitope was introduced close to the N-terminus of Slt22p and \textit{slt22-1p} (Fig. 4.5A) to aid the characterization of ATPase activities. The tagged wt protein is fully functional and can complement the chromosomal deletion of \textit{SLT22}. The presence of an HA-tag in the mutant protein did not exacerbate the ts phenotype (data not shown). Both tagged proteins were expressed from a high-copy plasmid in a \textit{\Delta SLT22} yeast strain; whole-cell splicing extracts were then prepared and immunoprecipitation was performed using monoclonal antibody 12CA5 (Kolodziej and Young 1991). Similar amounts of tagged protein (>200 KDa) were detected in the immunoprecipitates of extracts containing HA-Slt22p and HA-slt22-1p (but not that of wild-type extract) by silver staining (not shown) and by Western blotting (Fig. 4.5B), indicating that the \textit{in vivo} defect associated with \textit{slt22-1p} is unlikely due to instability of the mutant protein.

Immunoprecipitate containing approximately 10 ng of HA-Slt22p (as determined by silver staining) was used in an RNA-stimulated ATPase assay. Using immunoprecipitates from untagged wild-type extract as a control, only a basal-level of ATPase activity was detected in the absence of any exogenous RNA (Fig. 4.5C and D). I tested a range of RNAs for the stimulation of ATPase activity including yeast total RNA, poly(A)$^+$ RNA, tRNA, and synthetic snRNAs (U2, U4, U5 and U6). A smaller U2 snRNA (U2S; \textasciitilde 360 nt) containing only stems I and II was also used. Prior to ATPase assay, these snRNAs (250 ng, in excess, see below) were annealed separately or in mixtures (U2/U6, U2S/U6 and U4/U6). While low levels (3 - 4 fold) of stimulation were observed with all the snRNAs tested, the ATPase activity of HA-
Fig. 4.5. HA-tagged Slt22p and ATPase assays. A. HA-tagging of Slt22p. *top:* an SphI site was first introduced near the N-terminus resulting in the E5A mutation which did not confer any phenotypic defect (data not shown). *center:* schematics of HA-Slt22p (showing two copies of tags in opposite orientations, also see Fig. 4.2A). *bottom:* two copies of HA-coding sequences were introduced at the SphI site, one of which is inserted in the reverse orientation (italics), while the second one encodes HA-epitope (underlined). B. Immunoprecipitation/Western blot detection of HA-Slt22p and HA-slt22-1p. A protein band above the 200 kD marker was detected in HA-tagged samples but not in the untagged. C. Stimulation of ATPase activities of HA-Slt22p by synthetic snRNAs. D. Quantitation of RNA (250 ng each)-stimulated ATPase activities of HA-Slt22p (10 ng). E. Detection of specific U2/U6 crosslinks. 32P-labeled synthetic U2(S) RNA was annealed with unlabeled synthetic U6, U4 and U5 RNAs using the condition employed for ATPase assay. The mixtures were irradiated under UV light for time indicated and then resolved on a denaturing polyacrylamide gel. Arrows indicate crosslinks that are specific to U2/U6 RNAs.
Chapter 4

A

<table>
<thead>
<tr>
<th>SphI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG ACT GAG CAT GGA ACG AAG GAT AAG</td>
</tr>
<tr>
<td>ATG ACT GAG CAT GGA ACG AAG GAT AAG</td>
</tr>
<tr>
<td>MTEHERSTKDK</td>
</tr>
<tr>
<td>E→A</td>
</tr>
</tbody>
</table>

ΔSphI

<table>
<thead>
<tr>
<th>SphI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGACTGAGCATGGAACGTCTGGGACGTATGGGTATGCATGCA</td>
</tr>
<tr>
<td>MTEHERSGLGRMGHMHA</td>
</tr>
</tbody>
</table>

ΔSphI

| TACCCATACGACGCTGACTACGTTCATGCAACG |
| YPYDVYPYAHAT |

C

\[ \text{untagged} \]

\[ \text{HA-tagged} \]

D

<table>
<thead>
<tr>
<th>Free phosphate released (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no RNA</td>
</tr>
<tr>
<td>U2/U6</td>
</tr>
<tr>
<td>U25/U6</td>
</tr>
<tr>
<td>U4/U6</td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>UV irradiation (min)</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>unlabeled RNA</td>
<td>U6</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U4</td>
</tr>
<tr>
<td>U6 U4 U5</td>
<td>U6</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U4</td>
</tr>
<tr>
<td>U6 U4 U5</td>
<td>U6</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U4</td>
</tr>
<tr>
<td>U6 U4 U5</td>
<td>U6</td>
<td>U4</td>
<td>U5</td>
<td>U6</td>
<td>U4</td>
</tr>
</tbody>
</table>

32P-U2(S) RNA
Slt22p was stimulated ≥15 fold by pre-annealed U2/U6 and U2S/U6 snRNAs (Fig. 4.5C and D). Under the conditions employed in the ATPase assay, UV-crosslinks specific to U2/U6 snRNAs were detected (Fig. 4.5E), suggesting that the specific stimulation is likely due to formation of intramolecular structures between these two RNAs. Other RNAs tested showed no or very little stimulation (data not shown).

The specificity of stimulation by U2S/U6 snRNAs was characterized as a function of RNA amount (Fig. 4.6A) and in a reaction time of 60 min (Fig. 4.6B). With ~10 ng of tagged Slt22p, the ATPase activity increased as RNAs increased from 0 to 125 ng, but no further stimulation was observed at higher quantities. The strong stimulation by U2S/U6 snRNAs was higher than the sum of that by U2S and U6 snRNA alone and thus seemed to be highly specific (Fig. 4.6A). With excess of RNA (250 ng of U2S and U6 each), the maximal ATPase activity of HA-Slt22p was achieved within 20 - 30 min (Fig. 4.6B). The rate of ATP hydrolysis stimulated by U2S/U6 was estimated in the range of 40 - 50 moles P_i released per min per mole Slt22p, which is in the range of ATPase activities reported for Prp16p (Schwer and Guthrie 1991), eIF 4A (Griff et al. 1984), srmB (Nishi et al. 1988), and p68 (Hirling et al. 1989). A synthetic U2 snRNA [short version, U2S(11nt)] containing the 11nt substitution used in the original genetic screen was also used in the ATPase assay. Alone, this RNA showed a similar level of stimulation as the wt U2S. However, when this U2 RNA was annealed to wt U6, the level of stimulation was reduced by ~25% (Fig. 4.6C).

Tagged mutant protein (HA-slt22-1p) showed a similar basal level of ATPase activity compared to HA-Slt22p (Fig. 4.6C). A slightly reduced stimulation by U2S, U2S (11nt) or U6 snRNA was observed. In contrast to the wt protein, the maximal stimulation by both U2S/U6 and U2S(11nt)/U6 snRNAs was markedly reduced with mutant protein, and was only comparable to stimulation by U2 or U6 alone (Fig. 3.6C). Since the same amounts of wt and mutant proteins were used in these assays, the reduction in the RNA-stimulated ATPase activity of HA-slt22-1p can be attributed directly to the E909K mutation in the central RNA-
Fig. 4.6. Characterization of ATPase activity of Slt22p and slt22-1p. A. U2S, U6 and U2S/U6 snRNA-stimulated ATPase activities of HA-Slt22p (~10 ng) as a function of RNA amounts after incubation time of 60 min. B. Time course of U2S/U6-stimulated ATPase activities of HA-Slt22p and HA-slt22-1p. C. Comparison of ATPase activities of Slt22p and slt22-1p stimulated by wt and mutant (11nt) U2 and U6 snRNAs. Average ATPase activities were obtained from at least three independent experiments. Bars indicate standard deviations.
helicase region of the protein. Furthermore, the reduction of stimulation by U2S(11nt)/U6 but not by U2S(11nt) alone suggests that this reduction is likely caused by altered RNA structure(s) formed between mutant U2 and wt U6 snRNAs (see discussion).

4.2.5. *slt22-1* extract accumulates new splicing complexes.

As shown earlier (Fig. 4.1C), the *slt22-1* mutation blocked the splicing reaction before/at the first step. I examined whether and how the *slt22-1* mutation affects the assembly of splicing complexes. As reported previously (Cheng and Abelson 1987), during a 10 min reaction at both 25°C and 33°C, the assembly of splicing complexes in the wt extract proceeded as described: complex B (pre-spliceosome) → complex A2-1 (holo-spliceosome) → complex A1 (core spliceosome) → complex A2-2 (active spliceosome) (lanes 1 - 4 and 9 - 12 in Fig. 4.7A). After prolonged incubation (20 min), all the pre-splicing complexes were converted to the active spliceosome (Fig. 4.7B). Similar, but delayed, kinetics were observed in the *slt22-1* extract when the splicing reaction was performed at 25°C (lanes 5 - 10 in Fig. 4.7A). However, when the reaction was carried out at a nonpermissive temperature (33°C, without prior heat treatment), a new complex (X) was detected in the *slt22-1* extract after the onset of assembly (lanes 13 - 16 in Fig. 4.7A); i.e., complex X appeared after complexes B and A2-1, since it was not present at very early time (lane 13 in Fig. 4.7A). Following prolonged incubation (20 min, Fig. 4.7B), the only complexes present were A1-like, X and a second new complex, Y. Apparently, X and Y were derived from other complexes. Complex A1-like was found to contain U2, U5 and U6, but not U4 snRNAs, as determined by primer extension using RNA extracted from the complex on the native gel (Fig. 4.7C), thus is indeed related to complex A1 (Cheng and Abelson 1987; Tarn et al. 1993). However, complex X contained only U2 and U6 but not U4 or U5 snRNAs (Fig. 4.7C), and might represent an unusual splicing complex caused by malfunctioning of the mutant *slt22-1p* where the ATPase activity is not stimulated by U2/U6 snRNAs (Fig. 4.6C).
Fig. 4.7. Formation of a new splicing complexes in \textit{slt22-1} extract. A. Native gel electrophoresis of spliceosome assembly in \textit{wt} and \textit{slt22-1} extracts at 25°C and 33°C. Showing the kinetics of each complex in a time course of 10 min. B. Native gel electrophoresis of spliceosome assembly in \textit{wt}, \textit{slt22-1}, and \textit{prp2-1} extracts following prolonged incubation (20 min) at 33°C, showing the final complexes formed. C. Primer extension analysis of snRNA components of two complex formed in the \textit{slt22-1} extract at 33°C.
Since the kinetics of the appearance of complex Y (with similar mobility to that of complex H) was not clear, it has not been investigated further. It is possible that complex Y is a breakdown product of complex X.

A question arises as to whether complex X represents a transient intermediate splicing complex normally present in spliceosome assembly, but is not detected otherwise in wt extract. If so, complex X, once formed in slt22-1 extract, should be capable of re-introduction into the splicing pathway if functional Slt22p and other splicing factors are provided (Fig. 4.8A, right panel). A "chasing" experiment was devised to test this (Fig. 4.8). As a control, a prp2-1 extract was used to demonstrate that complex A1 (a functional intermediate) accumulated in the absence of Prp2p function can be activated to regain full splicing activity if a functional Prp2p is provided subsequently (Fig. 4.8A, left panel), since this factor is associated with spliceosome transiently prior to first step of splicing (Plumpton et al. 1994; Teigelkamp et al. 1994; Kim and Lin 1996). In this experiment, the amount of radio-labeled pre-mRNA was reduced to one-quarter of normal to ensure that all of it was incorporated into splicing complex(es) (Fig. 4.7B), preventing any de novo assembly of splicing complexes during the subsequent addition of the second extract. The prp2-1 extract was first incubated at 33°C for 20 min with labeled substrate at which point almost all the substrate has been converted to complex A1 without splicing activity (Fig. 4.7B, and Fig. 4.8A lane 2), as reported (Kim and Lin 1993; Teigelkamp et al. 1994). Then a second extract (half the amount), either wt or slt22-1, was added, and kept for an additional 20 min at the same temperature (Fig. 4.8B, lanes 7 and 8). This resulted in recovery of splicing activity, although such activity rescued by slt22-1 extract was much lower than that by wt extract. Prp2p activity provided by either extract was able to convert complex A1, formed during the initial incubation with the prp2-1 extract, to the active spliceosome. In contrast, following addition of wt or prp2-1 extract to a splicing reaction in slt22-1 extract at the non-permissive temperature (Fig. 4.8B, lane 3), no splicing activity was detected (Fig. 4.8B, lanes 5 and 6) suggesting that complexes accumulated in the slt22-1 extract at the non-permissive condition are "dead-end" (terminally malformed) complexes which can
not be rescued by functional Slt22p and other splicing factors. Thus complex X, containing only U2 and U6, might be a product of a default pathway triggered by a malfunctioning slt22-1p.

Fig. 4.8. Formation of dead-end complexes in slt22-1 extract. A. Schematic representation of the experimental design (p140). Labeled substrate was incubated with the first extract for 20 min at non-permissive temperature. Splicing complexes formed in prp2-1 (left) and slt22-1 (right) extracts are shown. By adding a second extract to the reaction, one can address whether active components from the second extract could rescue splicing complexes formed during the first incubation time, as such is the case for complex A1 formed in the prp2-1 extract. B. Complexes accumulated in slt22-1 extract are terminally blocked; i.e., they are "dead-end" complexes. Left: Standard splicing reactions were carried out at 33°C with wt, prp2-1, slt22-1 extracts, or prp2-1 plus slt22-1 extracts mixed prior to addition of substrate (lanes 1, 2, 3 and 4, respectively), showing the in vitro complementation (lane 4). Right: Splicing reactions with slt22-1 (lanes 5 and 6) and prp2-1 (lanes 7 and 8) extracts were allowed to proceed at 33°C for 20 min before aliquots of extracts (wt, lanes 5 and 7, and mutant, lanes 6 and 8) were added. Reactions were kept at 33°C for another 20 min. Splicing activity was only observed in the prp2-1 (lanes 7 and 8), but not in slt22-1 (lanes 5 and 6) extracts after such treatment. Precursor, intermediate (lariat intron-exon 2) and final products (mature mRNA and lariat intron) are indicated on the right side.
Chapter 4

A

1st extract
prp2-1

holo-
spliceosome

U7 U5
U6 U4
U2

1st extract
slt22-1

U7 U5
U6 U4
U2

complex A1

U6 U5
U2

prp2-1p

complex X

splicing

2nd extract

B

1st extract

20 min

20 min

1st extract

+ 32P-pre-actin

2nd extract

B

1st extract

2nd extract

B

1st extract

2nd extract

- 140 -
§4.3. Discussion

The synthetic lethal genetic screen was designed to isolate yeast splicing factors important for the function of U2 snRNA, particularly the 5' end region. It was also anticipated that such a screen would yield factors involved in U2/U6 interaction, since the original 11nt substitution of U2 snRNA could perturb U2/U6 helix II interaction (Fig. 2.1). Characterization of Slt22p indicated that the RNA-dependent ATPase activity associated with this large protein is linked to U2/U6 interaction(s). Mutation (E909K) in one of the conserved RNA helicase motifs in slt22-1p results in loss of U2/U6 stimulated ATPase activity (Fig. 4.6C) and blocks pre-mRNA splicing prior to the formation of the active spliceosome at the non-permissive temperature (Figs. 4.1C and 4.7A). An unusual complex (X) was accumulated at the expense of other normal complexes and the spliceosome assembly is terminally stalled (Fig. 4.8). The lack of U5 snRNA in complex X (Fig. 4.7C) provides evidence that one function of Slt22p is to stabilize indirectly the interaction between U5 snRNA and the rest of the core spliceosome. The genetic interactions between slt22-1 and mutations in U2 and U6 snRNAs (Fig. 4.1 and §3.2.1, §3.2.2) are consistent with the notion that U2/U6 helix II is the target of Slt22p. Taken together, these results suggest a potential role of helix II and/or 5' end of U2 snRNA in anchoring and assisting U5 snRNA to tether the two exons after the first catalytic step.

4.3.1. Slt22p is involved in U2/U6 snRNA interaction.

RNA-RNA interactions in the spliceosome undergo extensive conformational rearrangements to form the catalytic center and to accommodate RNA structures required for the two-step transesterification reaction of which the chemical nature is different for the first and second steps (Moore and Sharp 1992, 1993; Sontheimer et al. 1997; also see §1.3.2-2). The high thermodynamic stability of RNA structures (i.e., duplexes) renders the RNA folding process error-prone (Herschlag 1995). The high level of fidelity of pre-mRNA splicing can be
attributable at least in part to protein factors. In particular, RNA-dependent ATPases/RNA helicases may drive RNA conformational rearrangements in the spliceosome. Although DExD/H box proteins have been found in a wide range of organisms (Schmid and Linder 1992), the specific interaction between most RNA helicases and their RNA substrates is poorly understood. However, specific stimulation of ATPase activity by a particular RNA molecule is a strong indication that such an RNA is the substrate of the RNA helicase activity which is involved in unwinding of RNA secondary structure and/or maintaining the optimal conformation of the RNA molecule. The E. coli DbpA is the first DEAD box protein shown to display substrate (23S rRNA) specific stimulation of ATPase activity (Fuller-Pace et al. 1993). The function of the protein is implicated in establishing and/or maintaining the correct three-dimensional structure of the peptidyltransferase center in 23S rRNA; i.e., mutations that disrupt base-pairing in the stem structures of the center ablated stimulation of ATPase activity (Nicol and Fuller-Pace 1995). In yeast, preferential stimulation of ATPase activity by specific snRNA(s) has been only observed for two splicing factors, Prp5p (O’Day et al. 1996) and Slt22p (this study). The potential RNA targets for respective RNA helicases and their interactions are further suggested by complementary genetic studies.

Prp5p is involved in the formation of the pre-spliceosome by promoting the U2 snRNP and branchpoint interaction (Ruby et al. 1993; Wells and Ares 1994). Its ATPase activity is stimulated by synthetic U2 snRNA, and upon ATP hydrolysis, causes a conformational change in U2 snRNA (O’Day et al. 1996). A mutation in prp5 is synthetically lethal with substitutions in U2 snRNA that are important for the U2/branchpoint interaction (Yan and Ares 1996). Consistent with the idea that this protein is involved in maintaining proper conformation of U2 snRNA for its interaction with pre-mRNA at the branchpoint, mutations in other factors required in this step (prp9, prp11 and prp21) are synthetically lethal with prp5 (Ruby et al. 1993; Wells and Ares 1994). Overexpression of Cus1p, required for the integrity of a subdomain of U2 snRNA, is able partially to suppress the defect of prp5 in vivo (Wells et al. 1996). Targets for other two putative RNA helicases, Prp28p and Prp16p, were also identified.
by genetic means (see respective sections in Chapter 1). The function of Prp16p is linked to remodeling of the catalytic center for the second step (Schwer and Guthrie 1992), and its RNA-dependent ATPase activity has been implicated in the proofreading of products of the first step (Burgess and Guthrie 1993a, 1993b). In this model, ATP hydrolysis by Prp16p is in kinetic competition with a productive pathway (which leads to conformational changes necessary for the second step). The rate of ATP hydrolysis determines directly the outcome of proofreading; i.e., productive vs. discard pathway for the lariat-intron-exon 2 intermediate (Burgess and Guthrie 1993a, 1993b).

The RNA-dependent ATPase activity of Slt22p is likely to be involved in monitoring the integrity of U2/U6 helix II interaction. There are several lines of evidence that support this notion: 1) The ATPase activity associated with HA-Slt22p is preferentially stimulated by pre-annealed synthetic U2/U6 snRNAs in vitro. UV-crosslinks specific to U2/U6 snRNAs have been detected under conditions employed in the ATPase assay (Fig. 4.5E). Mutation E909K in the conserved GRAGR motif of RNA helicase, shown to be important for RNA binding, ATP hydrolysis and RNA unwinding for the prototypic RNA helicase, elF 4A (Pause et al. 1993), abolishes stimulated ATPase activity (Fig. 4.6C); 2) When a mutant U2 snRNA [U2(11nt)] carrying the original 11nt substitution used in the genetic screen (Fig. 2.1) was used in an ATPase assay, stimulation by U2(11nt)/U6 is reduced (Fig. 4.6C). Although the 11nt substitution in U2 snRNA disrupts the proposed helix II structure, I have noted that this mutant U2 and the 9nt substitution can potentially form alternative base-pairing interactions with U6 that show resemblance to helix II (Fig. 4.9A). However, in both alternative structures, the stem is disrupted by internal unpaired bulges. If ATPase activity is strongly stimulated by correctly folded RNA substrate of an RNA-dependent ATPase, changes in structural elements in the substrate should result in a reduction or loss of such an activity, as shown in the case of DbpA (Nicol and Fuller-Pace 1995). If this also holds for Slt22p, the reduction of U2(11nt)/U6 stimulation can be attributed to changes in the structural changes in the substrate (likely, helix II). However, other parts of the U2, U6 and/or U2/U6 structures may also contribute
Fig. 4.9. A. U2/U6 helix II interaction and formation of aberrant structures with mutant U2. It has been suggested that the formation of helix II precedes disruption of the U4/U6 interaction (top), and may serve to regulate formation of other RNA interactions (Brow and Vidaver 1995). It is noted that two mutations (11nt and 9nt) in U2 snRNA, that are synthetically lethal with slt22-1, may form alternative interaction with wt U6. Substitution G21C may affect the secondary structure of U2 stem I. B. Suppression of synthetic lethality of slt22-1 and U2-11nt by rss1-1. top: structure of mrz, noticing that the 13bp stem (a) introduced in the exon region of the 3' SS resembles helix II. rss1-1 was isolated as a suppressor of this mrz that blocks the second step of splicing (Lin and Rossi 1996). center: yeast strain used to test suppression of rss1-1. bottom: dominant suppression of synthetic lethality of slt22-1 and U2-11nt by rss1-1.
A

stem I

stem II

U2

helix II

S' exon

pre-mRNA

stem I

stem II

U4

S' exon

U6

ACAG

5'

3'

wt-U6 snRNA

GAAACUUr

UAACACAGAUUU

AUUCGUUr

81

70

51

100

110

10

51

U6 snRNA

GAACCUUr

UAACACAGAUUU

AUUCGUUr

81

70

51

100

110

10

51

11nt-U2 snRNA

GAAACUUr

UAACACAGAUUU

AUUCGUUr

81

70

51

100

110

10

51

9nt-U2 snRNA

GAAACUUr

UAACACAGAUUU

AUUCGUUr

81

70

51

100

110

10

51

G21C-U2 snRNA

GAAACUUr

UAACACAGAUUU

AUUCGUUr

81

70

51

100

110

10

51

B

mrz

3' exon

b

a

intron

slt22-1

SNR20::ΔHIS3

SNR20 or snr20-11nt

URA3

TRP1

LEU2

vector (pRS315)

U2-wt

U2-11nt

5-FOA His' Trp' Leu'
collectively to substrate specificity; and 3) Genetic analyses presented in §3.2.1 and §3.2.2 showed that slt22-1 is specifically synthetic-lethal with mutations in U2 and U6 snRNAs that perturb helix II interaction and that none of the mutations in other DEAD/H splicing factors are synthetically with the U2-11nt and -9nt (§3.2.1).

Additional evidence that Slt22p may unwind helix II comes from another independent genetic screen directed to isolate suppressors that could rescue a block of splicing by the introduction of a mutant hammerhead ribozyme (mzr) at the 3' splice site (Lin and Rossi 1996). The structure of mzr showed resemblance to that of U2/U6 intermolecular helices, including Ia, Ib and II (Fig. 4.9B). Two types of suppressors were isolated in the genetic screen (Lin and Rossi 1996): cis-suppressors, which disrupted or weakened the base-pairing interaction corresponding to helix II; and the trans-suppressor, rss1-1, allelic to slt22-1. Apparently, rss1-1 suppresses the block by unwinding the corresponding helix II region in mzr. The rss1-1 suppressor functions in a dominant manner, and shows synergistic suppression with cis-suppressors (Lin and Rossi 1996). Consistent with the proposition that Slt22p may also act to unwind helix II, I have observed that the rss1-1 mutation could also suppress the synthetic lethality of slt22-1 and U2-11nt substitution in a dominant fashion (Fig. 4.9B). The two mutant alleles of SLT22 [slt22-1 (E909K) and rss1-1 (G858R)], both mapped in the vicinity of the conserved GRAGR motif (Fig. 4.3A), but alter the properties of the protein quite differently. The slt22-1 mutation reduces the RNA-dependent ATPase activity such that it requires correct U2/U6 interaction to execute its additional functions in vivo (i.e., loss of function). The synthetic lethality between slt22-1 and U2-11nt (and 9nt) is likely caused by failure of the misfolded U2/U6 interaction to activate slt22-1p. On the other hand, rss1-1 is a gain-of-function mutation which may reduce its requirement for substrate specificity. However, the acquired promiscuity may escape the regulation of Slt22p in the spliceosome such that it confers severe growth defect in vivo (Lin and Rossi 1996). Consistent with this interpretation, suppression of mzr by rss1-1 is dominant, and the ts phenotype is recessive (Lin and Rossi 1996; data not shown).
Slt22p is distantly related to DEAD/H box proteins involved in pre-mRNA splicing. It contains several new motifs that are conserved only in a class of RNA-helicase like proteins (Fig. 4.2D). Slt22p is large. However, the C-terminal portion is a partial duplication of the central RNA-helicase domain, even though the RNA helicase motifs are not well conserved in this region. It is unlikely that this region has any RNA-dependent ATPase activity. Although this domain can be separated from the rest of protein (provided that the GKT and DAAH boxes are present in both halves), it is essential for the function of Slt22p (Fig. 4.4). The interactions between these two domains and their contribution to activities of Slt22p remain to be determined.

