Mutational analysis of the function and expression of Rpo26p, a subunit common to nuclear RNA polymerases in *Saccharomyces cerevisiae*

by

Shahrzad Nouraini

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

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ABSTRACT

MUTATIONAL ANALYSIS OF THE FUNCTION AND EXPRESSION OF Rpo26p, A SUBUNIT COMMON TO NUCLEAR RNA POLYMERASES IN SACCHAROMYCES CEREVISIAE

Shahrzad Nouraini, Degree of Doctor of Philosophy, 1997, Department of Molecular and Medical Genetics, University of Toronto

Eukaryotic RNA polymerases (RNAPs) are composed of up to 13 subunits, some of which are shared among the three nuclear RNAPs. I have isolated a collection of functionally defective mutant alleles of RPO26, a gene that encodes one of the shared polymerase subunits. I screened a randomly mutagenized library of RPO26 for synthetic lethality in combination with a temperature sensitive (ts) allele (rpo21-4) of the gene that encodes the largest subunit of RNAPII. Thirty-four mutant alleles were identified and divided into three classes based on their phenotype in the presence of RPO21: 5 ts, 12 null (inactive) and 17 neutral (no detectable growth phenotype). Twenty-nine alleles had mutations in the Rpo26 protein and five had mutations in the RPO26 promoter. One ts rpo26 mutant allele, rpo26-31, showed a notable reduction in the steady-state level of Rpo26p, Rpo21p and A190p (the largest subunit of RNAPI) at the non-permissive temperature. Furthermore, the amount of assembled RNAPI and RNAPII was notably reduced at high temperature. These results indicate that Rpo26p is required for stability of the largest subunits of RNAPI and RNAPII and for the assembly of these enzymes.

I isolated spontaneous suppressors of the ts phenotype of rpo26-31. One such suppressor mutation, pup3-1, resided in the PUP3 gene which encodes the catalytic subunit of the yeast 20S proteasome. The pup3-1 mutation allowed rpo26-31p to accumulate to wild-type levels. In contrast, in a pup3-1 strain, the ts phenotype of a number of RPO21 mutant alleles was not suppressed and the unstable rpo21-4 subunit did
not accumulate at high temperature. This result suggests that the steady-state levels of Rpo26p and Rpo21p are regulated independently.

Four of the *RPO26* promoter mutations mentioned above were alterations in a consensus binding-sequence for the transcription factor Abf1p. I have shown that purified Abf1p binds specifically to the *RPO26* promoter *in vitro*, and that the mutations in the Abf1p binding sequence abolish this binding. These promoter mutations reduced the level of expression of *RPO26* and shifted the start site of transcription upstream on the *RPO26* promoter.
"ATTITUDE isn't simply a state of mind... it is also a reflection of what we value. Attitude is more than just saying I can, it is believing you can. It requires believing before seeing, because seeing is based on circumstances, believing is based on faith. Attitude is so contagious especially when we allow it to turn our doubts of the past into passions of today and set the stage for our tomorrows. We have total ownership of our attitudes. No one else has the power to alter our attitudes without permission. Our attitude allows us to become more empowering than money, to rise above our failures, and accept others for who they are, and what they say. It is more important than giftedness and is the forerunner of all skills needed for happiness and success. Our attitudes can be used to build us up or put us down- the choice is ours. It also gives us the wisdom to know that we can't change events of the past. I am convinced that life is 10% what happens to me, and 90% how I respond to it ... and it is with this state of mind that I remain in charge of my ATTITUDES."

Source unknown
ACKNOWLEDGEMENT

I would like to thank my supervisor Jim Friesen for his support and encouragement. Jim has always inspired me with his adventurous nature and his never-ending energy and passion for science. I consider it a privilege to have been closely associated with him for the past seven years. I also thank the members of my supervisory committee, Henry Krause, Jack Greenblatt and Brenda Andrews for their support and interest in my graduate education. I specially thank and acknowledge Brenda for being a constant source of support and encouragement. She urged me to be assertive, supported me when I needed help and praised me for my achievements; I could not have asked for a better mentor!

I thank the members of Jim Friesen's lab, both past and present, for their friendship and also for their contribution to my graduate training. The past seven years has truly been a life experience; I would not change it for anything else.

I acknowledge the women's equality movement for their contribution to my personal growth. Reading the feminist literature taught me self-respect and encouraged me to follow my dreams no matter how bizarre they might seem in the eyes of others.