4.3.2. Function of U2/U6 helix II.

During maturation of the spliceosome, the formation of U2/U6 helix II may precede other U2/U6 interactions and stabilize the U4/U6 duplex to antagonize the premature formation of catalytically important RNA structures (§4.1). Since the formation of U2/U6 helix I and U6 Brow stem and that of U4/U6 stem I are mutually exclusive (Fig. 4.10), helix II must be unwound prior to activation of the spliceosome. The identification of Slt22p, an RNA-dependent ATPase (a putative RNA helicase), as a synthetic lethal mutant of U2 and U6 mutations that perturb helix II interaction is consistent with the above notion. It is tempting to suggest that unwinding of helix II by Slt22p is coupled with the dissociation of U4/U6 duplex and the formation of helix I and Brow stem (Fig. 4.10). Thus the unwinding of helix II, likely by Slt22p, is a crucial regulatory step in the activation of the spliceosome.

The potential role of Slt22p in dissociation of U4/U6 duplex has recently been suggested by Raghunathan and Guthrie (personal communication). Another allele of SLT22, brr2-1, was isolated in a genetic screen for cold-sensitive mutations that also affect pre-mRNA processing (Noble and Guthrie 1996). In a brr2-1 splicing extract, the release of U4 snRNP from
Fig. 4.10. A possible role of Slt22p in spliceosome assembly. After the formation of the holo-spliceosome (top right), the 5'-end of U2 snRNA may interact with the 3'-end of U6 snRNA, forming helix II (top), which may act to antagonize the premature formation of other RNA structures important for the subsequent steps, by stabilizing U4/U6 duplex (Brow and Vidaver 1995). The unwinding of helix II is thus a crucial step in the activation of the spliceosome. Likely it is coupled with the disruption of U4/U6 duplex, concomitant with the formation of other U2/U6 interactions (i.e., helices Ia and Ib).
U4/U6.U5 tri-snRNP is blocked, whereas in the wt extract, upon addition of ATP, U4 snRNP is readily released. The brr2-1 mutation is mapped in one of the conserved ATP binding (GKT) motifs. This particular mutation may reveal the potential coupling between unwinding of U2/U6 helix II and dissociation of U4/U6 duplex. However, this coupling is not a rate-limiting step, since helix II is not essential (see §4.1 for references). Energy released from ATP hydrolysis by Slt22p stimulated by U2 and U6 snRNAs (without forming appropriate helix II) is sufficient to promote disassembly of U4/U6 duplex. More recently, brr2-1 has been shown to be synthetically lethal with prp31-1, a mutation that affects the assembly at the formation of the holo-spliceosome (Weidenhammer et al. 1997) (J. Woolford et al. personal communication). Furthermore, slt22-1 is synthetically lethal with prp8-1 (Fig. 5.7), a mutation that affects spliceosome assembly at the same step (Brown and Beggs 1992), but not with mutations that affect the later stages of spliceosome assembly or activation (Table 3.4 and Fig. 3.4). These genetic interactions also support a role for Slt22p in spliceosome assembly.

Although helix II is not essential, once formed, it must be unwound to allow for dissociation of U4/U6. However, mutation E909K in slt22-1 may fail to unwind U2/U6 helix II under non-permissive conditions such that it may trigger a default pathway which leads to the dismantling of the spliceosome (Figs. 4.7 and 4.8; also see below).

4.3.3. Potential anchoring function of U2 snRNA in tethering U5 snRNP in the spliceosome.

What function does the 5'-end of U2 snRNA serve after helix II is resolved? Results presented in Chapter 3 indicate that it may interact with other factors identified in the genetic screen. The 5'-end of U2 snRNA, in association with some of the Slt factors (Sl(l5p/Prp17p, Sl(l7p/Slu7p and Slt21p/Prp8p), may play a role in the second step of splicing, a function
which overlaps that of U5 loop 1 (§3.3.1). Characterization of the \textit{slt22-1} mutation revealed another potential role for U2/U6 helix II and/or the 5'-end of U2.

Spliceosome assembly in the \textit{slt22-1} extract proceeded with normal, but slightly retarded, kinetics at 25°C. However, at a non-permissive temperature, a new complex was formed at the expense of other normal complexes. Since this complex was not detected during the initial incubation (i.e., 1 min), and its accumulation correlated with the reduction of other complexes (Fig. 4.7), complex X likely represents a divergence of spliceosome assembly, rather than a transient state in the normal pathway. This is in agreement with the observation that complexes that accumulated in the mutant extract were terminally stalled (Fig. 4.8). The divergence seems to occur during the transition from complex A2-1 (the holo-spliceosome) to complex A1 (the core-spliceosome). One of the final complexes accumulated, A1-like, indeed contained U2, U5, and U6 snRNAs, but not U4 (Fig. 4.7C). This temporal correlation with the dissociation of U4/U6 duplex in the assembly pathway is consistent with the notion that unwinding of U2/U6 helix II by Slt22p is coupled with the dissociation of U4/U6 duplex. The lack of U5 snRNA in complex X, however, is a surprising result (Fig. 4.7C).

Although mutation E909K abolishes the stimulated ATPase activity of slt22-1p by U2/U6 snRNAs at permissive temperature (Fig. 4.6C), it remains to be determined if other activities (e.g., RNA helicase) are affected. However, splicing defects associated with \textit{slt22-1} at 33°C seem to indicate that this mutation may alter the spliceosome assembly pathway prior to formation of the core-spliceosome. If slt22-1p fails to unwind helix II and/or perform other activities, instead of stalling spliceosome assembly in the midst of extensive conformational rearrangements (e.g., displacement of U1/5' splice site interaction by U6, dissociation of U4/U6 duplex etc.), it may trigger a default pathway. As a result, the spliceosome undergoes a process of active dismantling such that components of the splicing machinery can be recycled. Complexes X and Y (perhaps a further breakdown product of X) (Fig. 4.7) may represent different intermediates in this process.
The absence of U5 snRNA in complex X may indicate an important function provided by 5' end of U2 snRNA and/or 3' end of U6 in anchoring U5 snRNA likely after helix II is resolved by Slt22p. In a related experiment with HeLa extract, it has been observed that an oligonucleotide complementary to U5 snRNA (a region 3' to the loop 1) could induce the formation of a U1/U4/U5 complex (Ast and Weiner 1996), in contrast to complex X. This U1/U4/U5 complex may represent a transient step in spliceosome assembly during which the U1/5' splice site and U4/U6 interactions are being disrupted and displaced by U6/5' splice site and U2/U6 interactions, respectively. If U5 snRNA adopts an appropriate configuration (as induced by the complementary oligonucleotide), U5 snRNP will preferentially associate with U1 and U4 snRNPs that are in the process of dissociating from the rest of the spliceosome (i.e., U2, U6 snRNPs and pre-mRNA).

It has been proposed that the stem-loop structure of the U5 snRNA loop 1 region is analogous to subdomain ID3 of autocatalytic group II introns, which is essential for the 5'-splice-site recognition and tethering of the free 5'-exon (Newman and Norman 1992). However, unlike the loop region of group II ID3, which contains the exon-binding site (EBS1) complementary to the 3'-end of the 5'-exon (Jacquier and Michel 1987), the uridine-rich U5 loop 1 (Fig. 4.11) may interact with the two exons through non-canonical base-pairing, since the exon sequences at both splice sites are not conserved in pre-mRNAs. Additional RNA interaction(s) may be necessary to coordinate and stabilize the fragile U5/exon interaction. In the group II introns, a bulged region, α', at the bottom of ID3 stem-loop, interacts with a region in subdomain IB, α (Harriz-Kerr et al. 1993). Recently, an anchoring function for the tertiary α-α' interaction has been suggested (Hetzer et al. 1997). It has been shown that loop 1 of U5 snRNA is in close contact with the 5' splice site prior to the first catalytic step (Wassarman and Steitz 1992; Wyatt et al. 1992; Sontheimer and Steitz 1993; Newman et al. 1995). The results presented in this chapter (§4.2.5) suggest a role for the helix II region of U2 snRNA and/or U6 snRNA in anchoring U5 snRNA prior to the splicing reaction. It remains to be determined whether direct RNA base-pairing is involved in the anchoring interaction(s). However, it is
most likely that the interactions between the 5'-end of U2 snRNA with several Slt factors or the 3'-end of U6 snRNA may play an additional role in holding U5 snRNP to the rest of the active spliceosome. Three Slt factors, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8p, are related functionally to U5 loop 1 (Frank et al. 1992; Teigeskamp et al. 1995; Umen and Guthrie 1995b, 1995c), while Slt21p/Prp8p and Slt22p/Brr2p/Snu246p are both associated with U5 snRNP (Whittaker et al. 1990; Lauber et al. 1996).
§4.4. Materials and Methods

4.4.1. Yeast strains, genetic manipulation.

All yeast strains used in this study are derived from W303-1A and W303-1B. Genetic screen/characterization and other genetic manipulation (chromosomal deletion, two-step gene replacement etc.) are described in §2.4 and Guthrie and Fink (1991).

4.4.2. Preparation of yeast splicing extract, in vitro splicing assays and native gel electrophoresis.

Whole-cell splicing extracts were prepared according to Lin et al. (1985) with modifications. Synthetic $^{32}$P-labeled yeast pre-actin RNA was made from DNA template using T7 RNA polymerase with $\alpha$-$^{32}$P-UTP. In vitro splicing assays were performed by mixing 5 μl of extract with 5 μl substrate "cocktail" (containing ~250 cpm $^{32}$P-pre-actin RNA and 5mM ATP, 2mM MgCl$_2$ and other components, see Lin et al. 1985 for detail) in a 20 min time course at 25°C and 33°C. Heat treatment was done by incubating extracts at 37°C for 45 min in the absence of substrate. Reactions were stopped by adding equal volume of 100mM Na-phosphate buffer (pH 7.5), 0.5% SDS and 2 μg/μl proteinase K. After incubation at 37°C for 20 min, the mixture was extracted with phenol/chloroform and nucleic acids were precipitated by ethanol. The splicing substrate and products were resolved on a 5% denaturing polyacrylamide gel.

To assess spliceosome assembly, splicing reactions were performed as above, but with reduced amount of $^{32}$P-substrate (~150 cpm, unless otherwise indicated). Splicing reactions were stopped by mixing 5 μl reaction mixture with 5μl 2X loading buffer (10% glycerol, 6% PEG, 120 mM K-phosphate pH7.8, 6 mM MgCl$_2$, and 4 μg/μl heparin) followed by 5 min incubation
Native gel electro-phoresis was described by Cheng and Abelson (1987) with modifications. Aliquots of 5 μl of the final mixture were loaded on a 4% polyacrylamide gel (acrylamide:bis acrylamide = 80:1) containing 1X TEB. The native gel was run at 30V/cm at 4°C for ~4 hr in 1X TEB, 5 μM β-mercaptoethanol.

4.4.3. HA-Tagging of Slt22p.

To tag Slt22p, an SphI site was first created close to the 5‘-end of SLT22 gene by PCR mutagenesis, resulting in E5A change without any apparent effect on the function of Slt22p. DNA sequence coding the HA epitope was then introduced at this site by 2 oligonucleotides: oligo HA1: CATACCCATACGACGTCAGACTACGCTCATG, and oligo HA2: AGCGTAGTCTGGGACGTCGTATGGGTATGCATG. Different versions of the tagged genes, carried on pRS324 (TRP1 2μ), were introduced in a yeast strain carrying a chromosomal deletion of SLT22 gene, and tested for viability and detection by immunoprecipitation/Western blot. One of such tagged protein is shown in Fig. 4.5A. Note that an extra 11 amino-acid epitope (italic) was introduced upstream of the HA epitope (underlined) as a result of insertion of HA-coding sequence in the reverse orientation. The modified SLT22 is fully functional and readily detected by IPP/Western blot probed with 12CA5 antibody. The addition of HA-tag did not exacerbate the ts phenotype of slt22-1.

4.4.4. Immunoprecipitation and ATPase assays.

Splicing extracts were prepared from cells expressing only the tagged proteins. Wild-type extract containing untagged Slt22p was used as control. Extracts containing 2 mg of total proteins were subject to immunoprecipitation manipulation using monoclonal antibody 12CA5 (IgG from ascitic fluid, Boehringer Mannheim) and protein A sepharose beads (Koledzej and
Young 1991, with modification). Proteins bound to the beads, after extensive wash with buffer containing 250 mM NaCl, were dissolved on a 5% SDS-polyacrylamide gel. Following electroblotting, protein blot was probed with supernatant of 12CA5 cell culture and goat anti-mouse IgG conjugated to horseradish peroxides and detected by enhance chemiluminescent (ECL) kit (Kirkegaard & Perry Lab.). Approximately 20 ng of HA-Slt22p or HA-slt22-lp could be obtained by immunoprecipitation from 2 mg of starting materials (as determined by silver staining).

Synthetic snRNAs were made from T7-snRNA templates as described in McPheeters and Abelson (1992). The T7-U2(11nt) template was constructed with plasmid pGEM3Z (Promega). To remove unincorporated nucleotides, Microcon method was used following manufacturer's instruction (Amicon Inc.). Prior to ATPase reaction, RNAs were subject to annealing as follow: 65°C for 15 min, 42°C for 45 min, and then transferred to RT for 10 min. About 10 ng of HA-Slt22p was used in a single ATPase assay (Fuller-Pace et al. 1993) with 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT, 2 μM ATP and 12.5 nCi [γ-32P]ATP in a final volume of 25 μl containing 250 ng of each synthetic snRNA: U2, U2S (a shorter version of U2 snRNA containing only the 5' end stem I and II, ~360 nt), U4, U5 and U6, alone or in mixture (U2/U6, U2S/U6 and U4/U6). Reaction mixtures were incubated at 25°C for time indicated with constant shaking, and 1 μl of 100 μM EDTA and 1 mM ATP were than added to stop the reaction. Aliquots of 1.5 μl were loaded on PEI TLC plates, subsequently developed in 1M formic acid, 0.5 M lithium chloride. After Phosphor Image (Molecular Dynamics) quantitation, ATP hydrolysis, measured by release of free phosphate, was calculated from the percentage of free radioactive phosphate released, and background free phosphate resulting from incubation with immunoprecipitants of untagged wt extract was subtracted.
Chapter 5.

Role of Prp8p/Slt21p and U2 snRNA in the Formation of the Active Spliceosome and Recognition of the 3' Splice Site.
Abstract

Prp8p is a highly conserved splicing factor important for both steps of the pre-mRNA splicing reaction. Its action has been implicated in the recognition of both the 5'- and 3'-splice sites and the polypyrimidine tract in the pre-mRNA substrate and other RNA/RNA interactions in the spliceosome. A new allele of prp8-21/slt21 was isolated on the basis of synthetic lethality with an 11nt substitution in the yeast U2 snRNA that could perturb the U2/U6 snRNA helix II interaction. The prp8-21 mutation (Q1902K) is located in the polypyrimidine tract recognition domain, which is important for the 3' splice site selection (Umen and Guthrie 1996, Genetics 143:723). By genetic criteria, the synthetic lethality of prp8-21 is specific to mutations in the 5'-end of U2 snRNA, rather than U2/U6 helix II. Although no splicing defect was observed with a prp8-21 extract, activation of the spliceosome was blocked in an extract prepared from cells containing both prp8-21 and U2-G21C/C14G mutations. When a pre-mRNA substrate that lacks the polypyrimidine tract was used, spliceosome assembly was blocked at an early step in the prp8-21 extract. These results suggest that prp8-21 is defective primarily in its interaction with the polypyrimidine tract, and that U2 mutations exacerbate prp8-21 during the activation of the spliceosome. A potential role in the second step of splicing (3' splice site selection) for the 5' end of U2 snRNA has been suggested on the basis of genetic interactions with loop 1 of U5 snRNA (§3.2.3). prp8-21 was also synthetically lethal with slt17/slu7-100 and slu7-1, the corresponding protein of which is required for selection of the 3' splice site. Taken together, our results indicate that Prp8p is involved in mediating and coordinating recognition of the 3' splice site by several factors, including loop 1 of U5 snRNA, the 5' end of U2 snRNA and Slu7p, and that the interaction between Prp8p and U2 snRNA is required for the activation of the spliceosome.
55.1. Introduction

During spliceosome assembly, the splice sites in the pre-mRNA substrate are recognized and brought to close proximity by components of the spliceosome. The recognition of the 5' splice site (5' SS) and the branchpoint site (BPS) requires direct RNA-RNA interactions with U1 and U2 snRNAs, respectively (§1.3.1). In yeast, these two events and the recognition of the polypyrimidine tract (PyT, between the BPS and the 3' SS) are mediated and coordinated by a set of protein-protein interactions involving Prp40p (a U1 snRNP protein, bound to the 5' SS), BBP (bound to the BPS), Mud2p (bound to the PyT) and Prp11p (one component of SF3a, required for binding of U2 snRNP to the pre-mRNA) in the initial steps of spliceosome assembly (§1.2.1 and Fig. 1.4). Following addition of the U4/U6.U5 tri-snRNP, the initial base-pairing interaction between U1 and the 5' SS is displaced by interaction with the highly conserved ACAGAG motif in U6 snRNA (§1.3.2-1). The 3'-end of U6 snRNA also forms base-pairing interaction with the 5'-end of U2 snRNA; i.e., helix II. Results presented in the previous chapter and those of Brow and Vidaver (1995) indicate that the formation and the unwinding of this intermolecular helix may serve to regulate other RNA-RNA interactions that are important for the subsequent catalytic steps. The unwinding of helix II, likely by Slt22p, and the concomitant disruption of the U4/U6 duplex lead to the formation of additional U2/U6 interactions (i.e., helix I) and the U6 Brow stem which are responsible for the juxtaposition of the 5' SS and the BPS (§1.3.2). It is thus evident that RNA-RNA interactions and conformational rearrangements are highly coordinated in the spliceosome.

The selection/recognition of the 3' SS depends on both cis- and trans-acting elements. The 3' SS is usually separated from the BPS by a relatively short stretch of nucleotides which contains the PyT. The last two nucleotides, AG, of the intron are also conserved (Rymond and Rosbash 1992; Moore et al. 1993). Both the PyT and the AG dinucleotide are important for the efficient selection/recognition of the 3' SS (Patterson and Guthrie 1991; Umen and Guthrie...
Chapter 5

1995b, 1996; Luukonen and Séraphin 1997). A non-Watson-Crick interaction between the first and last nucleotides of the intron has been documented (Parker and Siliciano 1993; Chanfrau et al. 1994; Scaddena and Smith 1995). Furthermore, the spacing between the BPS and the 3' SS can also influence the utilization of the 3' SS (Chiara et al. 1997; Luukonen and Séraphin 1997). Although direct Watson-Crick base-pairing interaction does not seem to be involved in the selection/recognition of the 3' SS, two snRNAs in the active spliceosome, U2 and U5 snRNAs, are crosslinked to the exon region of the 3' SS prior to the second step (Sontheimer and Steitz 1993; Newman et al. 1995). The tethering function of U5 loop 1 in aligning the two exons for the second step has been demonstrated in yeast (O'Keefe et al. 1996; O'Keefe and Newman 1998). Mutations in U5 loop 1 can also influence the 3' SS selection (Newman and Norman 1992).

A functional role for the 5' end of U2 snRNA in the second step of splicing has been suggested by its genetic interactions with both U5 loop 1 (§3.2.3) and three Slt factors identified in the genetic screen (Chapter 2) that are also required for second step and/or 3' SS selection (§3.2.1). Slt21p/Prp8p is required for recognition of the 3' SS and the PyT (Umen and Guthrie 1995b, 1996). Both Prp8p and the human homologue, hPrp8p, bind to the 5' SS, BPS and 3' SS (Wyatt et al. 1992; MacMillan et al. 1994; Query et al. 1994; Teigelkamp et al. 1995; Umen and Guthrie 1995c; Chiara et al. 1996, 1997). It has been suggested that Prp8p may aid U5 snRNA in tethering and aligning the two exons for the second step (Teigelkamp et al. 1995; O'Keefe et al. 1996). Slt17p/Slu7p is involved in the 3' SS selection (Frank and Guthrie 1992; Umen and Guthrie 1995c; Brys and Schwer 1996). The other Slt factor, Slt15p/ Prp17p is required for efficient ligation of the two exons (Jones et al. 1995). However, Prp17p and Slu7p exert their functions prior to and following hydrolysis of ATP by Prp16p, respectively (Jones et al. 1995), and are related to the function of U5 loop 1 (Frank et al. 1995). Other protein factors required for the second splicing step include, Prp16p, an RNA dependent ATPase/RNA helicase, whose function has been implicated in remodeling of the spliceosome (Schwer and Guthrie 1992b) and proofreading of the first step reaction (Burgess and Guthrie 1993a), Prp18p (Horowitz and
Abelson 1993a, 1993b), and SSF1 (Ansari and Schwer 1994) (also see Table 1.2, and Umen and Guthrie 1995a for a review). It is clear that selection/recognition of the 3' SS is achieved by a collection of protein and RNA factors in sequential events. However, a question remains as to how these different events are coordinated temporally. In particular, tethering of the two exons by U5 loop 1 occurs only after the first splicing step (Newman et al. 1995; O'Keefe et al. 1996). It remains to be determined how this is connected to selection/recognition of the 3' SS by other factors, some of which may act prior to the second step.

Recently, Chiara et al. (1997) showed evidence that in mammalian pre-mRNA splicing, the PyT plays a central role in connecting these factors/events. As mentioned above, U2AF65 (the mammalian homologue of Mud2p) binds to the PyT in the initial step of spliceosome assembly. As assembly progresses, its binding is replaced by three U5 snRNP-associated proteins including hPrp8p, p110 and p116 (Chiara et al. 1997). It has been suggested that an interaction between the U2 snRNP, bound to the BPS, and the U5 snRNP is important for positioning U5 snRNA on the 3' SS. This notion is supported by the results presented in Chapter 3 (see §3.3.2 for detail). In this chapter, results of characterization of the slt21/prp8-21 mutation and its genetic interaction with U2 snRNA will be presented. The slt21/prp8-21 has been mapped to a domain in Prp8p that has been previously identified as being important for recognition of the PyT, which is involved in 3' SS selection (Umen and Guthrie 1996). However, defects associated with slt21/prp8-21 alone or in combination with a mutation in U2 block splicing prior to the first step during spliceosome assembly. My results suggest a possible role for Slt21p/Prp8p in coordinating recognition of the 3' SS during spliceosome assembly and in both steps of the splicing reaction. Furthermore, the interaction between the 5' end of U2 snRNA and Slt21p/Prp8p (and other factors) is required for activation of the spliceosome.
§5.2.  Results

5.2.1  slt21 is a new allele of prp8.

The synthetic lethal genetic screen (Chapter 2) yielded nine candidate slt mutants, one of which, slt21, when crossed to temperature sensitive (ts) prp strains, failed to complement prp8-1 at 37°C (Fig. 5.1A and Table 2.2). The original slt21 strain, containing a chromosomal disruption of SNR20 (the U2 snRNA gene), showed a ts phenotype at 37°C in the presence of a plasmid carrying the wt U2 gene. A low-copy (CENARS LEU2) plasmid carrying the wt PRP8 gene was introduced into this strain and resulted in full complementation of the ts phenotype and synthetic lethality (data not shown), suggesting that slt21 likely is a new allele of prp8.

To test this possibility and to identify the mutation at the PRP8 locus, a null allele of the PRP8 gene was created by deletion of the two internal 3.7Kb BglII fragments in the coding region (Fig. 5.1B), and then transformed into the original slt21 strain. None of the resulting transformants tested (>20) complemented the phenotypes mentioned above. The full-length gene encoding the mutant prp8 allele obtained by this gapping method was tested in a yeast strain carrying a chromosomal disruption of PRP8, and was found to confer a slow-growth phenotype at 37°C (Fig. 5.1B). Overexpression of this prp8 allele from a high-copy (2μ) plasmid did not suppress itself (data not shown). This new allele was then named as prp8-21.

Subsequently, the mutant allele was introduced by two-step gene replacement into the chromosome as the sole copy of PRP8 gene (Fig. 5.1C). On the chromosome, prp8-21 conferred a slow-growth phenotype at 37°C, but was otherwise indistinguishable from wt at other temperatures (Fig. 2.3). This strain was then crossed to a ΔSNR20 strain (i.e., with a chromosomal deletion of the U2 gene) to create haploids containing both prp8-21 and ΔSNR20 (with URA3 maintenance plasmid carrying SNR20). Synthetic lethality of prp8-21 and U2-11nt
Fig. 5.1.  *slt21* is a new allele of *prp8*. A. Complementation test. The original *slt21* strain (containing *SNR20Δ::HIS3*) was crossed to *prp* strains for complementation at 37°C. Shown is the 2-day growth of heterozygous diploids on YPD at 37°C. Note that *slt21/prp8-21* diploid displays a slow growth phenotype. B. Isolation of *prp8* sequence from *slt21* strain by gapping method. *Top:* A linearized CNEARS plasmid (*LEU2*) carrying the *PRP8* gene lacking the two internal *BglII* fragments (3.7Kb) was introduced into the original *slt21* strain. The gap was then filled by homologous recombination. The *prp8* plasmid (*prp8-21/original*) was then recovered. *Bottom:* *prp8-21* confers slow-growth at 37°C. The *PRP8*, *prp8-21/original*, a chimeric plasmid (*prp8-21/swapped*) containing *SphI-SpeI* fragment from *prp8-21/original* (see Fig. 5.3A) were introduced into a *ΔPRP8* strain and tested for growth phenotype. Shown is the 2-day growth of the resulting transformants in the absence of the wt *PRP8* maintenance plasmid at 37°C. C. Two-step gene replacement of *prp8-21*. Step 1. An integration plasmid carrying the full-length *prp8-21* gene was first introduced into a wt strain (containing *SNR20*) resulting the integration of *prp8-21* at the *PRP8* locus. Step 2. The extra copy of *PRP8* or *prp8-21* gene was excised through homologous recombination resulting *prp8-21* or *PRP8* gene on the chromosome, respectively. D. *prp8-21* is synthetically lethal with U2-11nt. The chromosomal *prp8-21* mutation was segregated genetically to the *SNR20Δ::HIS3* background to generate a yeast strain shown in top. Both U2-wt and U2-11nt were introduced into wt and *prp8-21* strains containing *SNR20Δ::HIS3*. Shown is 2-day growth at 30°C of the resulting transformants on medium containing 5-FOA.
was confirmed in such strains (Fig. 5.1D). Thus, the prp8-21 mutation is both necessary and sufficient to confer synthetic lethality with the U2 mutation.

5.2.2. Allele-specificity and U2 suppressors of synthetic lethality.

The synthetic lethality of prp8-21 with U2 snRNA is allele-specific with respect to mutations in the stem I region. In addition to the original 11nt substitution, a shorter version, an 9nt substitution (Fig. 3.1A) was also synthetically lethal with prp8-21. Among 25 single-nucleotide substitutions on both sides of stem I, only G21C showed synthetic lethality with prp8-21 at all temperatures, and C22G was partially synthetic-lethal (Table 5.1, and data not shown).

Results presented in Chapters 3 and 4 indicate that during maturation of the spliceosome, U2/U6 helix II is unwound, likely by Slt22p, in order to form other RNA interactions/structures which are important for the catalytic steps, and that the 5'-end of U2 snRNA may subsequently interact with other components of the spliceosome (§3.3.1 and §4.3.2). Nucleotides 21, 22, and 23 in U2 snRNA potentially can base-pair with nucleotide 61, 60 and 59 in U6 snRNA forming helix Ib (Fig. 1.6). However, this interaction has been demonstrated only partially between A56 (U6) and U23 (U2) (Madhani and Guthrie 1992; also see §1.3.3-2). Phenotypic asymmetry was observed in this part of U2/U6 interaction: while substitutions at positions 59, 60 and 61 in U6 confer deleterious growth defect in vivo (Madhani and Guthrie 1992) and inhibit splicing in vitro (Fabrizio and Abelson 1990), their U2 partners are more tolerant to mutations (with the exception of G21C, Madhani and Guthrie 1992). These results suggest that the function of the helix Ib portion is secondary and that either or both part(s) of helix Ib may interact separately with other components in the spliceosome for additional functions.
As shown in §3.2.2, the prp8-21 mutation is not synthetically lethal with mutations (U6-a, -b, -c and -d) in the 3' of U6 snRNA that affect helix II interaction. None of these U6 mutations suppressed synthetic lethality of prp8-21 with U2-11nt or U2-9nt (Fig. 3.2). These results indicate that Prp8p is not directly involved in U2/U6 helix II interaction. The asymmetric synthetic lethality may indicate a specific interaction between Prp8p and the 5'-end of U2 snRNA. The genetic interaction between prp8-21 and U2-G21C was explored further.

It has been observed that U2-G21C mutation can be suppressed partially by C14G, which restores the potential base-pairing between 21 and 14 position in the top part of stem I (D. Field and J.D. Friesen, unpublished observation). Genetic suppression of synthetic lethality of prp8-21 with U2-G21C mutation was tested in combination with substitutions at position 14. While neither the C14G or C14A substitution of U2 snRNA was synthetically lethal with prp8-21 (Fig. 5.2A), double-substitution of C14G/G21C was able to suppress partially the synthetic lethality of prp8-21 with G21C; i.e., the combination of prp8-21 and U2-C14G/G21C was viable at \( \leq 30^\circ C \), but temperature sensitive at \( \geq 33^\circ C \) (Fig. 5.2A and Table 5.1). Weaker suppression was observed with C14A/G21C, although base-pairing between residues 14 and 21 is not restored (Fig. 5.2A). In addition, one particular substitution of U19G suppressed partially the aforementioned synthetic lethality as well. However, substitution at the adjacent G20 position, G20C, failed to suppress synthetic lethality caused by U2-G21C and prp8-21 (Fig. 5.2B). Since the remaining slt mutations isolated in the genetic screen are synthetic lethal with U2-G21C (§3.2.1), three of them (slt11-1, slt17-1 and slt22-1) and \( \Delta SLT11 \) (see Chapter 6), were tested for suppression by substitutions C14G, C14A, U19G and G20C in combination with G21C of U2 snRNA, however, none was able to suppress (Table 5.1). Thus, suppression by second-site mutations in U2 snRNA is specific to synthetic lethality of prp8-21 with U2-G21C.
Fig. 5.2. U2 suppressors of synthetic lethality of *prp8-21* with U2-G21C. *prp8-21* is synthetically lethal with substitution G21C of U2 snRNA. The synthetic lethality was suppressed partially by second-site substitutions in U2 snRNA, C14G, C14A (A), and U19G (B), at 25°C (also see Table 5.1). Locations of G21C and second-site substitutions in U2 snRNA and in the context of U2/U6 interactions are shown in lower panels of A and B, and C respectively. Dots indicate the helix II region of U2 snRNA. An 11nt or 9nt substitution in this region is also synthetically lethal with *prp8-21*. 
Table 5.1.  U2 suppressors of the synthetic lethality of slt mutations with U2-G21C.

<table>
<thead>
<tr>
<th>U2 mutation</th>
<th>WT(^b)</th>
<th>(slt21)</th>
<th>(slt11-1)</th>
<th>(\Delta SLT11)</th>
<th>(slt17)</th>
<th>(slt22-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(sg@37^\circ C)</td>
<td>(ts\geq33^\circ C)</td>
<td>(ts\geq33^\circ C)</td>
<td>(ts@37^\circ C)</td>
<td>(ts\geq33^\circ C), (sg)</td>
</tr>
<tr>
<td>G21Cd</td>
<td>ts @ 35°C, sg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G21C/C14G</td>
<td>sg @37°C</td>
<td>+, (ts\geq33^\circ C)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C14G</td>
<td>wt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>G21C/C14A</td>
<td>ts\geq35°C, sg</td>
<td>+, (ts\geq30^\circ C)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C14A</td>
<td>wt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>G21C/U19G</td>
<td>ts @ 37°C</td>
<td>+, (ts\geq33^\circ C)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U19G</td>
<td>wt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>G21C/G20C</td>
<td>ts @ 37°C, sg @35°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G20C</td>
<td>wt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Notes:

a. U2 mutations were introduced into \(\Delta SNR20\) strains carrying slt mutation (e.g., Fig. 5.1D, top) and tested for synthetic lethality. Symbols: ++, no additive growth defect; +, partial suppression (growth defect as shown); -, synthetic lethal.

b. Growth defect of U2 mutations. Symbols: wt, wild-type growth; ts, temperature-sensitivity; sg, slow-growth.

c. Phenotype of slt mutation, also see Fig. 2.3.

d. Growth defect at 16°C was not tested.
5.2.3. *prp8-21* mutation is located in the polypyrimidine tract recognition domain in Prp8p.

To map the *prp8-21* mutation, restriction fragments of *prp8-21* and *PRP8* plasmids were exchanged *in vitro*, and the resultant chimeric genes were tested in the Δ*PRP8* strain for a slow-growth phenotype at 37°C and complementation of synthetic lethality of *prp8-21* and U2-11nt. The mutation was mapped to the 3.75 Kbp *SpeI-SphI* fragment (Fig. 5.3A). It was subsequently found that *prp8-21* contains a single G → A transition that results in mutation Q1902K near the C-terminus of Prp8p (Fig. 5.3B).

Mutation Q1902K is located in a domain that has been shown to be important for the recognition of the PyT (uridine tract in yeast) (Umen and Guthrie 1996). The polypyrimidine tract, located between the BPS and the 3' SS, is important for 3' SS election (Patterson and Guthrie 1991). However, when the PyT and 3' SS are duplicated, the proximal 3' SS will be used preferentially (Patterson and Guthrie 1991; Umen and Guthrie 1995b). In a genetic screen to identify *psf* (polyuridine splicing factor) mutants, *prp8-101/psf1* (E1960K) was isolated that abolished such preference (i.e., defective in recognition of the PyT) (Umen and Guthrie 1995b). Additional *prp8* mutations (7 in total) were isolated in the subsequent genetic screen (Umen and Guthrie 1996). All of these mutations fell into the ~140 a.a. domain near the C-terminus of Prp8p. *prp8-21* is located in the center of this domain, and the wt Q1902 amino acid is highly conserved (Fig. 5.3B). This location of the *prp8-21* mutation suggests that the mutant protein might be defective in recognition of the PyT.
Fig. 5.3. \textit{prp8-21} mutation (Q1902K) is located in the polypyrimidine tract recognition domain. \textbf{A.} \textit{prp8-21} is located in the \textit{SphI-SpeI} (-3.5Kb) fragment. The \textit{SphI-SpeI} fragments of \textit{PRP8} (open box) and \textit{prp8-21} (filled box) were exchanged \textit{in vitro}, and the resultant chimeric plasmids were tested for growth defect and complementation of synthetic lethality of \textit{prp8-21} with U2-11nt. The entire \textit{SphI-SpeI} fragment of \textit{prp8-21} was subcloned and sequenced in order to map the mutation. \textbf{B.} Functional domain structure of Prp8p and the polypyrimidine tract recognition domain. \textit{Bottom:} schematic representation of functional domains in Prp8p. The 3' splice site fidelity and the polypyrimidine tract recognition domains are described in Umen and Guthrie (1996). Sources of other domains: Prp40p-interacting (Abovich and Roshbash 1997): P.G. Siliciano, S.W. Ruby et al. personal communication; Snplp-interacting: S.W. Ruby et al. personal communication; 5' splice site binding: J.L. Reyes, M.M. Konarska personal communication. \textit{prp8-201} is also located near the C-terminus. \textit{prp8-1} (K2347D) is located near the C-terminal region. \textit{Top:} Amino-acid sequence alignment of the polypyrimidine tract recognition domain of Prp8p homologues from other species: \textit{Sc} = \textit{Saccharomyces cerevisiae}; \textit{Sp} = \textit{Schizosaccharomyces pombe}; \textit{Hs} = \textit{Homo sapiens}; \textit{Ce} = \textit{Caenorhabditis elegans}; \textit{Tb} = \textit{Trypanosoma brucei}. Amino-acids that are conserved in all five proteins are high-lighted.
A

Phenotype complementation of
at 37°C

Synthetic lethality

Wt

Slow-growth

- 1 Kb

PRP8

BglII BglII BglII BglII Sphi

Prp8-21

B

L (prp8-103, -105, -107)

Sc QPPEANNSSY ALPNDIKL F-PRP8TVVRV TvhKSHTVNVTATKAIKCIIF TLNPKCVRTIF KIIIDIIWA

Sp QPYCQSSSY AMPNIVQQL F-VADTNVRV TIfKPTXNL TTPKPhIAIF IFNPITQAPF KVIITITWA

Hs TPYNSSSY QILENQIIW F-PAADTVVRV TIKPEPHNL TTKPPhIAIF IFNPITQAPF KIIIDIIWA

Ce TPYTSQNY QILENQIIW F-PAADTVVRV TIKPEPHNL TTKPPhIAIF IFNPITQAPF KIIIDIIWA

Tb TIAAGTVNII AAMSEGMRW DTGSSATVT SEQPSAPGR KFRSEKAVL IFEPLAAK KIVETKFA

K (prp8-21)

Sc QPPELSQAK WTEPEVSAL VELLPKEEQP QKIIWTXKAM LDPL-EVHLLEPNTVAPRTE RJPFPSSAAMS

Sp QPPELSQAK WTEPEVSAL IELPPEEPQ QKIIWTXKGM LDPL-EVHLLEPNTVKGSE RQPFPQAIIK

Hs QPPELSQAK WTEPEVSAL IELPPEEQP QKIIWTXKDM LDPL-EVHLLEKNTVKGSE RQPFPQQACLK

Ce QPPELSQAK WTEPEVSAL IELPPEEPQ QKIIWTXKAM LDPL-EVHLLEPNTVKGSE RQPFPQAIMK

Tb QPPELSQAK WTEPEVSAL IELPPEEPQ QKIIWTXKAM LDPL-EVHLLEPNTVKGSE RQPFPQAIMK

G (prp8-102)

K (prp8-101)

N- (2,413)

Prp40p interaction
(N-terminal P-rich region)

Snp1p interaction
(-1,100 - 1,200)

Polypyrimidine tract
recognition
(1,631 - 1,970)

Splice site fidelity
(1,395 - 1,625)

Splice site binding
(C-terminus)

- 170 -
5.2.4. *In vitro* splicing defects.

A whole-cell splicing extract was prepared from *prp8-21* cells (obtained after two-step gene replacement, in the wt SNR20 background, Fig. 5.1C) to measure potential *in vitro* splicing defects due to the mutation. When *in vitro* splicing reactions were performed at 25°C or 33°C, the activity of the *prp8-21* extract was almost indistinguishable from that of wt (Fig. 5.4A, lanes 1 - 10). Following heat-treatment at 37°C for 60 min, both wt and *prp8-21* extracts (Δwt and Δ8-21) retained comparable splicing activities when assayed at 25°C (Fig. 5.4A, lanes 11 - 14). These results suggest that any splicing defect caused by *prp8-21* itself can not be detected at 25°C and 33°C, and is resistant to heat-treatment. This is consistent with the mild growth defect caused by the mutation (i.e., slow growth only at 37°C, Fig. 5.1B).

It would be ideal to determine the combinatorial splicing defect of *prp8-21* and a U2-mutation that generate synthetic lethality. However, since in the absence of wt-U2 maintenance plasmid cells harboring both mutations are lethal, it is impossible to prepare such a splicing extract with the materials available. Two separate splicing extracts were then made from yeast cells containing *prp8-21* and U2-G21C/C14G double mutations and were subjected to a series of *in vitro* splicing assays in order to gain understanding of the combinatorial defects caused by synthetic lethality of *prp8-21* with U2-G21C (which is suppressed by U2-G21C/C14G, Fig. 5.2A). The same results were observed with both extracts independently. Consistent with partial suppression observed (Fig. 5.2A), the *prp8-21* + U2-G21C/C14G extract (*prp8-21*) showed significantly lower splicing activity compared to that of wild type at 25°C (Fig. 5.4B, lanes 1 and 2). Heat-treatment at 37°C for 45 min, which led to loss of splicing activities of both *prp8-1* and *prp2-1* extracts (Fig. 5.4B, lanes 7 and 8), failed to inactivate the splicing activity of *prp8-21* extract when assayed at 25°C (Fig. 5.4B, lane 6). However, when *in vitro* splicing assay was performed at 33°C, activities of *prp8-21*, *prp8-1* and *prp2-1* extracts were
Fig. 5.4. **Splicing defects associated with prp8-21, and prp8-21 + U2-G21C/C14G extracts.** A. Splicing activity of the prp8-21 extract is comparable to that of wt. Splicing activities of wt and prp8-21 extracts were assayed using ³²P-labeled pre-actin substrate *in vitro* at 25°C (lanes 1-6) or 33°C (lanes 7-10) without prior treatment, or at 25°C with prior heat treatment at 37°C for 60 min (lanes 11-14). Precursor RNA, intermediates (exon 1, lariat intron-exon 2) and final products (mature RNA, lariat intron) were indicated in the middle. Note that lariat intron ran very close to the precursor in these gels. B. *prp8-21* (i.e., prp8-21 + U2-G21C/C14G) extract is resistant to heat inactivation, but is completely inactive at 33°C. Extracts of wt, prp8-21*, prp8-1 and prp2-1 were assayed for *in vitro* splicing activity at 25°C without (lanes 1-4) or with (lanes 5-8) prior heat inactivation treatment at 37°C for 45 min, and assayed at 33°C (lanes 9-12). Results of *in vitro* complementation of prp8-21* with prp8-1 and prp2-1 are shown in lanes 13 and 14. All reactions were incubated for 20 min. Note that prp8-21* extract retained comparable splicing activity following heat treatment (lane 6, cf. lane 2). C. Spliceosome assembly is blocked at 33°C at the formation of complex A1 in the prp8-21* extract. Similar assembly profile was observed at 25°C (data not shown). Scheme of spliceosome assembly is shown on the right.
all abolished, no preferential accumulation of either intermediate was observed (Fig. 5.4B, lanes 10 - 12). The prp8-21* extract could be complemented by prp2-1 but not by prp8-1 extract (Fig. 5.4B, lanes 13, 14).

Heat treatment has been used to inactivate a particular component of the splicing machinery that carries a thermolabile mutation susceptible to irreversible heat denaturation. It has been shown that such a heat treatment (37°C for ≥ 45min) inactivates irreversibly a splicing extract carrying either the prp2-1 (Kim and Lin 1993) or prp8-1 (Brown and Beggs 1992) mutation. However, the prp8-21 extract was resistant to the heat treatment (Fig. 5.4A, lanes 13 and 14), suggesting that the mutation (conferring only slow-growth at 37°C, Fig. 5.1B) is not susceptible to irreversible heat denaturation. D. Field et al. (unpublished observations) have shown that the U2-G21C extract displayed a splicing activity similar to that of the wt extract at both 25°C and 33°C. Thus, it is likely that the low level splicing activity of the prp8-21* extract (Fig. 5.4B, lane 2) is due to the combinatorial defect of the prp8-21 mutation and U2-G21C/C14G. Since the second-site mutation C14G suppressed partially the synthetic lethality of prp8-21 with U2-G21C (Fig. 5.2A), only a low level of splicing activity was detected in the prp8-21* extract (Fig. 5.4B, lane 2). Nevertheless, this residual activity was resistant to heat treatment (Fig. 5.4B, lane 6), suggesting that the combinatorial defect associated with prp8-21 and U2-G21C/C14G is not susceptible to irreversible heat denaturation. However, when in vitro splicing reactions were performed at 33°C, this residual activity was completely abolished (Fig. 5.4B, lane 10). These results, taken together, suggest that the possible combinatorial defect associated with prp8-21 and U2-G21C/C14G mutations may affect a certain step in spliceosome assembly (see below) such that the efficiency of this step is drastically reduced at the permissive temperature (i.e., partial suppression) but is completely blocked at the non-permissive temperature.

Native gel electrophoresis was then used to examine spliceosome assembly. In the wild-type extract, spliceosome assembly proceeded as described by Cheng and Abelson (1978): B →
A2-1 → A1 → A2-2, at both 25°C (not shown) and 33°C (Fig. 5.4C, lanes 1 - 4). However at 33°C, assembly in the prp8-21* extract was blocked at the transition from complex A1 (the core-spliceosome, without catalytic activity) to complex A2-2 (the active spliceosome, with catalytic activity) (Fig. 5.4C, lanes 5 - 8). Following 10 min incubation at 33°C, all the pre-splicing complexes (B and A2-1) were chased to complex A1 without any detectable formation of complex A2-2 (Fig. 5.4C, lane 8). The complex that runs at the position marked as A2 after a shorter incubation time, ≤ 5 min (Fig. 5.4C, lanes 5 and 6), represents A2-1, the holo-spliceosome. At 25°C, preferential accumulation of complex A1 was also observed after prolonged incubation in the prp8-21* extract (≥ 20 min, data not shown), consistent with the low level of splicing activity (Fig. 5.4B, lane 2).

An experiment was carried out to determine whether complex A1 which accumulates in the prp8-21* extract at 33°C is a functional intermediate of the normal assembly pathway. Following incubation of the prp8-21* extract with labeled substrate at 33°C for 20 min (i.e., after ≥90% of substrate was converted into complex A1), the splicing reaction was then transferred to 25°C and allowed for another 20 min incubation. A similar level of splicing activity, comparable to that obtained after incubation at 25°C for 20 min (Fig. 5.4B, lane 2), was restored after such a manipulation (Fig. 5.5A, lane 2), suggesting that complex A1 accumulated at 33°C is a functional intermediate. A similar result was obtained with a prp2-1 extract (Fig. 5.5A, lane 4). On the other hand, the activity of prp8-1 extract is extremely heat labile; i.e., no activity was detected after transfer to 25°C following the initial 20 min incubation at 33°C (Fig. 5.5A, lane 3).

Since hydrolysis of ATP by an RNA-dependent ATPase, Prp2p (DEAH protein), is involved in the transition from complex A1 to complex A2-2 (Kim and Lin 1993; Plumpton et al. 1994; Teigelkamp et al. 1994), I determined whether the actions of Prp8p and U2 snRNA during this transition are exerted before/concomitant with or after Prp2p. The prp8-21* and prp2-1 extracts were incubated with labeled substrate for 20 min at 33°C, and before
Fig. 5.5. Recovery of splicing activity in the prp8-21* extract. A. Wild-type and mutant (prp8-21*, prp8-1 and prp2-1) extracts were first incubated with \(^{32}\)P-labeled pre-actin substrate for 20 min at 33°C (non-permissive temperature for these mutant extracts) and then transferred to 25°C for an additional incubation for 20 min (lanes 1-4). Note that splicing activities of both prp8-21* and prp2-1 extracts were recovered after the second incubation. When glucose was added prior to the transfer to 25°C (to deplete NTPs), no splicing activity was recovered (lanes 5-8). B. Schematic representation of modes of action of Prp8p, U2 snRNA and Prp2p. During the transition from complex A1 to complex A2-2 (i.e., activation of the spliceosome), the interaction between Prp8p and U2 snRNA exerts its function prior to ATP hydrolysis by Prp2p.
transferring to 25°C, glucose (at the final concentrations of 2 and 5 mM) was added to the reaction and incubated for another 15 min. It has been shown that the addition of glucose to splicing reaction is able to deplete NTP molecules in the splicing extract (Horowitz and Abelson 1993). Both extracts, depleted of NTP, were then transferred to 25°C and incubated for an additional 20 min. Splicing in the prp2-1 extract is completely blocked (Fig. 5.5A, lanes 7 and 8). If ATP hydrolysis by Prp2p occurs prior to the block caused by prp8-21 and U2-G21C/C14G mutations; i.e., Prp8p and U2 snRNA exert their functions after Prp2p and does not need hydrolysis of ATP, splicing activity should be restored after shift to 25°C. However, the opposite was observed (Fig. 5.5A, lanes 5 and 6), suggesting that during the formation of the active spliceosome, Prp8p and U2 snRNA function prior to or concomitant with Prp2p (Fig. 5.5B). The loss of splicing activities in prp2-1 and prp8-21* extracts after the addition of glucose was due solely to the lack of NTPs, since activities were restored if both extracts were supplemented with extra ATP (at the final concentration of ≥5 mM, data not shown).

5.2.5. prp8-21 is defective in spliceosome assembly in the absence of the polypyrimidine tract.