My deepest appreciation goes to my husband, Bahram Shamsian, for believing in me even when I have doubts in myself. He is truly a one of a kind partner in life for his never ending love, encouragement, and support.

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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>3'</td>
<td>3 prime</td>
</tr>
<tr>
<td>5'</td>
<td>5 prime</td>
</tr>
<tr>
<td>5FOA</td>
<td>5-fluoro-orotic acid</td>
</tr>
<tr>
<td>6AU</td>
<td>6-azauracil</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair(s)</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
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<td>messenger RNA</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
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<td>ribosomal RNA</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>ts</td>
<td>temperature sensitive</td>
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CHAPTER I

STRUCTURAL AND FUNCTIONAL PROPERTIES OF RNA POLYMERASES
INTRODUCTION

Normal growth and differentiation depends on the transcription of cellular genes. This process is performed by the enzyme RNA Polymerase (RNAP) which, depending on its source, ranges in complexity from a single-subunit enzyme in bacteriophages to the four subunit *E. coli* and the 15 subunit eukaryotic enzymes. Multicomponent RNAPs are in a large part composed of two large subunits and a number of small molecular weight proteins. In eukaryotes, transcription is performed by three different RNAPs which have a number of their small subunits in common. The primary sequences of the two large subunits of multicomponent RNAPs are well conserved across species which has allowed drawing parallels between the information known about the *E. coli* RNAP subunits to that of the eukaryotic RNAPs. These structural conservations along with a large number of genetic and biochemical experiments have determined that the DNA- and RNA- binding sites and the catalytic center of multisubunit RNAPs are formed by the two large molecular weight subunits of these enzymes. Furthermore, work on characterization of the smaller subunits has implicated them in mediating the assembly of RNAP and the interactions with transcription factors and upstream activating factors. However, the functions of subunits that are common to eukaryotic RNAPs, which have no counterparts in the prokaryotic enzyme, remain elusive. The aim of the work presented in this thesis is to understand the functional role and the regulation of expression of the yeast common subunit, Rpo26p. Below, I present an introduction, in which I summarize what is known about the functional and structural properties of prototypical DNA-dependent RNAPs of the various types that have been most extensively studied.

I- STRUCTURE OF RNAPs

A- Single-subunit RNAPs

The RNAP from bacteriophage T7 is the most well studied of the single-subunit RNAPs which include RNAP from other bacteriophages and mitochondrial RNAP. T7
RNAP is a 99 kDa protein which can carry out a complete round of RNA synthesis from a T7 promoter without the need for auxiliary factors (Sousa, et al., 1993). The crystal structure of this enzyme has been determined (Sousa, et al., 1993). It contains a large cleft (15-25 Å wide, 25-40 Å deep and 60Å long) that is predicted to accommodate nearly two full turns of the double helix. This prediction is in good agreement with the observation that T7 RNAP protects ~20 bp of DNA from digestion by DNase I (Shi, et al., 1988) and intercalation by MPF-Fe²⁺ (Ikeda and Richardson, 1986). The T7 RNAP cleft includes a three-dimensional structure called the "polymerase fold", which is also present in the Klenow fragment of E. coli DNA polymerase I (Ollis, et al., 1985) and HIV-1 reverse transcriptase (Kohlstaedt, et al., 1992) (Fig. 1). The polymerase fold is topologically similar to a hand with features resembling the palm, thumb and fingers (Fig. 1). All single-subunit RNAPs share sequence homology in 11 colinear segments; the homology regions include three short stretches of amino acids, termed motifs A, B and C, that are common to all DNA-directed DNA and RNA polymerases (Delarue, et al., 1990). Motifs A and C reside in the floor of the DNA-binding cleft (the palm subdomain) in T7 RNA polymerase and motif B forms part of the finger subdomain (reviewed in Delarue, et al., 1990).

B- Multisubunit RNAPs

All eubacterial, archaeabacterial and eukaryotic nuclear RNAPs are multicomponent enzymes which require auxiliary factors to carry out a complete round of transcription. Below, I discuss what is known about multisubunit RNAPs.