As discussed in §3.3.1, the 5'-end of U2 snRNA, in association with Prp8p, Slt17p/Slu7p and Slt15p/Prp17p, may play a role in the second splicing step. The location of the prp8-21 mutation (Q1902K) in the PyT recognition domain of Prp8p (Fig. 5.3) suggests that this particular mutation is defective in 3'-splice site selection. However, in the prp8-21* extract, in vitro spliceosome assembly was blocked prior to the first step (Fig. 5.4C). It is not possible at this time point to attribute this defect either to U2-G21C/C14G alone or to the combinatorial effect of both prp8-21 and U2 mutations, since the splicing defect of U2-G21/C14G was not determined in this study. But, it is likely that the latter is the case -- neither prp8-21 nor U2-G21C/C14G conferred severe growth defect at ≤33°C (Fig. 5.1, and Table 5.1), and the U2-G21C extract displayed a similar level of splicing activity to that of the wt extract (Field et al.)
Fig. 5.6. *prp8-21* is defective in spliceosome assembly with a pre-actin substrate that lacks the polypyrimidine tract and the 3' splice site. A. The Ac/ΔClaI actin precursor RNA substrate. RNA sequence of the branchpoint site, the polypyrimidine tract (underlined) and the 3' splice site (the vertical bar indicates the 3' cleavage site) of pre-actin is shown on the top. When the T7-pre-actin template is linearized with EcoRI full-length pre-actin substrate is produced. When linearized with ClaI, which cuts immediately down stream of the branchpoint, the Ac/ΔClaI substrate is generated, which lacks the PyT and the 3' SS. The dot indicates the branchpoint adenosine. B. Spliceosome assembly with Ac/ClaI substrate in wt, *prp8-21* and *prp8-21* (i.e., *prp8-21* + U2-G21C/C14G) extracts. Note that in the wt extract, assembly was stalled at the formation of complex A1, whereas in both mutant extracts, it was blocked before the formation of complex A2-1. Schematic representation is shown on the right.
unpublished observations). It has been shown that both yeast Prp8p and its human homologue are crosslinked to the 3' splice site after the first step reaction (Wassarman and Steitz 1992; Sontheimer and Steitz 1993; Teigelkamp et al. 1995; Umen and Guthrie 1995c), however, sequences downstream of the branchpoint (including the PyT) are required for the formation of the active spliceosome (i.e. complex A2-2), but is dispensable for the functional/core spliceosome (i.e., complex A1) (Cheng 1994). I used a truncated pre-actin substrate (Ac/ΔClai) that lacks the PyT and the 3' splice site (Fig. 5.6A; Cheng 1994) to determine if the prp8-21 mutant extract is defective in spliceosome assembly prior to the first step reaction. It has been shown that with this truncated substrate spliceosome assembly is stalled at complex A1 without any splicing activity (Cheng 1994, Fig. 5.6B). In both the prp8-1 and the prp8-21* extracts, however, assembly was blocked at complex A2-1 (the holo-spliceosome) instead of complex A1 (Fig. 5.6B). This block is caused by the prp8-21 mutation, since the same was also observed in prp8-21 extract (with wild-type U2 snRNA) (Fig. 5.6B), even though it showed no detectable splicing defect when the full length substrate was used in splicing assays (Fig. 5.3A). These results suggest that the interaction between Prp8p and PyT and/or the 3' SS is required for the formation of the functional spliceosome (i.e., the core-spliceosome). The slt21/prp8-21 mutation may impair this interaction, which is only manifested in the absence of the PyT and/or the 3' SS.

5.2.6. prp8-21 is synthetically lethal with slu7-1, slt17/slu7-100.

In addition to prp8-21/slt21, five slt mutations were identified in the synthetic lethal screen (Chapter 2). Mutations in two of them, slt11-1 and slt22-1, block the first step of splicing in vitro, while two factors, Slt15p/Prp17p and Slt17p/Slu7p, are required for the second step. It is relevant to determine if prp8-21 exacerbates genetically any of the slt and other splicing mutations, in particular those which affect the second splicing step. Five slt mutations and slu4/prp17-2, slu7-1, prp2-1 and prp16-1, were segregated genetically into the
background of ΔPRP8 (Fig. 5.6A, see Material and Methods for detail). Three prp8 alleles, the original prp8-1, prp8-21/slt21, and prp8-101/psfl (Umen and Guthrie 1995b), were introduced into the resulting haploid strains and synthetic lethality was then tested by plasmid shuffling (Fig. 5.6A).

Although prp8-21 is viable at 25°C with all the mutations tested, it showed synthetic lethality at 30°C with only three mutations, slt17/slu7-100, slu7-1 and prp16-1 (Fig. 5.7B), all of which affect the second step of splicing. Similarly, prp8-101, shown to be defective in 3’ splice site selection (due to impaired binding to the 3’ SS, Umen and Guthrie 1995c), was synthetically lethal with mutations defective in the second step factors, prp16-301, prp17-1, prp18-1, slu4-1/prp17-2, slu7-1, slu7-ccss (Umen and Guthrie 1995c) and two mutations isolated in our screen, slt15/prp17-100, slt17/slu7-100 (Fig. 5.7B). On the other hand, prp8-1, which blocks splicing prior to the first step at the stage of recruitment of the U4/U6.U5 tri-snRNP (Brown and Beggs 1992), showed synthetic lethality only with slt22-1 (Fig. 5.7B, see §4.3.2 for discussion).

prp8-101 and prp8-21 were isolated in two different genetic screens, they share similar synthetic lethality with mutations that affect the second splicing step, in particular, both alleles of slu7, slu7-1 and slt17/slu7-100 (Fig. 5.7B). Moreover, both prp8 mutations are located in the domain that has been implicated genetically in recognition of the PyT (Fig. 5.3). However, prp8-21 is defective in spliceosome assembly prior to the first step (Figs. 5.4 and 5.6). These results suggest that recognition of the 3’ SS by Prp8p, U2 snRNA and other factor(s) occurs during spliceosome assembly. Since tethering of the two exons by loop 1 of U5 only occurs after the first step (O’Keefe et al. 1996), this potential interaction may be responsible for coordination of recognition of the 3’ SS by different factors prior to and following the first splicing step.
Fig. 5.7. Synthetic lethality of prp8-21 with slt17/slu7-100 and slu7-1. A. Representative yeast strain used to test synthetic lethality. slt and slu7 mutations and ASLT11 were segregated into the PRP8Δ::HIS3 background, as shown. After PRP8, prp8-21, prp8-101 and prp8-1 (carried on a LEU2, CEN-ARS plasmid) was introduced into these cells, the resultant transformants were tested for synthetic lethality by plasmid shuffling. B. 3-day growth of resultant transformants on medium containing 5-FOA at 25°C and 30°C. C. Summary of synthetic lethality of prp8 mutations with slt, slu and prp mutations. +: double mutations are viable; +/-: double mutations are viable at 25°C, but lethal at 30°C; -: double mutations are lethal at both 25°C and 30°C. DN: not determined.
§5.3. Discussion

A new allele of prp8-21 was isolated based on synthetic lethality with an 11nt substitution in the 5'-end of U2 snRNA. The mutation results in Q1902K change in a highly conserved amino acid in the polypyridine tract recognition domain of Prp8p (Fig. 5.3B), which is important for efficient selection of the 3' splice site (Umen and Guthrie 1996). Consistent with this, prp8-21 is synthetically lethal with slt17/slu7-100 and slu7-1 (Fig. 5.7B), the corresponding protein of which is involved in 3' SS selection (Frank and Guthrie 1992, Umen and Guthrie 1995c). Furthermore, when a pre-mRNA substrate lacking the PyT was used in an in vitro splicing assay, the defect associated with prp8-21 was evident (Fig. 5.6B). These results indicate that prp8-21 is likely to be defective in interaction with the PyT and in the efficient selection of the 3' SS. However, in an extract made from cells carrying both prp8-21 and U2-G21C/C14G mutations, splicing was blocked prior to the first step at the formation of the core-spliceosome (Fig. 5.4C). With the truncated substrate (Ac/AClal), prp8-21 blocked splicing the formation of the holo-spliceosome (Fig. 5.6B). These data suggest that the 3' SS is recognized by the 5' end of U2 snRNA, Prp8p and other factors during spliceosome assembly prior to being tethered by loop 1 of U5 snRNA.

5.3.1. Interaction between Prp8p and 5'-end of U2 snRNA.

The genetic interaction between prp8-21 and the 5'-end of U2 snRNA is specific. The supporting evidence includes: 1) prp8-21 is lethal only in combination with 11nt and 9 nt substitutions, and a single substitution G21C; i.e., the synthetic lethality with U2 mutations is highly allele-specific (Fig. 5.2A); 2) The synthetic lethality of prp8-21 with U2-G21C was suppressed by three second site mutations in U2 snRNA (Fig. 5.2). None of the synthetic lethality of the slt mutations (including slt11-1, ΔSLT11, slt17/slu7-100, and slt22-1) with U2-
G21C was suppressed by these suppressors (Table 5.1); and 3) Although the original 11nt substitution of U2 snRNA could potentially perturb U2/U6 helix II interaction (Fig. 3.9), substitutions in the U6 partner (i.e., U6-a, -b, -c and d) showed no synthetic lethality. Nor did any of these U6 mutations suppress the synthetic lethality of prp8-21 with U2-11nt (see §3.2.2). Together they suggest that the 5'-end of U2 (rather than U2/U6 helix II per se), in an unidentified form(s), may interact directly with Prp8p in the spliceosome. However, when does this interaction occur during the spliceosome assembly?

Prp8p is the most conserved splicing factor identified so far (Hodges et al. 1995, also see Fig. 4.3B for partial sequence alignment), and is involved in spliceosome assembly and the second step of splicing (see §5.1). This large splicing factor may be divided into several functional domains, including interaction with Prp40p (U1 snRNP protein), Snp1p (U1 70K homologue), 3' SS selection, PyT recognition, and 5' SS binding domains (see Fig. 5.3B for detail). This structural organization makes Prp8p an ideal candidate for regulation and coordination of the recognition of the splice sites and RNA/RNA interactions in the spliceosome. In a HeLa extract, the 5'-end region (i.e., stem I) of U2 snRNA is required for the interactions between U2AF65 (bound to the PyT during the formation of the early complex) and U2 snRNP to promote spliceosome assembly (Khellil et al. 1991). After binding to the BPS of pre-mRNA, the 5'-end of U2 snRNA undergoes conformational changes such that it becomes more accessible to RNase H digestion (Lamond et al. 1989; Barabino et al. 1989). A similar observation was also made in a yeast splicing extract (O'Day et al. 1996). This increased accessibility is probably necessary for the binding of U4/U6.U5 tri-snRNP, likely coupled to the formation of U2/U6 helix II (see §4.3.2). It is possible that Prp8p, which is associated with the U5 snRNP (Whittaker et al. 1990), may interact with the 5' end of U2 soon after the formation of the holo-spliceosome.

As discussed in §4.3.1, during the formation of the core-spliceosome, Slt22p may act to unwind U2/U6 helix II. This process might be coupled to the disruption of the U4/U6
interaction. Prp8p is also likely to be involved in the coupling of this process, since a mutation in U4 snRNA that blocks dissociation of U4/U6 can be genetically suppressed by a prp8 mutation (Li and Brow 1996; A.N. Kuhn, Z. Li, and D.A. Brow, personal communication). However, after resolution of helix II by Slt22p, the U2 part of helix II may interact with other component(s) of the spliceosome prior to the first step. Prp8p is likely to be one of these factors. The observed synthetic lethality of prp8-21 with specific U2 mutations may reflect their role in recognition of the 3' SS (including the PyT), which is required for the formation of the active spliceosome (see below). This suggestion is supported by several lines of evidence. In a splicing extract prepared from cells carrying double mutations of prp8-21 and U2-G21C/C14G, spliceosome assembly is blocked at the formation of the core spliceosome (complex A1, Fig. 5.4C) prior to ATP hydrolysis by Prp2p (Fig. 5.5). Furthermore, the prp8-21 mutation is located in the PyT recognition domain (Fig. 5.3), and was synthetically lethal with two second-step mutations, slt15/prp17-100 and slt17/slu7-100 (Fig. 5.7). The potential interaction between Prp8p (an U5 snRNP protein) and the 5'-end of U2 snRNA may play an additional role in tethering the U5 snRNP to the rest of the spliceosome prior to the first splicing step (see §4.3.3 for detail).

5.3.2. Prp8p/U2 snRNA interaction and selection of the 3' splice site.

The 3' SS is selected/recognized by several protein and RNA factors in a step-wise fashion (see §5.1 for detail). Although loop 1 of U5 snRNA plays an important role in tethering the two exons (O'Keefe et al. 1996 and reference therein), a direct canonical base-pair interaction is not involved in this function (Teigelkamp et al. 1995). Alteration of the AG dinucleotide in the intron at the 3' SS can be suppressed partially when complementarity between the first and second nucleotides of exon 2 and loop 1 of U5 snRNA is created. However, activation of other cryptic 3' splice sites was not suppressed by these U5 mutations (Newman and Norman 1992). These results suggest that U5 loop 1 is not the sole determining
factor in selecting/recognizing the 3' SS. Nevertheless, U5 loop 1 is important for the proper alignment of the two exons for the second catalytic step of splicing (O'Keefe and Newman 1998). This raises an important question as to whether and how the 3' SS is recognized prior to the second step.

A scanning mechanism for selection of the 3' SS in human was proposed on the basis of a strong preference for a proximal 3' SS to distal ones as a substrate for the second step. According to this model, following the first transesterification step, scanning initiates at the branchpoint and proceeds in a 5'-to-3' direction to the first AG dinucleotide (Smith et al. 1993 and references therein). However, early work on the PyT and AG dinucleotide revealed that the efficiency of the first step is often impaired by AG mutations (Reed and Maniatis 1985; Ruskin and Green 1985; Reed 1989), suggesting that recognition of AG could occur before the first step of splicing (§5.3.3).

In fact, a protein factor, AG100 was crosslinked to the last nucleotide of the intron at an early stage of spliceosome assembly. Subsequently, two more proteins, AG75 and hPrp8p, were crosslinked to the intron and exon regions of the 3' splice site before the second step (Chiara et al. 1996). In yeast, Prp8p was crosslinked to the 3' SS in a similar manner (Teilgelkamp et al. 1995; Umen and Guthrie 1995c). Mutations in the AG dinucleotide usually result in use of a downstream cryptic AG site(s) (Umen and Guthrie 1995b), but are suppressed by mutations in Prp8p (Umen and Guthrie 1996; also see Fig 5.3B). Another yeast factor, Slu7p, is also required to mediate selection of the 3' SS (Frank and Guthrie 1992), but its requirement for the second step depends on the distance between branchpoint and the 3' SS; i.e., Slu7p may act to bring the 3' SS to the BPS (Brys and Schwer 1996). Both Prp8p and Slu7p were identified in our genetic screen. Thus, the genetic interaction between these two protein factors and U2 snRNA mutations may indicate a role for the 5'-end of U2 in 3' SS selection. This is further supported by the genetic interaction (suppression and synthetic lethality) between mutations in loop 1 of U5 snRNA and substitutions at positions 21, 21 and
23 of U2 snRNA (§3.2.3). In particular, U23 and A30 of U2 snRNA have been shown to be crosslinked to the exon region of the 3' SS (Newman et al. 1995). One of them, U23 and two adjacent nucleotides (G26 and A27, in the helix Ia portion of U2/U6 interaction) are required in vitro for the second splicing step (McPheeters and Abelson 1992). Non-AG 3' splice sites can be suppressed by a substitution at another adjacent position, A25G, although the suppression is not allele-specific (Madhani and Guthrie 1994b). This nucleotide and G52 of U6 are implicated in a possible tertiary interaction (Madhani and Guthrie 1994b), and similar non-specific suppression of the 3' SS alterations by G52U was also observed (Less and Guthrie 1993). It is tempting to suggest that nucleotides adjacent to the helix Ia portion of U2 snRNA (i.e., nts 21 through 25), in association with other RNA(s) (e.g., U6 snRNA) may form an RNA structure that recognizes the 3' splice site (likely the intron region; i.e., AG dinucleotide, see Fig. 5.8). It remains to be determined what are the quantitative effects of U2 mutations (at nucleotides 21, 22 and 23) on wt and non-AG 3' SS mutations (see §7.2.1).

5.3.3. Recognition of the PyT and coordination of the two splicing steps.

Besides the highly conserved AG dinucleotide, the preceding PyT is another important element that influences the efficiency of 3' SS utilization. Usually, the branchpoint and the PyT both lie within a region of ~18 to 40 nt upstream of the 3' SS AG (Green 1998; Rymond and Rosbash 1992). In this configuration, mutations in the PyT often have no effect on 3' SS selection (Patterson and Guthrie 1991; Umen and Guthrie 1995b), and when the 3' SS is duplicated, the proximal one is used almost exclusively. However, when the AG dinucleotide is preceded by a strong polypyrimidine tract, the distal site can be used at the expense of the proximal one (Patterson and Guthrie 1991; Umen and Guthrie 1995b). The architecture of the yeast TUB3 intron is different in that the 3' SS is 139 nt away from the branchpoint, and the efficient splicing of this intron is highly dependent on the PyT and Prp8p (which recognizes the
former) (Umen and Guthrie 1995b). After an exhaustive genetic study, Luukkonen and Séraphin (1997) reached a conclusion that selection of the 3' SS depends critically on its distance from the branchpoint, but is not achieved by a simple leaky scanning mechanism. The function of the polypyrimidine tract and the strict distance requirement for the 3' splice site remain to be elucidated.

I propose the following: 1) The 3' splice site is recognized, in part, by factors (i.e., the 5'-end of U2 snRNA; see above) bound to the branchpoint. This imposes a spatial constraint on the optimal distance between the branchpoint and the 3' SS; and 2) The polypyrimidine tract, in association with Prp8p (Umen and Guthrie 1995b, 1996, also see Fig. 5.3B), is required to coordinate recognition of the 3' SS by branchpoint-bound factors and loop 1 of U5 snRNA. When the 3' SS is distant from the branchpoint, the interaction between Prp8p and the PyT is involved in bringing the 3' SS, likely through interaction with other protein factor(s) (e.g., Slu7p, Umen and Guthrie 1995c; Brys and Schwer 1996; Fig. 5.7), to the proximity of the branchpoint.

Although the PyT and the AG dinucleotide appear to be required only during the second step (Rymond and Rosbash 1985; Rymond et al. 1987; Patterson and Guthrie 1991; Umen and Guthrie 1995b), two types of introns can be distinguished on the basis of the AG dinucleotide requirement prior to the first step: AG-dependent (if the PyT is short, an adjacent AG is essential for the first step) and AG-independent (the AG is not essential in the presence of a long PyT) (Reed 1986). These two types of intron structures suggest cooperativity between recognition of the PyT and the 3' SS. At least in the AG-dependent splicing, (partial) recognition of the 3' SS prior to the first step is necessary for the coupling and coordination of the two steps. In fact, the PyT (and the 3' SS) are required for formation of the commitment complex (complex E) at the early stage of spliceosome assembly in both yeast (Legrain et al. 1988; Séraphin and Rosbash 1989) and human (Michuad and Reed 1993). This requirement seems to correlate with the sequential binding of several splicing factors, including U2AF65/Mud2p and Prp8p, to the PyT, and two other factors in to the 3' SS (see §5.1 and
Fig. 5.8. Recognition of the polypyrimidine tract (PyT) and the 3' splice site (3' SS) by multiple protein and RNA interactions. 

a. During formation of the early complex, the Pyt is recognized by Mud2p, which is also involved in cross-intron bridging. 
b. After the BP is recognized by U2 snRNA, Prp8p (in association with U4/U6.U5 tri-snRNP) is recruited into the splicing complex and may interact (indirectly) with Mud2p. 
c. During the maturation of the spliceosome, Mud2p/PyT interaction is replaced by Prp8p. 
d. In the active spliceosome, Prp8p may be responsible for coordinating recognition of splicing sites by snRNAs and proteins. The 3' SS is brought to the active center through Slt17p/Slu7p, and the intron region (i.e., the invariant AG dinucleotides) may be recognized by U2 snRNA (prior to being tethered by U5 loop 1, not shown). The 5'-end of U2, in an unidentified form, may also interact with both Slt21p/Prp8p and Slt17p/Slu7p.
Chapter 5

conserved elements in the intron
\(\downarrow\) nucleotides important for the 2nd step
\(\uparrow\) nucleotides important for 3' SS selection
\(\heartsuit\) nucleotides in U2 that interact genetically with U5 loop 1
- polypyrimidine tract
§5.3.2 for detail). In yeast the PyT is required for the formation of the active spliceosome (Cheng 1994; Fig. 5.6). It is possible that the interaction between Prp8p and the polypyrimidine tract is established at this stage (Fig. 5.8). The defect associated with prp8-21 and U2-G21C/C14G double mutation also blocked assembly at the formation of the core-spliceosome (Fig. 5.5). This may further suggest cooperativity between binding of Prp8p to the PyT and 5'-end of U2 snRNA to the 3' SS. The synthetic lethality is likely caused by the loss of this cooperativity (Fig. 5.8).

In the absence of the PyT, spliceosome assembly is stalled at the earlier step of formation of the holo-spliceosome in prp8-21 extract (Fig. 5.6), reiterating the interaction between Prp8p and the PyT in the early steps of spliceosome assembly. However, it is likely that at least one other protein factor is involved in mediating the Prp8p/PyT interaction. It will be interesting to determine if prp8-21 is also synthetically lethal with deletion of MUD2, which is not essential for viability (Liao et al. 1993). The polypyrimidine tract and its sequential interactions with Mud2p, Prp8p (and other protein factors) may serve to bridge recognition of the 5' SS and the branchpoint site (via Mud2, see above), and to couple the two steps of splicing (via Prp8p, acting to coordinate recognition of the 3' splice site by U2 snRNA bound to the branchpoint, and loop 1 of U5 snRNA).
§5.4. Materials and Methods

5.4.1. Yeast strains, plasmids, and genetic manipulation.

All yeast strains used this study are derived from W303-1A and W303-1B. Genetic screen/characterization and other genetic manipulation (chromosomal deletion, two-step gene replacement etc.) are described in §2.4 and Guthrie and Fink (1991). U2 mutant plasmids tested for allele specificity (Fig. 5.2 and Table 5.1) are provided by D.J. Field (also see §3.4.1) To construct chromosomal deletion of PRP8, the two internal BgIII fragments of PRP8 gene was deleted and replaced by the yeast HIS3 gene. The prp8-21 mutation was cloned by the gapping method (Fig. 5.1): a LEU2-marked PRP8 plasmid was first linearized by BgIII, and the fragment containing the vector and flanking PRP8 sequences was gel-purified and transformed into the original slt21 strain. The full-length plasmid was then recovered from the resultant transformants. prp8-1 and prp8-101 constructs were courtesy of J.D. Begs (Univ. Edinburgh) and C. Guthrie (UCSF), respectively.

To create ΔPRP8 strains in the background of slt, slu and prp mutations, mutant strains were crossed to ΔPRP8 strains, and the resultant diploids, carrying a maintenance plasmid (PRP8, URA3), were sporulated. After tetrad dissection, haploids containing both ΔPRP8 and slt mutation were obtained from non-parental ditype spores. Mutant prp8 alleles, carried on LEU2 CENARS plasmids, were then introduced into these haploids to test synthetic lethality by plasmid shuffling.

5.4.2. Preparation of yeast splicing extract, in vitro splicing assays and native gel electrophoresis.

See §3.4.2 for details.
Chapter 6.

Genetic Evidence that Slt11p is Involved in the snRNA Interactions for the Coordination of the Two Steps of the Splicing Reaction.
Abstract

Slt11p is a new splicing factor that contains zinc-binding motifs (two putative zinc-fingers) and a region homologous to the yeast ribosomal protein L25 (Rpl25p). Although SLT11 is essential for viability only at ≥ 33°C, Slt11p exerts its function immediately prior to the first splicing step and is required for efficient activation of the spliceosome. Genetic analysis reveals that Slt11p interacts with three snRNAs in the active spliceosome, U2, U5 and U6 snRNAs. In the absence of Slt11p, mutations in two regions of U2 snRNA and loop 1 of U5 snRNA cause lethality. Anotherslt mutation, slt17/slu7-100, is also synthetically lethal with ΔSLT11. Mutational analysis indicates that the N-terminal zinc-binding motifs are essential for Slt11p function, while the C-terminal portion (including large part of the Rbl25p-homology region) is dispensable for complementation of ΔSLT11. However, the integrity of the entire protein and the zinc-binding motifs is required for interactions with snRNAs and Slt17p/Slu7p. The genetic results suggest that Slt11p may act to modulate and coordinate RNA/RNA and RNA/protein interactions in the active spliceosome which are important for both steps of the splicing reaction.
§6.1. Introduction

Pre-mRNA splicing is almost certainly an RNA-mediated process and the RNA moieties of the spliceosome are largely, if not solely, responsible for the two catalytic steps of the transesterification reaction. Specific interactions between RNAs and proteins are also fundamental to this process. Protein factors exert their auxiliary functions in the recognition, selection and juxtaposition of the splice sites and in driving and coordinating RNA conformational changes during spliceosome assembly and catalysis. Based on their modes of action, protein factors involved in (direct) interactions with RNAs can be divided into two groups: RNA-dependent ATPase/RNA helicases and RNA-binding proteins. The functions of RNA helicases (DExD/H proteins) are involved directly in RNA conformational rearrangements. Energy released from hydrolysis of ATP is required to drive such changes (see §4.1 for detail and references). RNA-binding proteins, on the other hand, are involved in stabilizing and maintaining RNA conformations (Draper 1995).