1. Eubacterial RNAP. The RNAP from Escherichia coli is the prototype for eubacterial RNAPs. E. coli core RNAP is made up of four subunits termed β (151 kDa), β' (155 kDa), α (36.5 kDa) and ω (10 kDa). The latter subunit can be removed from RNAP preparations without changing known properties of the enzyme. E. coli core RNAP is not capable of promoter-specific transcription and requires the addition of the σ
Fig. 1. Three dimensional structure of the polymerase domain. The structures of the polymerase domains of T7 RNAP (A), HIV-1 reverse transcriptase (B), and Klenow fragment of DNA polymerase I (C). The topology of the polymerase domain resembles the shape of a hand, with features resembling thumb, palm and fingers. Accordingly, the regions of these RNA polymerases that correspond to the above features have been named the thumb, palm, and finger subdomains. This figure is a modified version of Fig. 3b, which appears in Sousa et al., 1993.
subunit (Burgess, 1976); \( \sigma \) and core RNAP form the holoenzyme. There are a number of different \( \sigma \) subunits in \textit{E. coli}. However, the subunit that is predominantly associated with the core enzyme during exponential vegetative growth is the 70kDa protein \( \sigma^{70} \). \( \sigma^{70} \) is the most extensively studied eubacterial \( \sigma \) factor and it forms the prototype for this class of proteins; therefore, I limit my comments to \( \sigma^{70} \).

All of the subunits in the holoenzyme, except \( \alpha \) which is present as a homodimer, have a stoichiometry of unity (Burgess, 1976). The three-dimensional structures of both the core (Polyakov, et al., 1995) (Fig. 2b) and holoenzyme (Darst, et al., 1989) (Fig. 2c) have been determined by electron microscopy of two-dimensional crystals formed on positively-charged lipid bilayers. Both contain a 25Å channel of 55 Å length, similar to the cleft found in single-subunit polymerases (Fig. 1). In the holoenzyme a thumb-like projection surrounds the 25Å channel, leaving one side of the channel open (Darst, et al., 1989) (Fig. 2c). In the core enzyme the thumb-like domain bends towards and closes the channel (Polyakov, et al., 1995) (Fig. 2b). It has been postulated that the \( \sigma \) subunit binds to the core polymerase and induces a conformational change in the enzyme so as to open the DNA-binding channel and to allow RNAP to bind to DNA. Since the \( \sigma \) subunit leaves the polymerase complex at the onset of the elongation phase of the transcription cycle, closing of the channel in the core enzyme may stabilize the RNAP-DNA complex during elongation (Polyakov, et al., 1995).

2. Archaebacterial RNAPs. Archaebacterial RNAPs are more complex than eubacterial RNAPs. The degree of the complexity depends on the subclass of the archaebacterial species:

- The RNAP from \textit{Solfolobus acidocaldarius} (a member of the sulfur-dependent archaebacterial class) has four large subunits B, A' (or A), A" (or C) and D, and nine smaller components (Zillig, et al., 1985).
Fig. 2. Three-dimensional structures of eubacterial and eukaryotic RNAPs. The structures of yeast RNAPII Δ4/7 (a) and *E. coli* core- (b) and holo-enzyme (c) have been determined by electron microscopy of two-dimensional crystals. The gross structure of multisubunit RNAPs is very similar to the polymerase domain of single subunit RNAPs in that it resembles the shape of a hand containing a 25 Å channel (labeled c) surrounded by thumb-like (labeled T) and finger-like (labeled D) projections. In the core *E. coli* RNAP (b) and yeast RNAPII Δ4/7 (a) the channel is closed; whereas, in *E. coli* holoenzyme (c) the channel is open on one side. The open and closed forms of the channel are thought to represent RNAP in the initiation and elongation mode of transcription, respectively. This figure is adapted from Fig. 5 of Polyakov et al., 1995.
The RNAP from *Methanobacterium thermoautotrophicum* (a member of methanogens and extreme halophile class) has four large subunits A' (or A), B', B", A" (or C) and D, and about three smaller subunits (Zillig, et al., 1985).

Western-blot analysis using anti-subunit antibodies has shown that subunits A', A" and D from *S. acidocaldarius* correspond to A', A" and D from *M. thermoautotrophicum* (Zillig, et al., 1985). Furthermore, subunits B' and B" from *M. thermoautotrophicum* are homologous to non-overlapping N-terminal and C-terminal portions, respectively, of subunit B in *Solfolobus acidocaldarius* (Zillig, et al., 1985). On the other hand, A' and A" are homologous to non-overlapping N-terminal and C-terminal fragments, respectively, of the largest subunit of *E. coli* RNAP. Similarly, archaeabacterial subunit B is homologous to the second largest subunit of *E. coli* RNAP. These observations have been borne out by cloning and sequence analysis of the genes encoding subunits of RNAP from *Solfolobus acidocaldarius, Methanobacterium thermoautotrophicum* and *Halobactereium halobium* (Langer, et al., 1995, Puhler, et al., 1989).