A number of RNA-binding splicing factors have been identified on the basis of homology to certain conserved motifs; i.e., RRM (consensus RNA recognition motif), KH (hnRNP K homology), zinc-finger, and zinc-knuckle etc. (Table 6.1). All these factors have been implicated in various steps of splicing pathway. Specific RNA targets have been identified, either genetically or biochemically, for some of them (Table 6.1). However, direct RNA-binding activities were demonstrated for only three yeast factors, BBP (branchpoint bridging protein) (Berglund et al. 1997), U2 B” (Tang et al. 1996), and Prp24p (Ghetti et al. 1995; Jandrositz and Guthrie 1995). In addition to RNA targets, many RNA-binding proteins also interact with other protein factors (Table 6.1). Multiple protein-protein interactions constitute a network that mediates and coordinates the recognition of RNA elements during spliceosome assembly. Two best-studied examples both involve RNA-binding proteins and their interactions with each other and with other factors. In the early steps of spliceosome assembly, the cross-intron bridging between the 5' splice site and the branchpoint (including the polypyrimidine
Table 6.1. RNA-binding proteins involved in pre-mRNA splicing in yeast.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>RNA target</th>
<th>Function and/or activity</th>
<th>Interaction with</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RRM (RNA-Recognition motif)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mud2</td>
<td>PyT</td>
<td>5' SS/BP bridging</td>
<td>BBP¹</td>
<td>a, b</td>
</tr>
<tr>
<td>Hsh49</td>
<td>--</td>
<td>SF3b</td>
<td>Cus1p¹</td>
<td>c</td>
</tr>
<tr>
<td>Prp24p</td>
<td>U4/U6 snRNA</td>
<td>U4/U6 annealing</td>
<td>--</td>
<td>d</td>
</tr>
<tr>
<td>U1 70K</td>
<td>U1 loop A</td>
<td>U1 snRNP</td>
<td>Prp8p¹</td>
<td>e</td>
</tr>
<tr>
<td>U1 A</td>
<td>U1</td>
<td>active U1 snRNA</td>
<td>--</td>
<td>f</td>
</tr>
<tr>
<td>U1 C</td>
<td>5' arm of U1</td>
<td>U1/5' SS interaction</td>
<td>--</td>
<td>g</td>
</tr>
<tr>
<td>U2 B”</td>
<td>stem IV of U2</td>
<td>U2 snRNP integrity</td>
<td>(Mud2p)²</td>
<td>h</td>
</tr>
<tr>
<td><strong>KH (hnRNP K homology)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBP</td>
<td>branchpoint</td>
<td>5' SS/BP bridging</td>
<td>Mud2p &amp; Prp40p¹</td>
<td>b</td>
</tr>
<tr>
<td><strong>Zn-fingers (C₂H₂)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prp2p³</td>
<td>--</td>
<td>RNA-dependent ATPase</td>
<td>Spp2p¹</td>
<td>i</td>
</tr>
<tr>
<td>Prp6p</td>
<td>--</td>
<td>U4/U6.U5 tri-snRNP integrity</td>
<td>--</td>
<td>j</td>
</tr>
<tr>
<td>Prp9p</td>
<td>(U2 snRNA)⁴</td>
<td>U2 binding</td>
<td>Prp21p¹</td>
<td>j, k</td>
</tr>
<tr>
<td>Prp11p</td>
<td>(U2 snRNA)⁴</td>
<td>U2 binding</td>
<td>Prp21p¹</td>
<td>k, l</td>
</tr>
<tr>
<td><strong>Zn-knuckle (CX₂CX₄HX₄C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBP</td>
<td>branchpoint</td>
<td>5' SS/BP bridging</td>
<td>Mud2p &amp; Prp40p¹</td>
<td>b</td>
</tr>
<tr>
<td>Slu7p</td>
<td>(3' SS)⁵</td>
<td>3' SS selection</td>
<td>Prp18p</td>
<td>m</td>
</tr>
</tbody>
</table>

Notes: 1. Direct binding  
2. Synthetic lethal interaction  
3. Zinc-funger like (CX₃CX₁⁶CX₆C)  
4. Based on genetic interactions  
5. Based on site-specific crosslinking  
6. Zinc-knuckle is not essential.

References: next page.
Table 6.1 (continued)

References:  

tract) is mediated through direct interactions between BBP (bound to the branchpoint) and both Prp40p (a U1 snRNP protein bound to the 5' splice site) and Mud2p (bound to the polypyrimidine tract) (Abovich and Rosbash 1997). In the subsequent step, Prp9p and Prp11p (Zn-finger proteins) both interact with Prp21p forming complex SF3a (Legrain and Chapon 1993; Legrain et al. 1993) which is important for binding of U2 snRNP to the pre-mRNA. Mutations in these factors are synthetically lethal with mutations in and adjacent to the stem II region of U2 snRNA (Ruby et al. 1993; Wells and Ares 1994; Yan and Ares 1996). Human homologues of SF3a bind directly to the anchoring region upstream of the branchpoint and function to tether U2 snRNP to pre-mRNA (Champion-Amau and Reed 1994; Gozani et al. 1996). The functional duality (RNA recognition and protein-protein interaction) of RNA-binding proteins is critical to the regulation of pre-mRNA splicing, since it provides various RNA-RNA interactions with a mechanism of cooperativity in both spatial and temporal terms.

Slt11p is a new splicing factor isolated in the genetic screen for factors important for the 5' end of U2 snRNA. It contains Zn-finger like motifs and sequences homologous to ribosomal protein L25. Although the RNA-binding activity of Slt11p remains to be determined, results of genetic analyses are consistent with the suggestion that Slt11p is an RNA-binding protein. *SLT11* is not essential for viability at permissive temperature; a chromosomal deletion confers ts phenotype at ≥33°C. However, the protein is required for the efficient activation of the spliceosome. Genetic analyses revealed that this protein is important for all three snRNAs present in the active spliceosome and another protein factor Slt17p/Slu7p involved in the 3' splice site selection. Mutational studies indicate that the N-terminal zinc-binding motifs and the central region are critical for the interactions of Slt11p with both spliceosomal snRNAs and Slt17p. Results presented in this chapter suggest that Slt11p may act to mediate RNA/RNA interactions in the active spliceosome and to coordinate the two-step splicing reaction in association with other splicing factors.
§6.2. Results

6.2.1. *SLT11* encodes a new splicing factor containing zinc-binding motif.

*SLT11* is identical to *YBR065c*, which encodes a protein of 364 amino acids (41 kD, Fig. 6.1A). Slt11p can be divided into three regions. The N-terminal region (amino acids 1 - 150) contains a zinc-binding motif, possibly two zinc-fingers (CX2CX17CX2CX23CX2CX6CX2C) (Fig. 6.1C). Zinc-finger and other zinc binding motifs have been identified as nucleic acid binding motifs in several transcription factors (e.g., TFIIB, TFIID, see §6.3.2) and splicing factors (Prp9p, Prp11p, BBP, and Slu7p, see Table 6.1). The central region (amino acids 151 - 300) of Slt11p shows overall homology to the yeast ribosomal protein L25 (Rpl25p) (27% identity, 40% similarity, Fig. 6.1B). The yeast ribosomal protein L25 binds to the 26S rRNA (El-Baradi et al. 1985). The C-terminal end is unique and highly charged due to a stretch of lysine residues. Slt11p is a basic protein with estimated pI of 9.7.

BLAST searches did not reveal any homologue in the data base. However, the N-terminal proximal Zn-finger is similar to that present in the yeast transcription factor TFIIB (Colbert and Hahn 1992) (Fig. 6.1C). Moreover, the entire N-terminal region, including both putative Zn-fingers, shows high homology (35% identity and 53% similarity) to the corresponding region of a *C. elegans* protein with unknown function (Fig. 6.1C). In particular, the size and spacing of two putative zinc fingers are well conserved between both proteins. In addition, the central region of the *C. elegans* protein contains a conserved RNA recognition motif present in proteins such as the yeast polyadenylated RNA-binding protein Pub1p, splicing factors [e.g., mouse alternative splicing factor ASF, human SF2 and hnRNP A1] (Fig. 6.1C). Although the C-terminal half of the *C. elegans* protein is unique, its domain structural organization resembles that of Slt11p; i.e., an RNA-recognition motif (vs. homology to a ribosomal protein, in the case of Slt11p) preceded by a zinc binding domain (Fig. 6.1D). In addition, the N-terminal Zn-binding motif (likely two Zn-fingers) in Slt11p shares extensive
A

\[
\begin{array}{c}
\text{CC..CC...CC..CC} \\
\text{Rpl25p homology} \\
\text{KKK}
\end{array}
\]

MNDEINEPPP NICEQCLGDE ANIRMTKIPQ GSEEKICTLP FTLYHFKTSK RSNNIIKTLL 60
CVRQATARNI COCCMLDSRW HIPIQLRDHL ISLVNEENVM TEEAKNDMMK RFLSLKVKKL 120
GGAQITSDPS EADNVDKKL NILLRATDSG PSTPLIKNTT ALYKNEKGAN EVKNLEKYS 180
VDISHILKLN PLNESFKNPS STKSFPLYNK DASIPEDWKT DTVSQLGIK KWKDGNSLSL 240
IVNHKAKCGG LRFQSSELGE RFVSKISETL VTPKGLKRGV LLLDFRIFI IPWSSGFSA 300
SGTNTAENI KLSLSLNKLI QLEGLSFPT KSTDNAKNDK KKTSKVHKS RSKKSKPRAN 360
KLTI 364

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Start</th>
<th>Stop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt1lp</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>Rp125p</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Protein</th>
<th>Start</th>
<th>Stop</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFIIB</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Slt1lp</td>
<td>10</td>
<td>69</td>
</tr>
<tr>
<td>T11G6.8</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>Protein</th>
<th>Start</th>
<th>Stop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt1lp</td>
<td>150</td>
<td>364</td>
</tr>
<tr>
<td>C. elegans T11G6.8 protein</td>
<td>150</td>
<td>300</td>
</tr>
</tbody>
</table>

- 199 -
Fig. 6.1. **Structure and homology of Slt11p.**

A. Schematic representation and amino acid sequence of Slt11p. Slt11p is identical to Ybr065cp. Cysteine residues in the putative Zn-fingers are underlined.

B. Amino acid sequence alignment of the central region of Slt11p with yeast ribosomal protein L25 (Rpl25p, accession number P04456).

C. Amino acid sequence alignment of the N-terminal Zn-fingers with corresponding regions in yeast TFIIIB (accession number P29056) and a C. elegans protein of unknown function (T11G6.8, accession number Z69384).

D. Overall structural similarity between Slt11p and the C. elegans protein T11G6.8 that contains an RNA recognition motif (RRM). Amino acid sequence alignment of the N-terminal regions (shaded) of both proteins is shown in C. The amino acid sequence of the RRM is shown at the bottom with conserved residues underlined. The two bolded glycine residues are absolutely conserved in a class of (putative) RNA-binding proteins.

E. Amino acid sequence alignment of two putative Zn-fingers present in Slt11p, and human (accession number T81062), *Drosophila melanogaster* (AA392128), rice (D47797), mouse (W85575) and C. elegans T11G6.8 (Z69384) proteins. Arrowhead indicate cystine residues. For amino acid sequence alignment in B, C and E, identical amino acids are indicated by black boxes, and conserved ones by dark grey ones.
sequence homology with several eukaryotic proteins with unknown function (Fig. 6.1E). It is possible that they may represent a new class of Zn-binding motif.

6.2.2. \textit{SLT11} is essential for viability at elevated temperatures.

In the first attempt to construct a chromosomal deletion of \textit{SLT11}, the central portion in the gene that corresponds to the Rpl25p homologous region was deleted and replaced with the yeast \textit{HIS3} gene (deletion \textit{a}). After being introduced into the wt diploid strain, His\textsuperscript{+} transformants were confirmed by PCR for the deletion of \textit{SLT11}. After sporulation, tetrad dissection was performed. In all cases, four viable spores were recovered at 25°C and 30°C (Fig. 6.2B), two of them were His\textsuperscript{+} and ts at \(\geq33\)°C while the other two are His\textsuperscript{-} and wt (data not shown). In order to eliminate the possibility that the N-terminal Zn-binding domain could provide sufficient functions at \(<33\)°C for viability, the N-terminal portion of the gene was removed in addition to deletion \textit{b} (Fig. 6.2C). Four viable spores (at 25°C and 30°C) were recovered upon tetrad dissection of heterozygous diploids of \(\Delta SLT11/SLT11\) (Fig. 6.2C). Similar to deletion \textit{a}, progeny spores carrying the chromosomal deletion of \textit{SLT11} (deletion \textit{b}, His\textsuperscript{+}) were ts at \(\geq33\)°C. Thus, \textit{SLT11} is not essential for viability at temperatures below 33°C. A chromosomal deletion of \textit{SLT11} confers ts phenotype at \(\geq33\)°C. At temperatures lower than 33°C, \(\Delta SLT11\) strains grew normally.

6.2.3. Deletion of \textit{SLT11} blocks pre-mRNA splicing prior to the first step.

A splicing extract made from the original \textit{slt11-1} cells was defective in splicing (Fig. 2.5). In order to determine if splicing was impaired in the absence of Slt11p, extracts were prepared from yeast cells with deletion \textit{a} and deletion \textit{b} (Fig. 6.2). Since the same observations were made with both extracts, results of deletion \textit{b} (i.e., completion deletion, also see Fig. 6.11 for the
SLT11 is essential for viability at ≥33°C. A. Restriction map of the SLT11 locus. The ORF is marked to illustrate the corresponding regions in Slt11p. B. Chromosomal deletion a of SLT11 (in which the central region is deleted) (left) and result of tretad dissection of ΔSLT11/SLT11 diploid grown at 30°C (right). C. Chromosomal deletion b of SLT11 (in which the N-terminal and central regions were deleted) (left). Result of tetrad dissection of ΔSLT11/SLT11 diploid (grown at 30°C) is shown on the right. Individual progeny were determined for ΔSLT11 (His+) and growth defect (bottom). Note that all His+ (ΔSLT11) progenies are ts at ≥33°C.
absence of Slt11p in this extract) are presented here. Consistent with the defect associated with slt11-1 (Fig. 2.5, lanes 2 and 5), at a permissive temperature (25°C), the in vitro splicing activity of the ΔSLT11 extract (Fig. 6.3A, lanes 4-6) was much lower than that of the wt (Fig. 6.3A, lanes 1-3). Prolonged incubation (60 min) resulted in an accumulation of more splicing products in the ΔSLT11 extract (Fig. 6.3A, lanes 14-16), whereas maximum splicing activity in the wt extract was achieved in 20 - 30 min (Fig. 6.3A, lanes 11-13). At 30°C and 33°C, while splicing activities were detected in the wt extract (Fig. 6.3A, lanes 7 and 9), the activity of the ΔSLT11 extract was completely abolished (Fig. 6.3A, lanes 8 and 10). No detectable activity was observed even after 60 min at 33°C (Fig. 6.3A, lanes 17 and 18). The ΔSLT11 extract was complemented by both prp2-1 and slt22-1 extracts at non-permissive temperatures (data not shown). Thus, Slt11p is a bona fide splicing factor. However, its activity is not essential for the splicing reaction at a permissive temperature. In the absence of Slt11p, the rate of the splicing reaction is impaired at the permissive temperature and the process is blocked prior to the first splicing step at the non-permissive temperatures.

Native gel electrophoresis was performed to determine if spliceosome assembly was defective in the ΔSLT11 extract at permissive and nonpermissive temperatures. At 25°C the profile of spliceosome assembly in the ΔSLT11 extract was similar to that of wt (data not shown). Virtually the same assembly profile was observed at 30°C for both the wt and the ΔSLT11 extracts (Fig. 6.3B); i.e., appearance of complexes B and A2-1 at an early time, and eventual accumulation of complex A2-2 (as in the case of the wt extract). Complex A1 was barely detected in the wt extract, but was relatively abundant in the ΔSLT11 extract (Fig. 6.3B, lane 5). Due to the low resolution of native gels, it is difficult to determine, based on mobility, whether the final complex in the ΔSLT11 extract is identical to complex A2-2. However, the kinetics of spliceosome assembly seems to indicate that in the ΔSLT11 extract it followed B → A2-1 → A1 → A2-2 (or A2-2 like). This result suggests that in the absence of Slt11p, pre-mRNA splicing is only affected during the activation of the spliceosome immediately prior to the first splicing step (Fig. 6.3C). Apparently, without Slt11p the rate of activation is reduced
Fig. 6.3.  **In vitro splicing defect associated with ΔSLT11.** A. Radio-labeled pre-actin mRNA substrate was used for *in vitro* splicing reaction with extracts of wt (lanes 1-3, 7 and 9) and the ΔSLT11 (lanes 4-6, 8 and 10, as indicated by -slt11 or -11) cells at 25°C (lanes 1-6), 30°C (lanes 7 and 8) and 33°C (lanes 9 and 10) for a 20 min incubation time. After prolonged incubation at 25°C (i.e., 60 min), no further accumulation of splicing products was observed in wt extract (lanes 11-14), whereas an increase of the splicing products was observed in the ΔSLT11 extract (lanes 14-16). However, no appreciable splicing products were detected in the ΔSLT11 extract after prolonged incubation at 30°C (lanes 17 and 18). The polyacrylamide gel shown on the right was broken during the process of fixing, which resulted in the distortion of mobility of the mature splicing product.  B. Spliceosome assembly in wt and ΔSLT11 at 33°C. The final complex accumulated in the ΔSLT11 extract runs at the same or similar position as complex A2-2. See text for details.  C. Schematic representation of yeast spliceosome assembly. It is likely that Slt11p is involved in activation of the spliceosome, which becomes impaired and heat-labile in the absence of the protein.
and is also heat-labile (see Fig. 6.3A). Thus, Slt11p is required for efficient activation of the spliceosome prior to the first splicing step.

6.2.4. Genetic interactions with U2, U6 and U5 snRNAs.

In the ΔSLT11 extract, the activation of the spliceosome becomes rate-limiting at the permissive temperature (Fig. 6.3A, lanes 14 - 16). Since Slt11p contains a Zn-finger-like motif that has been implicated in RNA binding function, it may act to stabilize and coordinate RNA-RNA interactions that are important for the splicing reaction, as indicated by the splicing defect associated with ΔSLT11 (Fig. 6.3). In the absence of Slt11p, mutations in these RNAs could have a drastic effect on splicing and cellular viability. I tested mutations in three snRNAs present in the active spliceosome, U2, U5 and U6, for synthetic lethality with ΔSLT11.

a) Genetic interactions with U2 snRNA. Using a yeast strain carrying both SLT11 and SNR20 deletions (with SNR20 carried on a URA3-marked plasmid, Fig. 6.4A left), mutations in the 5' end of U2 snRNA were tested, including substitutions in or adjacent to the U2/U6 helix II region and single nucleotide substitutions at 21, 22, 23 and other positions (Fig. 6.4B). While most of these mutations conferred little or mild growth defect on their own at 25°C and 30°C (also see Fig. 3.1B, Fig. 6.4C and Table 3.2), some of them became lethal in the ΔSLT11 background. Substitutions in the helix II region of U2 snRNA, the original 11nt and 9nt substitutions, and shorter ones (Ω1 and Ω2, corresponding to the two halves of the 9nt substitution, constructed in this study), were all synthetically lethal with ΔSLT11 (Fig. 6.4B). However, a 3nt substitution (Ω3) in the 3' adjacent region (i.e., positions 14, 15 and 16) showed no synthetic lethality with ΔSLT11. In addition, most single nucleotide substitutions at 21, 22 and 23 position (with the exception of C22U and U23A) were lethal in combination with ΔSLT11 (Fig. 6.4B and C). Other substitutions tested (U19G, G20C, C14A and C14G) showed no synthetic lethality (data not shown). Thus Slt11p is important for the function of two
Fig. 6.4. Synthetic lethality of ΔSLT11 with mutations in U2 and U6 snRNAs.

A. The yeast strains used for testing synthetic lethality of ΔSLT11 with either U2 (left) or U6 mutations (right). U2 and U6 mutations, carried on TRP1- and LEU2-marked plasmids respectively, were introduced into the respective strains. The resultant transformants were tested for synthetic lethality by plasmid shuffling on 5-FOA-containing medium (indicated by arrows).

B. Summary of synthetic lethality of ΔSLT11 with U2 and U6 mutations. ΔSLT11 is synthetically lethal (at 25°C and 30°C) with mutations in both U2 and U6 components of helix II interaction (right +: viable; -: synthetic lethal). Mutations in the helix Ib region of U2 snRNA, were also lethal in combination with ΔSLT11. Shown on the left is U2/U6 interaction with relevant regions highlighted. Open circles indicate positions at which substitutions are not synthetically lethal with ΔSLT11.

C. Genetic interactions between ΔSLT11 and U2 mutations in the helix Ib region. Most single nucleotide substitutions were lethal in the absence of Slt11p (left), while they conferred mild growth defect in the SLT11 background (right).
particular regions of U2 snRNA: the 5'-end helix II and the helix Ib (i.e., nucleotides 21, 22 and 23) regions (Fig. 6.4B). The synthetic lethality of Ω1 and Ω2 substitutions with ΔSLT11 is specific, since neither was lethal with two other slt mutations tested, slt17/slu7-100 and slt21/prp8-21 (data not shown).

b) Genetic interaction with U6 snRNA. Phenotypic asymmetry has been observed for mutations in the U2 and U6 parts of helix II interaction (Field and Friesen 1996). While 11nt and 9nt substitutions in U2 snRNA confer little or mild growth defects, the corresponding 9nt substitution in U6 snRNA partner is lethal by itself and is not suppressed by compensatory 9nt substitution in U2 snRNA (Field and Friesen 1996). Shorter substitutions (i.e., 3nt substitutions a, b, c, and d, as described in Field and Friesen 1996, Fig. 6.4B) were chosen to test for synthetic lethality with ΔSLT11. While substitution U6-a (at the most 3' end) showed no synthetic lethality with ΔSLT11, the other three were all lethal in the absence of Slt11p (Fig. 6.4B). These preliminary results raise the possibility of potential involvement of Slt11p in U2/U6 helix II interaction. It remains to be determined if substitutions Ω1 and Ω2 of U2 snRNA can be suppressed by compensatory mutations in U6 snRNA in the ΔSLT11 background (provided that such U6 mutations are not lethal by themselves). However, neither the 11nt or 9nt substitution was suppressed by any of the 3-nt substitutions of U6 snRNA tested (data not shown). Similar results were obtained with slt11-1 (§3.2.2).

c) Genetic interactions with U5 snRNA. Since the helix Ib region in U2 snRNA was implicated in the function of loop 1 of U5 snRNA possibly in recognition of the 3' splice site (§3.3.2 and §5.3.2), and Slt11p is important for the U2-helix Ib region (Fig. 6.4C), I tested if Slt11p is also functionally important for loop 1 of U5 snRNA. A yeast strain containing both ΔSLT11 and ΔSNR7 (the U5 snRNA gene) (Fig. 6.5A) was constructed, and two sets of U5 loop 1 mutations were introduced into this strain for testing synthetic lethality by plasmid shuffling. As a control, slt17/slu7-100 was also tested.
Fig. 6.5. Genetic interactions between ΔSLT11 and mutations in loop 1 region of U5 snRNA. 
A. The yeast strain containing double deletions of SLT11 and SNR7 is shown on the left. Mutant U5 gene (snr7) carried on a LEU2 CEN-ARS plasmid was introduced into the strain. Synthetic lethality was then tested by plasmid shuffling on 5-FOA-containing medium. The secondary structure of yeast U5 snRNA is schematically shown the right. 
B. Substitutions at nucleotide 98 in loop 1 are lethal in the absence of Slt11p. U98C/sup is an intragenic suppressor of U98C (which confers lethality by itself), containing A84U and ΔG113. 
C. Deletions and insertion (+1U) in loop 1 are lethal in the absence of Slt11 at 30°C. Note that slt17/slu7-100 was included as control in both B and C. Asterisks indicate synthetic lethality. 
For B and C, shown is 2-day growth on 5-FOA-containing medium. 
D. Summary of mutations in other regions of U5 snRNA that are not synthetically lethal with ΔSLT11.
The two sets of U5 mutations include single nucleotide substitutions at position 98 (Fig. 6.5B) and deletions/insertion in loop 1 (Fig. 6.5C). An intragenic suppressor of U98C, U98C/A84U/ΔG113 (U98C/sup, Fig. 6.5B), was used to replace U98C, which confers lethality by itself (unpublished observations). Both substitutions U98A and U98G conferred a severe growth defect at 25°C and 30°C, compared to wt and U98C/sup. All three substitutions were synthetically lethal with ΔSLT11 at both 25°C and 30°C, whereas slt17/slu7-100 showed synthetic lethality with U98G, but had no additive growth defect with U98G or U98C/sup (Fig. 6.5B). The two deletions, ΔC94C/ΔC95 and ΔU96/ΔU97, on their own, confer a mild growth defect at 25°C and 30°C (Fig. 6.5C) and are ts at ≥33°C (data not shown). In the absence of Slt11p, U5-ΔC94/ΔC95 became lethal at both temperatures, and U5-ΔU96/ΔU97 lethal at 30°C, but barely viable at 25°C (Fig. 6.5C). The combination of slt17/slu7-100 with either deletion impaired severely growth at both temperature, but was nevertheless viable (Fig. 6.5C). The difference between ΔSLT11 and slt17/slu7-100 is particularly apparent at 30°C. A single insertion at the U-rich region of loop 1 confers a mild growth defect only at 37°C (data not shown), however, insertion of two nucleotides in the same region leads to lethality at all temperatures (Fig. 6.5C). Although the single insertion conferred normal growth in the ΔSLT11 cells at 25°C, it caused lethality at 30°C, while slt17/slu7-100 showed no additive growth defect with this insertion at both temperatures (Fig. 6.5C). For both ΔSLT11 and slt17/slu7-100, synthetic lethality with U5 mutations is restricted to loop 1. Substitutions in the two bulged regions at the bottom of stem 1 (substitution at C112, and substitutions ω1 through ω4) and in stem 3 (ψ substitutions ψ1 through ψ3) showed no synthetic lethality with ΔSLT11 or slt17/slu7-100 (Fig. 6.5D and data not shown).

In summary (Fig. 6.6), the results of genetic analyses indicate that Slt11p is important for all three snRNAs present in the active spliceosome. The genetic interaction between ΔSLT11 and U5 mutations is specific; i.e., only mutations in the loop 1 region showed synthetic lethality (Fig. 6.5), whereas two regions (helix II and helix Ib, Fig. 6.4B) in U2 snRNA depend on Slt11p,
Fig. 6.6. Summary of mutations in U2, U5 and U6 snRNAs that are lethal in the absence of Slt11p. The function of Slt11p is likely to be exerted immediately prior to the first catalytic step (Fig. 6.3). Although SLT11 is not essential for viability at ≤33°C, mutations in three snRNAs present in the active spliceosome become lethal in the absence of Slt11p. Since some of these mutations lie in the residues or structures that are important for either step of splicing, RNA-RNA interactions are illustrated in context of the two step reaction. The U2 and U6 parts of the U2/U6 helices Ib and II are indicated by shading. The function of U2/U6 helix II interaction may be exerted prior to the splicing reaction, and may be resolved by the action of Slt22p (see §4.3.2). A. Prior to the first step, loop 1 of U5 snRNA is in close contact only with the 5' exon. Although U2/U6 helix Ib is shown unwound, it is not clear if this is true prior to the first step. Protein factors (including Slt21p/Prp8p) are involved in the recognition of the 3' splice site (via the polypyrimidine tract) at this stage. It is possible that the 5'-end of U2 snRNA plays a role in recognizing this site concomitantly or subsequently. B. After the first step, the two exons are tethered by loop 1 of U5 snRNA. The spliceosome undergoes a process of remodeling to accommodate structures required for the second step. It is possible that the helix Ib region of U2 snRNA is also involved in tethering the exons (see §5.3.3, Fig. 5.8). Residues and tertiary interactions that are important for this step are highlighted (see §1.3.2-3). Slt11p may function, in association with other factors, to coordinate the remodeling of RNA structures for the second step. Genetic interactions between U2 and U5 are described in §3.2.3 and Fig. 3.3). Site-specific crosslinks between the first nucleotid of the 3' exon and U23 and A30 nucleotides of U2 are described in Newman et al. (1995).
Mutations that are synthetically lethal with ΔSLT11

* Mutations that are NOT synthetically lethal with ΔSLT11

- Mutations that are synthetically lethal with ΔSLT11

* Mutations that are NOT synthetically lethal with ΔSLT11

- branchpoint adenosine
- polypyrimidine tract
- conserved elements in the intron
- nucleotides important for the 2nd step
- tertiary interaction between U32(U2) and G52(U6) important for the 3' SS selection
- tertiary interaction between terminal nucleotides of the intron
- genetic interactions between U2 and U5
- site-specific cross-linking
the latter of which (i.e., helix Ib) is functionally related to loop 1 of U5 snRNA (§3.2.3). Since only four 3-nt substitutions in the 3' end of U6 snRNA were tested in this study, I can not conclude that the interaction between U6 snRNA and Slt11p is restricted to the 3'-end region. However, these genetic results raise a distinct possibility that Slt11p might be involved directly in, but not restricted to, U2/U6 helix II interaction. Alternatively, both U2 and U6 parts of helix II may interact with other RNAs and/or protein factors after helix II is resolved by the potential RNA helicase activity of Slt22p, and Slt11p is required for these additional interactions (see below).

6.2.5. Interaction with Slt17p/Slu7p.

In addition to mutations in three snRNAs, the original slt11-1 was also synthetically lethal with slt15/prp17-100, slt17/slu7-100, slu4/prp17-2, slu7-1 and prp16-1 (§3.2.5). When ΔSLT11 /deletion a (Fig. 6.2B) was tested, it showed synthetic lethality with all aforementioned mutations, but not with slt21/prp8-21, slt22-1 or prp2-1 (data not shown). For the ΔSLT11 /deletion b (Fig. 6.2C), slt17/slu7-100 was chosen for further study, since Slt17p/Slu7p, containing a Zn-knuckle (potential RNA binding motif), is required for the 3' splicing site selection important for the second step of the splicing reaction (Frank and Guthrie 1992; Jones et al. 1995; Brys and Schwer 1996) and its function is also genetically linked to loop 1 of U5 snRNA (Frank et al. 1992).

It was anticipated that slt17/slu7-100 might cause lethality in the background of ΔSLT11/deletion b. After slt17/slu7-100 and ΔSLT11 strains were mated, a URA3, CEN-ARS plasmid carrying SLT11 gene was introduced into the resulting heterozygous diploid so that ΔSLT11 slt17/slu7-100 progeny haploids could be recovered following tetrad dissection (Fig. 6.7A). Following sporulation, three types of tetrads were obtained: (1) those containing four 5-FOA resistant (5-FOAR) spores; (2) those containing two 5-FOA sensitive (5-FOAS) and two 5-
Fig. 6.7.  

**slt17/slu7-100 is synthetically lethal with ΔSLT11.** A. Scheme of genetic manipulation to obtain ΔSLT11 slt17/slu7-100 double mutant strain. A plasmid (URA3 CEN-ARS) carrying SLT11 (pSLTI1) was introduced in the ΔSLT11/SLT11 slt17/SLT17 heterozygous diploid so that following sporulation and tetrad dissection, double mutant progeny (indicated by hatched ovals) in the NPD and T tetrads were aviable in the presence of the SLT11 plasmid. If the combination of ΔSLT11 and slt17 is lethal, these progeny are sensitive to 5-FAO, as shown in B. Asterisks indicate double mutant progeny, in which only slt17 was observed phenotypically (due to presence of the SLT11 plasmid). The SLT11 URA3 plasmid was absent in other progeny.
FOAr; (3) those containing one 5-FAO\textsuperscript{S} + three 5-FOAr. They correspond to parental ditype (PD), non-parental ditype (NPD) and tetratype (T) tetrads, respectively (Fig. 6.7B). The 5-FOA\textsuperscript{S} spores in NPD and T tetrads were progeny that inherited lethal combination of ΔSLT11 and slt17/slu7-100 mutations such that their viability depended on the SLT11 gene on the URA3 plasmid.

In summary, although SLT11 is not essential for viability at ≤30°C, the lack of Slt11p exacerbates mutations in U2, U5 and U6 snRNAs and Slt17p/Slu7p. These results suggest that Slt11p is involved in the functions of these snRNAs and Slt17p/Slu7p. While Slt11p is required for activation of the spliceosome, Slt17p/Slu7p is required for 3' splice site selection important for the second splicing step. The interactions between these two factors is important for the functions of snRNAs in the spliceosome. Since both proteins contain similar Zn-binding motifs and both are putative RNA-binding proteins, it is likely that these snRNAs and protein factors interact with each other in the spliceosome. The synthetic lethality between ΔSLT11 and mutations in U2, U5 and U6 snRNAs and Slt17p/Slu7p provided genetic means to dissect functional domains of Slt11p; i.e., to determine whether different regions of Slt11p mediate different interactions or these regions of Slt11p are integral parts of the protein and function cohesively.

6.2.6. Deletional analyses of Slt11p.

Slt11p can be divided into three regions on the basis of amino acid sequence motif/homology (Fig. 6.1A): the N-terminal putative Zn-fingers, the central region homologous to Rpl25p, and the C-terminal K-rich region. Truncations of Slt11p were made in order to determine: a) whether the N-terminal Zn-finger region is sufficient for Slt11p function, and b) how the other two regions contribute to the function of N-terminal region. Three C-terminal truncations were constructed (Fig. 6.8A): ΔA, containing the central region in addition to the N-
Fig. 6.8. Deletional analysis of Slt11p. A. Truncations of Slt11p. *top:* SLT11-containing genomic fragment and the corresponding protein. See Fig. 6.1A for structural organization of Slt11p. *bottom:* three truncations of Slt11p. PCR method was used to construct these truncations. To facilitate cloning, BamHI sites were introduced in primers 2, 3, and 4. The 3' untranslated region was first cloned as a BamHI-BglII fragment (PCR product with primers 4 and 5). For truncation ΔA, the entire 5' end half of the gene was amplified as a BglII-BamHI fragment (PCR product with primers 1 and 3) and ligated to the 3' half, whereas truncation ΔC was constructed with primers 1 and 2. Truncation ΔB was caused by a PCR error that introduced a stop codon at amino acid 178. B. Complementation of ΔSLT11. *top:* the yeast strain carrying ΔSLT11. *bottom:* complementation of ts phenotype of ΔSLT11 by three truncations.
terminal region, ΔB, a short version of ΔA in which a stop codon was introduced at amino acid 178 (Y/TAC→Z/TAA) (due to a PCR error in the construction of ΔA), and ΔC, only the N-terminal region. These three deletions were tested for complementation of ΔSLT11 (deletion b, Fig. 6.2C) and synthetic lethality of ΔSLT11 with mutations in U2 and U5 snRNAs and slt17/slt7-100.

a) Complementation of ΔSLT11. Since a chromosomal deletion of SLT11 confers ts growth at ≥33°C, these three deletions were tested in the ΔSLT11 strain for complementation of ts defect at non-permissive temperatures (Fig. 6.8B). The N-terminal Zn-finger portion alone, ΔC, was able to complement at ≤35°C. With the addition of a small portion (26 amino acids) of the central region, deletion ΔB complemented almost fully; i.e., it conferred only slow growth at 37°C. However, deletion ΔA failed to complement ΔSLT11 at ≥33°C. Subsequent biochemical results revealed that this was due to the instability of ΔA in the cells (see §6.2.8 and §6.2.9). Thus the N-terminal region can fold properly and provide at least partial function of Slt11p. This conclusion is consistent with the finding that this region of the recombinant His-Slt11p forms a highly stable domain (resistant to partial proteolysis, unpublished observations, in collaboration with C. Koth). The K-rich region and most of the central region seem to be dispensable for the function of Slt11p, provided that the stability of the truncated protein is maintained. For this reason, only truncations ΔB and ΔC were used in the subsequent analyses.

b) Complementation of synthetic lethality of ΔSLT11 with mutations in U2, U5 and slt17/slt7-100. Although both truncations ΔB and ΔC were (partially) functional (Fig. 6.8B), the U2-11nt and 9nt substitutions in helix II region were lethal in the presence of either truncations (Table 6.2). The same is true for substitutions C22A and C22G (Table 6.2). However, both truncations were able to rescue partially the lethality of other substitutions at G21 and U23 positions of U2 snRNA (Table 6.2).

Of six mutations in loop 1 of U5 snRNA, two single substitutions (U98A and U98G) and two double-deletions (ΔC94/ΔC95 and ΔU96/ΔU97) were not rescued by either
Table 6.2. Genetic analysis of truncations of Slt11p -- complementation of synthetic lethality of ∆SLT11 with U2, U5 and slt17/slu7-100 mutations.

<table>
<thead>
<tr>
<th>Slt11 truncations</th>
<th>Slt11p</th>
<th>Slt11ΔBp</th>
<th>Slt11ΔCp</th>
<th>vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>U2 mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9nt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>11nt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>G21A</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>G21C</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G21U</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>C22A</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C22G</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C22U</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>U23A</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>U23G</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U23C</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>U5 mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U98A</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U98G</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U98C/sup</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>∆C94/∆C95</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>∆U96/∆U97</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>+1 U</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>slt17/slu7-100</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: ++: wt growth; +: mildly slow growth; +/-: slow growth; -: no growth
truncation. A single nucleotide insertion (+1 U) was rescued partially by ΔB or ΔC, while U98C was rescued partially only by ΔB (Table 6.2).

Although Slt11ΔBp is almost fully functional, it rescued partially the synthetic lethality of ΔSLT11 with slt17/slu7-100. Truncation ΔC failed to do so at all temperatures (Table 6.2).

Results of genetic analyses are largely consistent with the idea that the N-terminal portion of Slt11p is a functional domain which is partially sufficient to complement ΔSLT11. However, the rest of the protein is also important. In particular, a fully functional Slt11p is likely required to rescue U2 substitutions of 9nt, 11nt, C22A and C22G, and several U5 loop 1 mutations.

6.2.7. Mutational analysis of N-terminal Zn-fingers

The N-terminal Zn-binding motifs may represent two Zn-fingers. If the cysteine residues in these motifs are indeed involved in binding of zinc and are important for Slt11p, one may anticipate that (C → A) mutations at these residues could drastically impair the function of Slt11p. Assuming that the two adjacent pairs of cystines are involved in the zinc-binding, six mutations were constructed, four of which contained single pair change of C → A (Z1, Z2, Z4, and Z5), and two combinations of the two adjacent pairs (Z3 and Z6) (Fig. 6.9A). They were tested for complementation of ΔSLT11 and synthetic lethality of ΔSLT11 with both U2 mutations and slt17/slu7-100. Three of them, Z1, Z2 and Z3, were also tested for complementation of synthetic lethality of ΔSLT11 with U5 mutations.

a) Complementation of ΔSLT11. Consistent with the notion that these cystine residues are required for chelating zinc ions important for the function of Slt11p, all six mutations were found defective in complementing the ts phenotype of ΔSLT11. In particular, mutations in the two central pairs of cystines (Z2 and Z4) rendered Slt11p inert, while mutations (Z2 and Z5) in
Fig. 6.9. Zn-finger mutations of Slt11p. A. The four pairs of conserved cysteines may represent two fingers, assuming that the two adjacent pairs (i.e., M1+M2, M3+M4) are involved in chelating one zinc ion. C → A change was introduced in each pair (Z1, Z2, Z3 and Z4) or in combination (Z3 and Z6). B. Complementation of ts phenotype of ΔSLT11 by Zn-finger mutations, using the yeast strain shown in Fig. 6.8B. C. Two possible arrangements of zinc-finger motifs.
the other two pairs conferred ts phenotype at 37°C (Fig. 6.9B). Combination of mutations in the two adjacent pairs of cystines (Z3 and Z6) yielded no additive defect.

b) Complementation of synthetic lethality of ΔSLT11 with U2, U5 mutations and slt17/slu7-100. U2 substitutions of 9nt, 11nt, C22A and C22G remained lethal in combination with any of the six Zn-finger mutations (Table 6.3). While Z2 and Z3 failed to complement the synthetic lethality of ΔSLT11 with the remaining U2 mutations (except G21U), other Zn-finger mutations rescued partially the synthetic lethality (with exception of Z4 and Z6, which were lethal with U23G, Table 6.3). Of three Zn-finger mutations tested in combination with four U5 mutations in loop 1, Z1 complemented partially the synthetic lethality of all four U5 mutations, whereas Z2 and Z3 failed to do so (Table 6.3). Three Zn-finger mutations, Z1, Z2 and Z5, were partially functional in complementing of the synthetic lethality of ΔSLT11 with slt17/slu7-100, while the other three were not.

In summary, the mutational analysis (Tables 6.2 and 6.3) revealed that the integrity of Slt11p is essential for the function of the helix II region and the C22 position of U2 snRNA. Substitutions in either region (except C22U) are lethal in combination with any of the truncation or the Zn-finger mutations. Two truncations (ΔB and ΔC) and most Zn-finger mutations (Z1, Z4, Z5, and Z6) are partially functionally in combination with other U2 mutations. There seems to be a correlation between the function of a mutant protein to complement ΔSLT11 and its ability to rescue U2 mutations in the ΔSLT11 background. Complementation of lethality of slt17/slu7-100 mutation in the ΔSLT11 background requires at least part of the central region (Rpl25p homology) (Table 6.2), but the Zn-finger region is also important. For U5 mutations, a mixed pattern was observed.
## Table 6.3. Genetic analysis of C → A mutations of Slt11p -- complementation of synthetic lethality of ΔSLT11 with U2, U5 and slt7/slu7-100 mutations.

<table>
<thead>
<tr>
<th>mutations</th>
<th>WT</th>
<th>Z1</th>
<th>Z2</th>
<th>Z3</th>
<th>Z4</th>
<th>Z5</th>
<th>Z6</th>
<th>vector</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>°C</strong></td>
<td>25</td>
<td>30</td>
<td>33</td>
<td>25</td>
<td>30</td>
<td>33</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td><strong>wt</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td><strong>U2 mutations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9nt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11nt</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G21A</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G21C</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G21U</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>C22A</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22G</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22U</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>U23A</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>U23C</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>U5 mutations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U98C/sup</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ΔC94/ΔC95</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ΔU96/ΔU97</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+1 U</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>slt7/slu7-100</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Note:** ++: wt growth; +: mildly slow growth; +/-: slow growth; -: no growth
6.2.8. **Expression and purification of His-Slt11p in *E. coli*, and production of rabbit anti-His-Slt11p antibody.**

To explore biochemically the roles of Slt11p in the pre-mRNA splicing, in particular protein-RNA and protein-protein interactions involving Slt11p, antibodies against Slt11p are required. To this end, recombinant Slt11p was expressed in *E. coli* and purified.

Bacterial expression vector pET16 (Invitrogen) was used for the expression construct (see §6.4.7 for detail). DNA sequences encoding the full Slt11p and Slt11ΔAp open reading frames were cloned in pET16b which introduced a 6-histidine tag near the N-termini in order to facilitate purification by means of Ni²⁺ affinity chromatography.

After induction, a low level of expression of proteins of predicted sizes, ~45Kd for His-Slt11p and ~36 Kd for His-slt11ΔAp, was observed (Fig. 6.10A, lanes 5 and 6). The full-length recombinant protein was found to be in soluble form (data not shown) while the truncated protein was insoluble and aggregated as inclusion bodies (Fig. 6.10A, lanes 8). It is likely that the same truncated protein is unstable in yeast and thus fails to complement ΔSLT11 (Fig. 6.8B, also see below).

The full-length soluble His-Slt11p was purified under native conditions by means of Ni²⁺ affinity chromatography (Fig. 6.10B). Total soluble proteins from *E. coli* expressing His-Slt11p were first mixed with Ni²⁺-beads and loaded on a column. His-Slt11p was bound to the resin efficiently and was not detected in the flow through (Fig. 6.10B, lanes 1 and 2). Following extensive washes and final washes with 25 mM and 100 mM imidazole to eliminate non-specifically bound host proteins (Fig. 6.10B, lanes 3 through 9), the column was then eluted with 200 mM imidazole (Fig. 6.10B, lanes 10 through 12). Approximately 7.5 mg of His-Slt11p was obtained from 1 liter *E. coli* culture. After dialysis against PBS to remove imidazole, His-Slt11p was sent to the animal center at University of Toronto for the production of rabbit
Fig. 6.10. Expression and purification of His-Slt11p. A. Expression of His-Slt11p and -Slt11ΔAp in *E. coli*. Total bacterial protein extracts were prepared before (-) and after (+) the induction, resolved on a 12.5% SDS-PAGE and stained with Coommasie blue. Soluble and insoluble fractions of His-Slt11ΔAp extract are shown in lanes 7 and 8, respectively. Triangles indicate His-tagged proteins expressed after induction. B. Purification of His-Slt11p using Ni++ affinity chromatography under native condition. load: soluble fraction of His-Slt11p extract used as starting material. FT: flow-through. lanes 3 - 9: fractions collected after extensive washes with 25 and 100 mM imidazole. lane 10 - 15: eluates. C. Purification of His-Slt11ΔAp using Ni++ affinity chromatography under denaturing conditions. load: insoluble fraction of His-Slt11ΔAp extract. lanes 1 - 6: fractions collected after extensive washes. lanes 7 - 11: eluates.
antibodies. The truncated protein was also purified under denaturing conditions using 8M urea pH 4.5 (Fig. 6.10.C, see §6.4.7 for detail).

### 6.2.9. Detection of Slt11p in total protein extracts and splicing extract.

Rabbit anti-His-Slt11p antibody was produced according to standard procedures. As determined by Western/protein blot analysis, the titer of the serum sample collected prior to the final exsanguination reached 1/5,000; i.e., Slt11p was detected in wt yeast splicing extract containing total protein of 10 mg with diluted rabbit antiserum at 1/5,000 dilution (Fig. 6.11A, lane 2, and data not shown). However, a signal corresponding to Slt11p was not detected in a splicing extract prepared from a ΔSLT11 strain, while background signals remained unchanged (Fig. 6.11A, lane 3).

Total protein extracts were prepared from ΔSLT11 cells carrying a plasmid that expresses full length wt, ΔA, ΔB and ΔC Slt11p (Fig. 6.11B) or from cells with a vector alone. The presence of Slt11p in these cells was determined by SDS-PAGE/Western protein blot assays. A full-length (~41kD) and truncated (~20kD) Slt11p proteins were detected in cells that carried a wt SLT11 and slt11ΔB genes, respectively (Fig. 6.11A, lanes 4 and 5). The predicted size of Slt11p with deletion ΔA is ~35kD, but was not detected in cells carrying slt11ΔA (Fig. 6.11, lane 6). Since the same truncated protein (His-tagged) is not soluble when expressed in E. coli (Fig. 6.10A, lanes 7 and 8), it is likely that this protein is not stable in yeast cells; i.e., the failure of Slt11ΔAp to complement the ts phenotype of ΔSLT11 (Fig. 6.8B) is likely to its instability in vivo. On the other hand, Slt11ΔC is partially functional (Fig. 6.8B and Table 6.2), but was not detected with the antibody (Fig. 6.11, lane 7). However, this is not due to the instability of the truncated protein in vivo. When recombinant His-Slt11p was probed with an anti-His-tag antibody, a large fraction of degradation products (~24kD) (representing the stable N-terminal domain) was detected (data not shown), however, it was not detected when probed
Fig. 6.11. Western/protein blot detection of Slt11p. lane 1: 50 ng of His-Slt11p, lanes 2: wt splicing extract (~10 mg), lane 3: ΔSLT11 splicing extract (~20 mg), lanes 4 - 8: total protein extracts (~20 mg total proteins) from cells as indicated. Proteins were resolved on a 12.5% SDS-PAGE and electroblotted to a nylon membrane, and probed with rabbit anti-His-Slt11p antibody (1/5,000 dilution) and goat anti-rabbit IgG conjugated with horseradish peroxidase (1/10,000 dilution).
with antibody produced (Fig. 6.11A, lane 1). Taken together, these results suggest that the antibody produced does not recognize the N-terminal region of Slt11p. Truncation ΔC represents a stable domain of Slt11p that contains the Zn-binding motifs; it is also the domain that retains the minimal function of Slt11p (unpublished observations, in collaboration with C. Koth).
§6.3. Discussion

Slt11p is the second new splicing factor isolated in the genetic screen on the basis of synthetic lethality with a U2 mutation that can perturb the U2/U6 helix II interaction. It is a putative RNA-binding protein with Zn-binding motifs in the N-terminal region. Although SLT11 is not essential for cellular viability at permissive temperatures, a chromosomal deletion confers growth defect at ≥ 33°C (Fig. 6.2C). In the absence of Slt11p, the activation of the spliceosome is impaired, and is heat-labile (Fig. 6.3), suggesting that the protein exerts its function immediately prior to the first splicing reaction. Genetic analyses indicate interactions of Slt11p with three snRNAs in the active spliceosome (see Fig. 6.6 for summary). In particular, mutations in two regions (helix II and helix Ib) in the 5'-end of U2 snRNA become lethal in the absence of Slt11p, even though most of these mutations confer little or mild growth defect themselves (e.g., Fig. 6.4C). It has been proposed in the previous chapter (§5.3.2) that the nucleotides 21, 22 and 23 of U2 snRNA may play a role in the second step; i.e., it may interact with the highly conserved AG dinucleotide in the exon region of the 3' splice site (Fig. 5.8). The genetic interaction between Slt11p and U2 snRNA may suggest a potential role of Slt11p in the second step. Consistently, slt17/slu7-100 is lethal in the absence of Slt11p, and the original slt11-l is synthetically lethal with several other second-step mutations (§3.2.5, Fig. 3.6). Taken together, these results suggest that Slt11p may regulate and coordinate RNA-RNA and RNA-protein interactions in the active spliceosome which are important for both steps.

6.3.1. Possible functions of Slt11p.

The presence of zinc-binding motifs and a central region with homology to ribosomal protein L25 indicates that Slt11p may be an RNA-binding protein. If so, a question arises as to what is the RNA target for this protein. Our genetic analyses failed to pinpoint such an RNA
target. On the contrary, the genetic data (Figs. 6.4 and 6.5) seem to suggest that this protein may interact with three snRNAs in the active spliceosome. There are two possibilities: a) Slt11p may recognize specifically the intermolecular RNA-RNA interaction(s); e.g., U2/U6 helix II (see below); and/or b) Slt11p may act as an RNA chaperone to facilitate RNA conformational changes in the catalytic center of the spliceosome. These two possibilities are not mutually exclusive, although experimental data seem to favor the second one slightly.

If Slt11p is a specific RNA binding protein, the intermolecular U2/U6 helix II interaction could be a potential target, since in the absence of Slt11p, mutations in both U2 and U6 parts of the helix II interaction are lethal. In particular, two U2 mutations (\(\omega_1\) and \(\omega_2\)) that represent the two halves of 9nt substitution remain lethal, while the adjacent \(\omega_3\) mutation is not synthetically lethal with \(\Delta SLT11\) (Fig. 6.4B). It will be interesting to determine whether \(\omega\)-corresponding mutations in U6 are lethal in the absence of Slt11p, and whether restoration of mutationally disrupted helix II can suppress the original lethality conferred by either the U2 or the U6 mutation (see §7.2.4 for detail). If this is the case, Slt11p may exert its function during the spliceosome assembly, since helix II is likely formed in spliceosome assembly (§4.3.2). In addition, or alternatively, Slt11p may possess non-specific RNA binding activity and act as an RNA chaperone in the active spliceosome (Fig. 6.12A). If so, Slt11p may facilitate RNA conformational changes in the catalytic center of the spliceosome by preventing misfolding or by resolving misfolded RNA structures, a function that is analogous to protein chaperones. It may also act to stabilize RNA conformations in the spliceosome. The broad genetic interactions of Slt11p with all three snRNAs in the active spliceosome support this idea.

As discussed in §1.1.2, RNA-binding proteins aid RNA-based catalytic reactions by accelerating proper folding of biologically active RNA structures, since RNA folding is an error-prone process in the absence of protein factors (Herschlag 1995). The same could be true for pre-mRNA splicing. Prior to the splicing reaction, RNA-RNA interactions must be converted into catalytically active configurations. Although RNA components alone may be sufficient for
Fig. 6.12. Role of Slt11p in activation of the spliceosome and its interactions with other components in the spliceosome. A. Slt11p may function as an RNA chaperone that facilitates and coordinates RNA conformational changes during the activation of the spliceosome. Slt11p may facilitate RNA productive conformational changes by preventing misfolding of inactive conformations. In the absence of Slt11p, these inactive conformational changes may be in competition kinetically with the productive conformation, which results in the reduction in the rate of activation. Furthermore, the process of activation may become heat-labile. B. A summary of interactions of multiple RNA-RNA, RNA-protein and protein-protein interactions in the spliceosome. Slt11p may act to mediate and coordinate various interactions.
spliceosomal activation, protein factors are required to achieve a high level of efficiency and precision. Slt11p could be one of such factors. When Slt11p is absent in the yeast splicing extract, activation of the spliceosome is kinetically retarded at a permissive temperature and is completely impaired at an elevated temperatures (Fig. 6.3). It is possible that without protein factors (e.g., Slt11p) and the proper protein-protein interactions, productive conformational changes are in kinetic competition with the formation of alternative structures (Fig. 6.12A). However, given enough incubation time, sufficient splicing activity can be achieved, whereas in the presence of such a factor, maximum activity is observed in a short period of incubation time (Fig. 6.3A, lanes 11 through 16). But at higher temperatures, formation of the active conformation may not be favored thermodynamically over other competing structures. Furthermore, mutations in RNAs which are involved in the conformational changes can drastically alter the thermodynamics of RNA folding such that the alternative structures are strongly favored over the active conformation. Protein factors (e.g., Slt11p) and their interactions are absolutely required under the circumstance. The observed synthetic lethality of ΔSLT11 with mutations in U2, U5 and U6 snRNAs may reflect the RNA chaperone function of Slt11p in activation of the spliceosome. For both U2 and U5 snRNAs, only mutations in specific regions are lethal in the absence of Slt11p (Figs. 6.4 and 6.5). These regions may constitute part of the catalytic center; i.e., loop 1 of U5 snRNA is involved in tethering the two exons, and the helix Ib region of U2 snRNA may interact with the exon region of the 3' splice site (Fig. 6.6).

Although the N-terminal portion of Slt11p almost complements fully the chromosomal deletion of SLT11 (Fig. 6.7), efforts to dissect Slt11p failed to reveal any domains that may interact with snRNA(s) specifically (Tables 6.2). On the contrary, it seems that different portions of Slt11p may act together to exert its function. Results of genetic analyses (Tables 6.2 and 6.3) are consistent with the idea that Slt11p may act as an RNA chaperone, rather than an specific RNA-binding protein.
6.3.2. Zinc-fingers vs. zinc-ribbon.

Slt11p contains four pairs (M1 to M4) of CX2C clusters in the N-terminal region, which may represent two putative zinc-fingers. The first Zn-finger shows similarity to that in yeast transcription factor TFIIIB, with respect to the spacing between the conserved CX2C pairs (Fig. 6.1C). The Zn-finger motif was first identified as an RNA binding motif in another transcription factor TFIIIA, required for the transcription of 5S rRNA gene (for a review, see Pieler 1995). TFIIIA contains nine Zn-fingers (CX4CX12HX2H) in tandem repeats. It has been shown that these Zn-fingers are involved directly in DNA and RNA binding. Interestingly, the first three fingers are found to contribute to the overall DNA-binding activity (Darby and Joho 1992; Theunissen et al. 1992; Del Rio and Setzer 1993), while the three center fingers govern RNA binding (Theunissen et al. 1992; Clemens et al. 1993; Rollins et al. 1993; Setzer et al. 1996). However, the Zn-fingers in TFIIIA are different from those two in Slt11p (Fig. 6.13A). The Zn-fingers in Slt11p may represent a new class of Zn-binding motif present in a group of eukaryotic proteins (Fig. 6.1E). For Slt11p, these Zn-fingers are important for its function (Fig. 6.9, Table 6.3).

If the N-terminal portion of Slt11p forms two Zn-fingers as shown in left panel of Fig. 6.9C; i.e., two adjacent pairs of CX2C are involved in chelating zinc, changing either of them (C → A) should confer similar defects. However, mutational analyses revealed that the two center pairs (M2 and M3) are more susceptible to mutation. This observation suggests an alternative Zn-finger structure in which the two central pairs of CX2C are involved in chelating the same zinc ion and forming a structure (CX2CX23CX2C) (Fig. 6.9C, right) similar to zinc ribbon (CX2CX24CX2C) present in the eukaryotic transcriptional elongation factor TFIIS, shown to bind to ssDNA (Qian et al. 1993a, b). In this arrangement, the two remaining pairs of CX2C (M2 and M3) may also coordinate the second zinc ion. Since the N-terminal portion of Slt11p forms a highly stable domain (resistant to partial proteolysis, unpublished observations, in collaboration with C. Koth), and is also almost fully active (Fig. 6.8), it can be expressed and
purified to determine its nucleic-acid-binding activity, and ultimately, the structure of this potential RNA-binding protein in the absence or presence of RNA.

6.3.3. Functional duality of Slt11p: coupling and coordination of the two steps of splicing.

Most RNA-binding proteins involved in pre-mRNA splicing interact with other protein factors in addition to RNA targets. It is proper to suggest that protein-protein interactions based on RNA-binding proteins may provide cooperativity for RNA-RNA interactions in the spliceosome (see §6.1 and Table 6.1 for details). Slt11p may act to modulate RNA-RNA structures in the active spliceosome that are important for both steps of the splicing reaction. It may also interact with other protein factors, in particular, those which are involved in the second step. These protein-RNA interactions (Fig. 6.12B) may reflect a mechanism for efficient coupling and coordination of the two-step reaction in which the functional duality of Slt11p plays an essential role.

Both the original slt11-l mutation and ΔSLT11 (deletion a) are synthetically lethal with mutations in several second-step factors: slt15/prp17-100, slu4/prp17-2, slt17/slu7-100, slu7-1, and prp16-1 (Fig. 3.6 and data not shown). Although ΔSLT11 (deletion b) has been shown to be synthetically lethal with slt17/slu7-100, it is most likely that it is also lethal with these mutations. It remains to be determined if Slt11p interacts directly with Slt17p/Slu7p (see §7.2.4 for detail). However, genetic analyses (Tables 6.2 and 6.3) failed to reveal a domain in Slt11p that is specific for interaction with Slt17p/Slu7p. On the contrary, Slt11p-Slt17p interaction is affected by deletions and Zn-finger mutations that also affect Slt11p-snRNA interactions; i.e., interactions of Slt11p with both snRNA and protein(s) are functionally interdependent. If Slt11p acts as an RNA chaperone in the spliceosome (Fig. 6.12A), its interactions with Slt17p/Slu7p and other second-step factors may coordinate the actions of
RNA and protein factors for the second splicing step, since selection/ recognition of the 3' splice site is achieved by several factors sequentially (see §5.1). Slt11p may also facilitate the RNA conformational changes involved in the remodeling of the spliceosome for the second splicing step. Although SLT11 is not essential for viability at <33°C (Fig. 6.2), mutations that perturb the network of RNA-RNA, RNA-protein and protein-protein interactions (Fig. 6.12B) are exacerbated in the absence of Slt11p. Multiple protein and RNA interactions in the spliceosome are advantageous and may constitute a built-in "insurance" mechanism so that the effect of a mutation in one component can be compensated by additional interactions.
§6.4. Materials and Methods

6.4.1. Yeast strains and plasmids.

All yeast strains were derived from W303-1A and -1B (Mata or Matα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100). slt17, ASNR20 and ASNR7 strains are described elsewhere (§2.4.2 and §5.4.1). snr7 (U5) and snr20 (U2) mutations are described in §3.4.1.

U2-ω mutations, and U5 α and ψ mutations were constructed using Quick-changeTM site-directed mutagenesis (Stratagene, catalog #200518) method (also see §6.4.6). U6-a, -b, -c and -d mutations were described in Field and Friesen (1996). U5 mutations, ΔC94/ΔC95, ΔU96/ΔU97, +1U, +2Us, C112A, C112G, and C112U, are courtesy of A.J. Newman (MRC Laboratory of Molecular Biology, UK).

6.4.2. Chromosomal deletion of SLT11.

For deletion a (Fig. 6.2B), the internal SacI-HindIII fragment, which corresponds to the region homologous to Rpl25, was deleted and replaced by the yeast HIS3 gene. PCR mutagenesis was used to create a BamHI site 5' to the ATG codon of SLT11 ORF. The HIS3 gene was inserted at this BamHI site and the HindIII site near the 3' end to generate deletion b (Fig. 6.2C). Both deletion constructs were linearized and introduced into wt diploid (LP112). The resulting His⁺ transformants were screened by PCR for disruption at SLT11 locus. SLT11/ΔSLT11 diploids were sporulated and tetrad dissection performed. Four viable spores were obtained in both cases, two of which were His⁺ and ts at ≥33°C. Wild-type SLT11, on a CEN-ARS plasmid, complemented fully the ts phenotype of all His⁺ progenies, thus confirming chromosomal deletion of SLT11.
6.4.3. Preparation of splicing extract and \textit{in vitro} splicing assays.

Whole cell splicing extracts were prepared from YDX1138 (deletion a) and YDX1186 (deletion b) according Lin et al. (1987) with modification. \textit{In vitro} splicing reaction and native gel electrophoresis were performed as described in §3.4.2.

6.4.4. Genetic analysis.

The following is a list of the relevant yeast strains used in genetic analysis:

- YDX1186-1: \textit{Mat} \textalpha{} \textit{SLT11}::\textit{HIS3} \textit{ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100}
- YDX1191-1: \textit{Mat} \textit{SLT11}::\textit{HIS3} \textit{SNR20}::\textit{HIS3} \textit{ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100} \{\textit{SNR20 URA3 CEN-ARS}\}
- YXU52-1: \textit{SLT11}::\textit{HIS3} \textit{SNR7}::\textit{HIS3} \textit{ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100} \{\textit{SNR7 URA3 CEN-ARS}\}
- YDX92-1: \textit{SLT11}::\textit{HIS3} \textit{SNR20}::\textit{HIS3} \textit{SNR6}::\textit{HIS3} \textit{ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100} \{\textit{SNR20 SNR6 URA3 CEN-ARS}\}
- YXU92-4: \textit{SLT11}::\textit{HIS3} \textit{SNR6}::\textit{HIS3} \textit{ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100} \{\textit{SNR6 URA3 CEN-ARS}\}
- YXU84: \textit{Mat}/\textit{Mat} \textalpha{} \textit{SLT17}/\textit{SLT17} \textit{SLT11}/\textit{SLT11}::\textit{HIS3} \textit{ade2-1/ade2-1,his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100} \{\textit{SLT11 URA3 CEN-ARS}\}
- YXU84-1: \textit{SLT17} \textit{SLT11}::\textit{HIS3} \textit{ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100} \{\textit{SLT11 URA3 CEN-ARS}\}
6.4.5. Construction of Truncations.

A PCR method was used for the construction of two truncations of slt11 (Fig. 6.8A). First, a BamHI site was introduced immediately 3’ end of TGA of SLT11 ORF such that the 3’ non-coding region was obtained on a BamHI-BglII fragment (~0.6Kb) and cloned at the BamHI site in pRS315 (LEU2 CEN-ARS). To construct truncations ΔA and ΔC, BamHI sites were introduced in SLT11 ORF at positions corresponding to amino acids 150 and 318 respectively. The 5’ portions of gene, including the promoter region and ORF corresponding to truncations ΔA and ΔC contained on two BamHI-BglII fragments, were cloned at the BamHI site in the above plasmid.

6.4.6. PCR-based site directed mutagenesis.

Quick-change™ site-directed mutagenesis (Stratagene, catalog #200518) method was used to introduce Cyt → Ala changes at two Zn-finger motifs near the N-terminus of Slt11p. Briefly, the desired mutation was first introduced in two ~30 nt oligonucleotides, both of which were annealed to the plasmid carrying wt SLT11 gene. Pfu DNA polymerase extended the primers resulting in nicked circular strands in which the mutation was incorporated. The methylated, nonmutated parental plasmid template was then digested with DpnI. Mutated plasmid, in the nicked circular form, was transformed into XL1-Blue E. coli competent cells. Plasmid DNA was then prepared from the resulting transformants. An example is given as follows:

To change C13 and C15 to alanines, mutations were introduced in two complementary oligonucleotide primers, XI-17 (5’ CCCAATATAGCTGAGCA\textbf{AGCTTTAGGAT}GAT) and XI-18 (5’ ATCACCTAAA\textbf{AGCTTGCTCAGCTATATTGGG}) [bolded nucleotides indicate C→A changes, underlined nucleotides created a convenient HindIII site (silent mutations) which was
used to identify mutated plasmids]. About 50 ng of SLT11 plasmid (2.1KbBglII fragment of SLT11 in pRS315, total size ~7.2Kb) was mixed with primers XI-17 and XI-18 (125 ng each) in a total volume of 50μl containing 1X reaction buffer, 200μM dNTPs (50μM each) and 2.5U of Pfu DNA polymerase. PCR was performed as follows: segment 1 (1 cycle, 95°C for 2 min → 55°C for 1 min → 68°C for 17 min) and segment 2 (20 cycles, 95°C for 30 sec → 55°C for 1 min → 68°C for 17 min). Reaction mixture were then transferred to a fresh tube and 10U of DpnI added for digestion at 37°C for 1 hr. One microliter of the digest was used to transform 50μl aliquots of XL1-Blue supercompetent cells. Plasmids isolated from the transformants were examined for the presence of the new HindIII site (introduced in the two original primers). The mutation was confirmed by DNA sequencing.

6.4.7. Expression and Purification of His-Slt11p.

The ORFs of Slt11p and Slt11ΔA were cloned in bacterial expression vector, pET16b, which introduces 6 histidine residues at the N-terminus of the protein (Novagen). The expression constructs were used to transform host BL21 (DE3). The expression of the yeast gene is driven by T7 promoter which is transcribed by T7 RNA polymerase. The latter is under the control of lac promoter. When bacterial culture reached an OD600 between 3-5, IPTG was added to a final concentration of 0.4 mM. After 2.5 hr of induction, an aliquot was collected to determine protein expression level and solubility of the recombinant protein. The remaining cells were pelleted by centrifugation and frozen at -70°C. Ni++ affinity chromatography was used to purify bacterially expressed recombinant proteins.

The His-Slt11p was found to be soluble in E. coli lysates and, thus a native purification method was used. The cell pellet (of 2 liter culture) was resuspended in sonication buffer (50mM Na-phosphate buffer pH 8.0, 300mM NaCl, at 2.5 volumes per gram of pellet), and sonicated on ice. Cell debris and insoluble proteins were removed by centrifugation at 12,000g
for 20 min. The supernatant was mixed with 50% slurry of Ni-NTA resin (Qiagen, 1/4 volume of resin per volume of lysate) previously equilibrated with sonication buffer. The His-tagged protein was allowed to bind to resin with stirring at 4°C for 60 min. The resin/lysate mixture was then loaded on a 1.6 cm diameter column and washed with sonication buffer (≥20 column volume at the flow rate of ~0.5 ml/min). The column was then washed with wash buffer (50 mM Na-phosphate buffer pH 6.0, 300 mM NaCl and 10% glycerol, ≥20 volume, or until OD₂₈₀ of flow-through was ≤0.01). To elute the protein, step gradients of imidazole in wash buffer (25 mM, 100 mM, 200 mM and 300 mM, equal volume for each, 1X column volume) were used at the same flow rate. The eluate was collected at ~1 ml/fraction and analyzed on SDS-PAGE.

His-Slt11ΔAp was insoluble in bacteria and formed inclusion bodies. It was purified using a denaturing method. The insoluble fraction that contained His-Slt11ΔAp inclusion bodies was collected and resuspended in buffer A (6M GuHCl, 0.1M Na-phosphate, 0.01M Tris-HCl, pH 8.0, 5 ml/gram pellet). Following centrifugation (10,000g for 20 min), the supernatant was collected and mixed with 0.5 vol of 50% slurry of Ni-NTA resin pre-equilibrated with buffer A, an stirred at 4°C for 1 hr. The mixture was then loaded to a 1.6 cm column and washed with 10 column volume of buffer B (8M urea, 0.1M Na-phosphate, 0.01M Tris-HCl, pH 8.0) and buffer C (8M urea, 0.1M Na-phosphate, 0.01M Tris-HCl, pH 6.3) until OD₂₈₀ was ≤0.01. The column was then eluted with 1X column volume of buffer D (8M urea, 0.1M Na-phosphate, 0.01M Tris-HCl, pH 5.9), followed 2X volume of buffer E (8M urea, 0.1M Na-phosphate, 0.01M Tris-HCl, pH 4.5).


Splicing extract was prepared as described in §3.4.2. Total yeast protein extracts were prepared according to Foiani et al. (1994). Extracts containing ~15 mg of total yeast protein
were resolved on a 12.5% SDS-PAGE and electroblotted to immobilon-P membrane (Millipore). The membrane was first probed with a 1/5,000 dilution of rabbit anti-His-Slt1lp antibody and then a 1/10,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase, and detected by enhanced chemiluminescent (ECL) kit (Kirkegaard & Perry Lab.).
Chapter 7.

Summary and Future Directions
§7.1. Summary.

Pre-mRNA splicing is a highly coordinated process. During spliceosome assembly, individual RNA/RNA interactions are connected through protein/protein interactions. After all five snRNAs are brought to the pre-mRNA substrate, the spliceosome undergoes extensive RNA conformational rearrangements in order to form the catalytic center in which the splice sites are brought to close proximity and juxtaposed properly, and RNA structures important for the transesterification reaction are assembled. The intermolecular base-pairing interactions between U2 and U6 snRNAs, in particular helix Ia, are responsible directly for bringing the 5' splice site and the branchpoint to close proximity and may form part of the catalytic center (see Fig. 1.5 and §1.3.2). However, the function of U2/U6 helix II interaction is less-well understood. The invariant loop 1 region of U5 snRNA is required for the tethering of the two exons in the second step of splicing (O'Keefe and Newman 1998 and references therein). It interacts with the 3' exon only following the first step. Very little is known about interactions that hold U5 snRNA in the active center prior to the second step -- U5 loop 1 is in close contact with the 5' exon before the first step. Although selection/ recognition of the 3' splice site requires other components of the spliceosome, it is not clear how these factors are connected to U5 loop 1, or whether and how the 3' splice site is recognized prior to the second step.

The genetic screen described in Chapter 2 was designed to identify yeast splicing factors that are important for the U2/U6 helix II interaction and/or the 5' end of U2 snRNA. Six slt mutations were isolated based on synthetic lethality with an 11nt substitution in the 5' end region of U2 snRNA which could also perturb the helix II interaction. Two of the corresponding SLT genes encode new splicing factors, Slt11p and Slt22p. While the functions of both proteins are related to RNA/RNA interactions in the spliceosome, the RNA-dependent ATPase activity of Slt22p is involved directly in U2/U6 helix II interaction during spliceosome assembly (Chapter 4). However, subsequent genetic analyses revealed that the 5'-end of U2 snRNA is
Fig. 7.1. A summary of possible functions of Slt factors in the splicing pathway.
not restricted to helix II interaction with the 3'-end of U6 snRNA, and that at least two Slt factors, Slt21p/Prp8p and Slt17p/Slu7p, interact specifically with the U2 snRNA (Chapter 3). These specific genetic interactions indicate that U2/U6 helix II is unwound, likely by the RNA helicase activity of Slt22p. Since the 3'-end of human U6 snRNA is involved in the disruption of U4/U6 snRNA duplex (Brow and Vidaver 1995), it is possible that U2/U6 helix II may serve to antagonize the premature formation of helix I and Brow stem (Fig. 4.10), both of which are important for the subsequent catalytic steps.

In the slt22-1 mutant extract, a new splicing complex, X, was formed at the expense of other normal complexes. Complex X was found to contain only U2 and U6 snRNAs, but not U5 snRNA (Chapter 4). Consistent with the idea that Slt22p may represent a crucial regulatory step in the initiation of the splicing reaction, these results suggest that U2/U6 helix II and/or the 5'-end of U2 snRNA may play a role in anchoring U5 snRNA to the rest of the spliceosome. The potential interaction between U2 and U5 snRNPs was suggested two Slt factors, Slt15p/Prp17p and Slt17p/Slu7p, both of which have been shown to interact genetically with loop 1 of U5 snRNA (Frank et al. 1992). Genetic interactions (synthetic lethality and allele-specific suppression) between mutations in nucleotides 21, 22 and 23 of U2 snRNA and loop 1 of U5 snRNA were observed (Fig. 3.3C). Taken together these results suggest a potential role for this region of U2 snRNA in selecting/recognizing the 3' splicing site (e.g., interacting with the intron region of the 3' splice site, Fig. 5.8), a function related to those of loop 1 of U5 snRNA, Slt17p/Slu7p and Slt21p/Prp8p.

Slt21p/Prp8p is the most highly conserved splicing factor yet identified. It is involved in spliceosome assembly and the second step of splicing. The mutation corresponding to slt21 is located in the polypyrimididine tract recognition domain in Prp8p (Umen and Guthrie 1996), important for 3' splice site selection (Chapter 5). slt21/prp8-21 was also synthetically lethal with mutations in two factors required for selection of the 3' splice site, loop 1 of U5 snRNA (Chapter 3) and Slt17p/Slu7p (Chapter 5). However, a splicing defect associated with
slt21/prp8-21 was not apparent in the splicing reaction with a full-length pre-mRNA substrate in vitro. When a truncated substrate lacking the polypyrimidine tract and the 3' splice site was used in the splicing reaction, slt21/prp8-21 was found to be defective in spliceosome assembly. In a splicing extract prepared from prp8-21 + U2-G21C/C14G double mutants, spliceosome assembly with the full-length substrate was also blocked prior to the first step (Chapter 5). Taken together, these results suggest that 1) the interaction between Slt21p/Prp8p and the polypyrimidine tract is established in spliceosome assembly; 2) the potential interaction between U2 and the 3' splice site is also established prior to the first; and 3) these interactions may persist through the first step. The interactions of Slt21p/Prp8p with U2 snRNA, Slt17p/Slu7p and other factor(s) may represent part of a mechanism for the coordination of the two steps of the splicing reaction. Since Slt21p/Prp8p is associated with U5 snRNP, its interactions with the polypyrimidine tract and U2 snRNA prior to the second step may provide the basis to anchor/position U5 snRNA in the spliceosome.

Slt11p is a putative RNA binding protein containing zinc-binding motifs (likely, two Zn-fingers) and a region with homology to the yeast ribosomal protein L25 in the N-terminal and central portions, respectively. Although SLT11 is not essential for viability at permissive temperatures (≤30°C), in the absence of Slt11p, in vitro splicing reaction is impaired without an apparent affect on spliceosome assembly, suggesting that this splicing factor acts immediately prior to the first step; i.e., at the step of spliceosome activation. Genetic analyses indicate that Slt11p may function as an RNA chaperone which mediates and coordinates RNA/RNA and RNA/protein interactions (Fig. 6.12) in the active spliceosome required for the catalytic steps (Chapter 6). Slt11p, Slt15p/Prp17p, Slt17p/Slu7p, Slt21p/Prp8p and Prp16p may act as two overlapping functional groups in the spliceosome (Chapters 3 and 6). Since all the other factors in these two groups are required for the second step of splicing, their interactions with Slt11p and snRNAs (U2 and U5, at least) are important for the efficient coupling and coordination of both steps of the splicing reaction.
§7.2. Future directions.

Results presented in this thesis suggest several new interactions in the spliceosome. The RNA-dependent ATPase activity of Slt22p is involved in U2/U6 helix II interaction. It is possible that the action of Slt22p may couple the unwinding of helix II with the disruption of U4/U6 duplex, as originally proposed by Brow and Vidaver (1995). The slt21/prp8-21 mutation identified in our genetic screen is likely defective in the selection of the 3' splice site. More significantly, the 5'-end of U2 snRNA may play a role in the second step of splicing, in particular, positions 21, 22 and 23 may interact with the highly conserved AG dinucleotide in the exon region of the 3' splice site. On the other hand, Slt11p is a new splicing factor, and its roles in pre-mRNA splicing need to be characterized biochemically in order to understand the potential mechanism for coupling and coordination of the two steps of the splicing reaction. Given the complexity of the splicing pathway, these new interactions and/or functions are likely to be more complicated than proposed. Nevertheless, they should be explored experimentally. A few of these experiments are considered as follows.

7.2.1. A potential role for the 5' end of U2 snRNA in the second step of splicing

Several lines of evidence that support this idea include: 1) mutations in the 5' end of U2 snRNA are synthetically lethal with three splicing factors important for the second step, Slt15p/Prp17p, Slt17p/Slu7p and Slt21p/Prp8 (Chapter 3); 2) some of these U2 mutations (i.e., in positions 21, 22 and 23) are synthetically lethal with or suppress mutations in loop 1 of U5 snRNA (Chapter 3); and 3) nucleotides U23 and A30 of U2 snRNA are in close contact with the 3' exon (Newman et al. 1995). It is proposed in Chapter 5 that nucleotides U23 and C22 of U2 snRNA may interact directly with the AG dinucleotide in the 3' splice site. The experiment shown in Fig. 7.2 is designed to test this direct interaction by genetic means. It is known that mutations in the AG dinucleotide can block the correct selection of the 3' splice site.
Fig. 7.2. Genetic experiments designed to test the potential interactions between the intron region of the 3' splice site and the 5' end region of U2 snRNA. A. ACT1-CUP1 fusion genes that carry mutations in the intron region of the 3' splice site (described in Umen and Guthrie 1996). The selection of wt 3' splice site produces an in-frame transcript of CUP1 that confers resistance to copper in the medium. However, mutations in the 3' splice site reduce the cleavage at the correct cleavage site (as indicated by the relative sizes of triangles at the appropriate cleavage sites) and activate cryptic sites that produce out-frame transcripts. This reduces the resistance to copper. Numbers in the parentheses indicate the highest concentrations of copper in the medium at which CUP1Δ yeast strain carrying indicated ACT1-CUP1 fusion gene can survive. B. Yeast strain used for genetic screen for U2 suppressor of mutations in the 3' splice site. In the presence of wt U2 snRNA, a mutant ACT1-CUP1 fusion gene (carrying mutation in the intron region of the 3' splice site, as indicated by N in C) will generate sensitivity to copper. C. The nucleotides (20 - 24) in U2 snRNA which will be randomized in the mutant U2 library. These positions are shown in the context of possible RNA/RNA interactions in the spliceosome following the first step. Nucleotides 21, 22 and 23 in U2 snRNA have been shown to interact genetically with loop 1 of U5 snRNA (see Chapter 4), not shown in this figure for clarity. D. A library of mutant U2 genes containing mutations at positions 20 through 24 (see C) will be introduced into the yeast shown in B and the resultant transformants will then be selected for resistance to copper in the presence (i.e., dominant suppressors) or absence (i.e., recessive suppressors) of wt U2 in combination with ACT1-CUP1 genes carrying mutations in the intron region of the 3' splice site, based on increased resistance to copper.
nucleotides important for the 2nd step
\( \Delta \) nucleotides important for 3\' SS selection
nucleotides in U2 that interact genetically with U5 loop 1
polyuridine tract

A
\[
\begin{align*}
\text{ACT1} & \quad \text{UAG} \quad \text{CUP1} \quad \text{cup}^r \ (2 \text{ mM}) \\
\text{ACT1} & \quad \text{GAG} \quad \text{CUP1} \quad \text{cup}^s \ (0.1 \text{ mM}) \\
\text{ACT1} & \quad \text{UGG} \quad \text{CUP1} \quad \text{cup}^s \ (0.05 \text{ mM}) \\
\text{ACT1} & \quad \text{UGG} \quad \text{CUP1} \quad \text{cup}^s \ (0.075 \text{ mM})
\end{align*}
\]

B
\[
\begin{align*}
\Delta \text{CUP1} & \quad \text{SNR20A::HIS3} \\
\text{SNR20} & \quad \text{URA3} \\
\text{URA3} & \quad \text{LEU2}
\end{align*}
\]

C
\[
\begin{align*}
\text{U6} & \quad \text{U2} \\
5' \text{ exon} & \quad 3' \text{ exon}
\end{align*}
\]

D
\[
\begin{align*}
\Delta \text{CUP1} & \quad \text{SNR20A::HIS3} \\
\text{SNR20} & \quad \text{URA3} \\
\text{URA3} & \quad \text{LEU2} \\
\text{U2 library} & \quad \text{wt-U2} \\
\Delta \text{CUP1} & \quad \text{SNR20A::HIS3} \\
\text{SNR20} & \quad \text{TRA1} \\
\text{TRA1} & \quad \text{LEU2}
\end{align*}
\]

- 251 -

recessive suppressor

dominant suppressor
The inhibitory effects of mutations in the last three nucleotides (UAG) of the intron in a sensitive ACT1-CUP1 fusion gene (Lesser and Guthrie 1993b; see Fig. 7.2A for detail) have been assessed quantitatively (Umen and Guthrie 1996). Three of the mutations (Fig. 7.2A) described in that study (Umen and Guthrie 1996) are available and can be used to screen for U2 snRNA suppressors.

A yeast strain containing a chromosomal deletion of SNR20 in the ΔCUP1 background (with a SNR20 URA3 maintenance plasmid) should be constructed (Fig. 7.2B). In the presence of wt U2 snRNA, wt and mutant ACT1-CUP1 genes would be introduced into this strain to assess the effects of mutations shown in Fig. 7.2A, as determined by their sensitivity to copper in the medium. A library of snr20 will be constructed to contain randomized nucleotides in positions 20 through 24 (Fig. 7.2C). This library will be introduced into the above strain carrying one of the mutant ACT1-CUP1 fusion gene, and the resultant transformants will then be selected for suppressors based on increased resistance to copper (Fig. 7.2D). In a similar manner, the effects of other U2 mutations (in particular, the 11nt and 9nt substitutions) on mutations in the 3' splice site or polypyrimidine tract (Umen and Guthrie 1995a; see Fig. 7.4A) can also be determined. If results of these experiments are informative, the genetic suppressor approach should be extended to more mutations in the 3' splice site in order to establish whether direct basepairing interaction between U2 and the intron region of the 3' splice site is involved. Primer extension analyses will be used to demonstrate that the observed genetic suppression correlates with the correct or enhanced selection of the 3' splice site.


During the maturation of the spliceosome, the disruption of U4/U6 duplex (Fig. 7.3A) is a critical regulatory step. However, very little is known about the splicing factors involved in
this step. One of the RNA elements important for the disruption of human U4/U6 duplex lies in the 3' end of U6 snRNA (Brow and Vidaver 1995), which forms helix II with the 5'-end of U2 snRNA. Indirect experiments suggest that the formation of helix II may stabilize the U4/U6 duplex and that the energy released from switch between alternative conformations, instead of the action of an RNA helicase, is sufficient to disrupt the duplex (Brow and Vidaver 1995). It has been proposed in Chapter 4 that the formation of helix II precedes other U2/U6 interactions in spliceosome assembly and that the action of Slt22p, an RNA-dependent ATPase/RNA helicase, may couple the unwinding of helix II with the disruption of the U4/U6 duplex. Consistent with this idea, the slt22-1 mutation is synthetically lethal with prp8-1 (Fig. 5.7). It has also been observed that brr2, a mutant allele of slt22-1, is synthetically lethal with prp31-1 (J.L. Woolford, Jr., personal communication). Both prp8-1 and prp31-1 mutations affect spliceosome assembly at the step during or after the recruitment of the U4/U6.U5 tri-snRNP.

It is known that mutations in the 3'-end of yeast U6 snRNA that disrupt the U2/U6 helix II interaction confer lethality (Field and Friesen 1996). To explore the potential for this region of U6 in the disruption of the U4/U6 duplex, the 5' end of U4 snRNA will be extended to form basepair interaction with the 3' end of U6 snRNA (Fig. 7.3B). In this hyperstabilized U4/U6 duplex, the extended U4/U6 helix is in direct competition with the formation of U2/U6 helix II. The growth phenotype of this particular U4 mutation will be determined. If it confers lethality or a severe growth defect, slt22 suppressors can be isolated (Fig. 7.3C) -- if Slt22p is involved in coupling of unwinding of helix II with U4/U6 duplex, mutations in Slt22p with less stringent substrate specificity may unwind the extended U4/U6 helix and thus suppress the U4 mutation. The suppressor approach can also be used to isolate other splicing factors involved in the step. On the other hand, if this U4 mutation confers mild or no growth defect, synthetic lethality between mutant U4 and slt22 can be sought (Fig 7.3C). In either case, a library containing mutant slt22 will be constructed. Since SLT22 is a large gene, it is necessary
to divide the entire gene into several smaller regions. Particular attention should be given to the RNA-dependent ATPase domain (i.e., domain II, Fig. 4.1A).

Fig. 7.3. Genetic experiments designed to test the potential role for Slt22p in coupling of the unwinding of U2/U6 helix II and the disruption of U4/U6 duplex. A. RNA/RNA interactions in the holo-spliceosome. The formation of U2/U6 helix II may precede other U2/U6 interactions (i.e., helices Ia and Ib, as indicated by blocks with different shading in U2 and U6 snRNAs, also see Fig. 4.10). Helix II may stabilize the U4/U6 duplex and in doing so may also antagonize the formation of other RNA interactions important for the subsequent events. It is possible that the unwinding of helix II is coupled with the disruption of U4/U6 duplex such that the energy released for the former may serve the latter, as proposed by Brow and Vidaver (1995). B. Proposed U4 mutation that affects the U2/U6 helix II interaction. A mutant U4 snRNA [U4 snRNA (5' ext)] with an extended 5' end will be constructed. The extended region contains an 11 nt sequence that can basepair with the 3' end of U6 snRNA. The extended U4/U6 interaction is in direct competition with the formation of U2/U6 helix II, as shown. C. Suppression and synthetic lethality screen. A yeast strain with double deletions of SLT22 and SNR14 (the U4 snRNA gene) will be constructed which carries a maintenance plasmid of both SLT22 and SNR14. The mutant U4 will be tested for growth defect in the presence of SLT22 (on a TRPI-marked plasmid). If it confers lethality or severe growth defect, slt22 suppressors will be sought that can suppress U4 snRNA (5' ext.) (left). However, if the mutant U4 is viable or confer mild growth defect, mutations in slt22 will be isolated that are synthetically lethal with the mutant U4 (right). In either case a mutant slt22 library will be constructed. The RNA helicase domain (i.e., domain II, Fig. 3.1A) will be subject to random mutagenesis.
Chapfer 7

is lethal

U4 (ext.) is viable, i.e., suppression

U6 snRNA (3'-end)

U2 snRNA (5'-end)

U4 snRNA (5' ext)

if 5-FOA5; i.e., U4(Ext.) is lethal

if 5-FOA5; i.e., U4(Ext.) is viable

- 255 -
7.2.3. Slt21p/Prp8p

Results presented in Chapter 5 suggest that *slt21/prp8-21* is likely to be defective in recognition of the polypyrimidine tract. This should be tested directly by genetic means. A yeast strain containing a chromosomal deletion of *PRP8* in the *ΔCUP1* background will be constructed (Fig. 7.4A). The effects of *PRP8, prp8-1, prp8-101* and *slt21/prp8-21* on splicing of *ACT1-CUP1* transcript containing a modified disruption of the U4/U6 duplex. Consistent with this idea, the *slt22-1* mutation is synthetically lethal with *prp8-1* (Fig. 5.7). It has also been observed that *brr2*, a mutant allele of *slt22-1*, is synthetically lethal with *prp31-1* (J.L. Woolford, Jr., personal communication). Both *prp8-1* and *prp31-1* mutations affect spliceosome assembly at the step during or after the recruitment of the U4/U6.U5 tri-snRNP.

It is known that mutations in the 3'-end of yeast U6 snRNA that disrupt the U2/U6 helix II interaction confer lethality (Field and Friesen 1996). To explore the potential for this region of U6 in the disruption of the U4/U6 duplex, the 5' end of U4 snRNA will be extended to form basepair interaction with the 3' end of U6 snRNA (Fig. 7.3B). In this hyperstabilized U4/U6 duplex, the extended U4/U6 helix is in direct competition with the formation of U2/U6 helix II. The growth phenotype of this particular U4 mutation will be determined. If it confers lethality or a severe growth defect, *slt22* suppressors can be isolated (Fig. 7.3C) -- if Slt22p is involved in coupling of unwinding of helix II with U4/U6 duplex, mutations in Slt22p with less stringent substrate specificity may unwind the extended U4/U6 helix and thus suppress the U4 mutation. The suppressor approach can also be used to isolate other splicing factors involved in the step. On the other hand, if this U4 mutation confers a mild or no growth defect, synthetic lethality between mutant U4 and *slt22* can be sought (Fig 7.3C). In either case, a library containing mutant *slt22* will be constructed. Since *SLT22* is a large gene, it is necessary to divide the entire gene into several smaller regions. Particular attention should be given to the RNA-dependent ATPase domain (i.e., domain II, Fig. 4.1A).
Fig. 7.4. **A. prp8 mutations and 3' splice site competition.** top: A yeast strain containing a deletion of PRP8 in the background of ΔCUP1 will be constructed. Mutant ACT1-CUP1 fusion genes, containing duplicated 3' splice sites will be introduced into the strain. below: In the presence of PRP8, the proximal 3' splice site will be selected preferentially (as indicated by the relative sizes of the triangles). This will produce an out-frame CUP1 transcript, and thus confers sensitivity to copper in the medium. However, mutation in prp8-101 (see Fig. 5.3B) loses such preference resulting in an increase in the selection of the distal 3' splice site which produces in-frame CUP1 transcript. The yeast strain thus becomes resistant to copper (Umen and Guthrie 1995b). Using this strain and mutant ACT1-CUP1 fusion gene shown above, slt21/prp8-21 mutation will be tested for defect in the 3' splice site selection. Other experiments are considered in the text. **B. Genetic interaction between ΔMUD2 and prp8 mutations.** left: a yeast strain used to test synthetic lethality between ΔMUD2 and prp8 mutations (including prp8-1, slt21/prp8-21, prp8-101). right: If synthetic lethality is observed, deletions of Mud2p will be constructed to test the minimal domain(s) that is(are) able to rescue the synthetic lethality.
polypyrimidine tract and duplicated 3' splice sites (+T PyDOWN, Fig. 7.4B, Umen and Guthrie 1995b) will be tested. It has been shown that the proximal 3' splice site is preferentially selected in the PRP8 background which results in an out-frame mature transcript and thus sensitivity to copper. However, prp8-101, defective in the recognition of the polypyrimidine, loses such a preference so that the distal 3' splice site is used to generate an in-frame transcript, thus gives rise to resistance to copper. If slt21/prp8-21 is also defective in the recognition of the polypyrimidine tract, similar copper resistance will be observed with the fusion reporter mentioned above. Furthermore, slt21/prp8-21 may exacerbate mutations in the 3' splice site (see Fig. 7.2A); i.e., in combination with these mutant reporter genes, slt21/prp8-21 may reduce the resistance to copper, as it has been shown to be the case for prp8-101 (Umen and Guthrie 1995b). If the predicted resistance to copper is observed with slt21/prp8-21, primer extension will then be used to demonstrate the loss of preference of the proximal 3' splice site.

The defect associated with slt21/prp8-21 is exerted in spliceosome assembly suggesting that recognition of the polypyrimidine tract by Prp8p occurs during assembly. Since Mud2p is involved in recognition of the polypyrimidine tract early in assembly (Abovich et al. 1994), its interaction (likely indirect) with Prp8p may be required for spliceosome assembly and maturation. One may observe synthetic lethality between slt21/prp8-21 and AMUD2. If so, truncations of Mud2p (Fig. 7.4B) will be tested for complementation of such synthetic lethality to determine the minimal domain(s) of Mud2p which is required for interaction with Prp8p in spliceosome assembly. These genetic interactions, if observed, will suggest a role of Prp8p in coordinating the selection/recognition of splice sites in spliceosome assembly and the two steps of the splicing reaction.
7.2.4. Slt11p

Slt11p may function as an RNA chaperone in the active spliceosome to coordinate a number of RNA/RNA interactions that are important for both steps of the splicing reaction (Chapter 6). One potential target of Slt11p is the U2/U6 helix II interaction. It has been shown in Chapter 6 that mutations (ω1, ω2) corresponding to the two halves of the U2-9nt substitution are synthetically lethal with \( \Delta S L T 1 1 \) (Fig. 6.4). It remains to be determined if the ω-corresponding mutations in the 3' end of U6 snRNA are synthetically lethal with \( \Delta S L T 1 1 \), as in the case for three other U6 mutations (U6-b, -c, and -d, Figs. 3.2 and 6.4). If so, mutual suppression of these U2 and U6 mutations should be determined (Fig. 7.5A). Should Slt11p be involved directly in the U2/U6 helix II interaction, such mutual suppression will be observed. However, it is possible that Slt11p is involved in other RNA/RNA interactions which require both regions of U2 and U6 snRNAs. In this case, a suppressor screen (Fig. 7.5B) may shed light on these additional interactions.

Since the \( \Delta S L T 1 1 \) extract is defective in pre-mRNA splicing, in vitro complementation by the recombinant His-Slt11p will be examined. It will be interesting to determine if the final complex that accumulates in the \( \Delta S L T 1 1 \) extract at the non-permissive temperature is a functional intermediate or a terminally stalled complex in which RNA conformational changes are kinetically trapped in the inactive forms. If the former is the case, addition of recombinant Slt11p after the formation of the complex should rescue the splicing activity. Otherwise, Slt11p can only rescue the splicing activity when it is added prior to the addition of the pre-mRNA substrate. Truncations (ΔB and ΔC, Fig. 6.8) and Zn-finger mutations (Z2 and Z4, Fig. 6.9) of Slt11p should also be tested for in vitro complementation.

The anti-His-Slt11p antibody produced in this study should be purified using affinity chromatography. Once purified, it can be used to determine if Slt11p is associated with any snRNP(s), and if it interacts with the pre-mRNA substrate in spliceosome assembly and the
Fig. 7.5. A. A potential role for Slt11p in U2/U6 helix II interaction. left: A yeast strain containing deletions of SLT11 (not essential at ≤30°C), SNR20 and SNR6 (both are essential) has been constructed. It also carries a maintenance plasmid with both SNR20 and SNR6 genes. right: Two mutations in the 5' end of U2 snRNA (U2-ω1 and -ω2) have been shown to be synthetically lethal with ΔSLT11. Three 3rd substitutions in the 3' end of U6 (U6-b, -c and -d) are also synthetically lethal with ΔSLT11 (Fig. 6.4B). U6 mutations (U6-χ1 and -χ2) corresponding to U2-ω1 and -ω2 will be constructed and tested for synthetic lethality with ΔSLT11, using the yeast shown on the left, in the presence of wt U2. Combinations of U2-ω1 and U6-χ1, U2-ω2 and U6-χ2 will be tested for mutual suppression of synthetic lethality observed with either U2 or U6 mutation. B. Genetic screen for suppressors of synthetic lethality of U2-ω mutation with ΔSLT11. Since either U2-ω mutation is lethal in the absence of Slt11p, ΔSLT11 cells carrying both U2-wt (on the URA3 plasmid) and U2-ω (on a TRP1 plasmid) will be screened for spontaneous mutations that rescue the lethality of U2-ω in the absence of U2-wt; i.e., resistance to 5-FOA in the medium.
intermediates/substrates in the splicing reaction. In particular, site-specific crosslinking between Slt11p and the 3' splice site should be examined in various mutant extracts (prp9-1, prp8-1, slt22-1, slt21/prp8-21, prp2-1, slt17/slu7-100, and prp16-1, each of these mutations blocks splicing at a different stage in the splicing pathway) in order to determine the early stage at which Slt11p makes contact with the 3' splice site. Similarly, the kinetics of interactions of Slt11p with U2 and U5 snRNAs (if the protein is not associated with either snRNP) can be determined. Furthermore, direct protein/protein interaction between Slt11p (and mutant Slt11p proteins) and Slt17p/Slu7p will be examined. Results of these experiments will be interpreted in the context of the coupling and coordination mechanism for the two steps of the splicing reaction.

Partial proteolysis of His-Slt11p revealed a stable domain that contains the N-terminal Zn-binding motifs (corresponding to either Slt11ΔB or Slt11ΔC) (in collaboration with C. Koth, data not shown). This minimal domain is almost fully functional in complementing the ts phenotype of ΔSLT11 (Fig. 6.8). The RNA-binding properties will be studied using full-length and minimal His-Slt11p proteins, and Zn-finger mutant proteins. The minimal Slt11p domain, in the absence and presence of RNA, will then be used for structural studies.

7.2.5. A possible connection between pre-mRNA splicing and nuclear transport.

In course of this study, a spontaneous suppressor was isolated that rescues the synthetic lethality of ΔSLT11 with U2-11nt substitution. The suppressor itself confers a severe growth defect (slow-growth at ≤30°C and ts at ≥33°C). The corresponding wild-type gene was cloned by complementation, and it is identical to RAT7, which encodes a 159kD nucleoporin, a component of the nuclear pore complex (Gorsch et al. 1995; Kraemer et al. 1995). Rat7p is involved in export of poly(A)+ RNA from nucleus to the cytoplasm (Gorsch et al. 1995; Kraemer et al. 1995). This suppressor suggests a possible connection between pre-mRNA
splicing and nuclear transport. Genetic suppression by the rat7 mutation has been extended to synthetic lethality of ΔSLT11 with other U2 mutations, and two other slt mutations, slt21/prp8-21 and slt22-1, with U2 mutations (data not shown). Thus, it seems that the suppression is relatively general. One possible mechanism for such non-specific suppression is provided here. Although very little is known about the fate of pre-mRNA transcripts when the splicing machinery is completely blocked, preliminary studies indicated that in yeast, these precursor transcripts are exported to the cytoplasm by default (Legrain and Rosbash 1989). If this is the general phenomenon, and/or if components of spliceosome assembled under nonpermissive condition (due to a mutation in one of these components) are exported to the cytoplasm, it is possible to suppress the splicing mutation by blocking nuclear export such that the precursor transcripts and/or the splicing components are retained in the nucleus for another round of splicing (thus enhancing the efficiency of pre-mRNA splicing). According to this interpretation, only rat7 mutations that are defective in nuclear export at permissive temperatures should be able to suppress synthetic lethality of slt mutations with U2 mutations. Several rat7 mutations have been acquired from C.N. Cole (Dartmouth Medical School, Hanover NH). Some of them are defective at the permissive temperature, while others are defective only at non-permissive temperatures (Gorsch et al. 1995). These rat7 mutations can be tested for suppression of synthetic lethality of ΔSLT11 with U2 mutations using the yeast strain shown in Fig. 7.6. Furthermore, the fate of intron-containing transcripts after splicing is blocked can be studied using in situ hybridization of particular intron-containing transcripts. The transport of splicing factors can be studied using GFP (green fluorescent protein)-tagged protein factor(s).
Fig. 7.6. Suppression of synthetic lethality of ΔSLT11 with U2-11nt substitution by rat7 mutations. A yeast strain containing deletions of SLT11 (not essential for viability at ≤30°C), RAT7 and SNR20 (both are essential) has been be constructed. The maintenance plasmid (URA3-marked) carries both RAT7 and SNR20 genes. To test suppression by rat7 mutations, plasmids carrying the mutant U2 (11nt substitution) and rat7 mutations will be introduced into the strain. The resultant transformants will be tested for 5-FOA resistance; i.e., an indication of viability of U2-11nt mutation in the background of ΔSLT11.
References


References


References


References


Séraphin, B. and M. Rosbash. 1991. The yeast branchpoint sequence is not required for the formation of a stable U1 snRNP-pre-mRNA complex and is recognized in the absence of U2 snRNA. *EMBO J.* 10:1209-1216.


References


Teigelkamp, S., M. McGarvey, M. Plimpton, and J.D. Beggs. 1994. The splicing factor PRP2, a putative RNA helicase, interacts directly with pre-mRNA. *EMBO J.* 13:888-896.


Umen, J.G. and C. Guthrie. 1995c. Prp16p, Slu7p, and Prp8p interact with the 3’ splice site in two distinct stages during the second catalytic step of pre-mRNA splicing. RNA 1:584-597.