3. Eukaryotic RNAPs. Eukaryotic nuclear transcription is performed by three different RNAPs, each of which is responsible for the transcription of a separate class of genes. These polymerases have been distinguished on the basis of differences in chromatographic behavior, salt sensitivity, template specificity, sensitivity to the toxin α-amanitin, nuclear localization, subunit composition and gene-specificity (reviewed in Roeder, 1976). RNAPI transcribes rRNA genes; RNAPII, which is most sensitive to α-amanitin, synthesizes mRNAs and some small nuclear RNAs (snRNAs); and RNAPIII is responsible for the transcription of genes encoding 5SRNA, tRNAs and some snRNAs. RNAPs purified from a wide variety of eukaryotic organisms are composed of two large subunits and a number of small components, an organization similar to that found in
eubacterial RNAPs (Roeder, 1976). Several observations have established that some of the smaller subunits are common among the three RNAPs; 1) identical molecular weights (Engelke, et al., 1983), 2) migration distances in two-dimensional polyacrylamide gels (Buhler, et al., 1976, D'Alessio, et al., 1979, Guilfoyle, 1980, Valenzuela, et al., 1976), 3) identical peptide maps (Buhler, et al., 1976, Dahmus, 1981a, Dahmus, 1981b), and 4) antigenic cross reactivity among these subunits (Buhler, et al., 1980, Guilfoyle, et al., 1984, Sentenac and Hall, 1982). This was confirmed later by cloning of the genes encoding these subunits in *Saccharomyces cerevisiae*, and demonstrating the presence of the product of one gene in all three RNAPs (Woychik, et al., 1990).

The RNAPs from *Saccharomyces cerevisiae* are typical of eukaryotic nuclear RNAPs. The subunit structures of these polymerases are shown in Tables 1-3. Five subunits are shared (Rpo25, Rpo26, Rpo28, Rpo210α, Rpo210β) among all three polymerases and two are common (AC40 and AC19) between RNAPI and RNAPIII (Reviewed in Archambault and Friesen, 1993). Some of the RNAP subunits dissociate from these enzymes *in vitro* under certain purification conditions as outlined in Tables 1-3. Dissociation of these subunits does not affect the catalytic activity of these enzymes, and in some cases a specific regulatory role has been defined for these subunits (see below). Eukaryotic RNAPs have been studied independently by a number of different groups, leading to three different systems of nomenclature for each subunit. I have not adhered to one system of nomenclature and the name that I have used for each subunit is in accord with that used by the group(s) whose work I report. The name of each subunit and the three systems of nomenclature are illustrated in Tables 1 to 3.

The three-dimensional structures of yeast RNAPI and RNAPII have been determined by electron microscopy of two-dimensional lipid crystals at 30 Å (Schultz, et al., 1993) and 16 Å (Darst, et al., 1991) resolution, respectively. The RNAPII preparation used for crystallization is termed RNAPII Δ4/7 since it lacks the two dissociable subunits of this enzyme, Rpo24p and Rpo27p (Darst, et al., 1991). The
Table 1. Subunit structure of *S. cerevisiae* RNAPI

<table>
<thead>
<tr>
<th>Size</th>
<th>Subunit name</th>
<th>Gene</th>
<th>RNAP</th>
<th>Disruption</th>
<th>Dissociable?</th>
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1There are three separate systems of nomenclature for the subunits of yeast RNAPs
Table 2. Subunit structure of *S. cerevisiae* RNAPII

<table>
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<tr>
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<th>Disruption</th>
<th>Dissociable?</th>
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<td>Ingles <em>et. al.</em>, 1984</td>
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<td><em>RPB3</em></td>
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<td>No</td>
<td>Sweetser <em>et. al.</em>, 1987</td>
</tr>
<tr>
<td>32</td>
<td>B32 Rpo24</td>
<td>Rpb4</td>
<td><em>RPB4</em></td>
<td>conditional</td>
<td>Yes</td>
<td>Kolodziej &amp; Young, 1989</td>
</tr>
<tr>
<td>27</td>
<td>ABC27 Rpo25</td>
<td>Rpb5</td>
<td><em>RPB5</em></td>
<td>I, III</td>
<td>lethal</td>
<td>Woychik &amp; Young, 1990</td>
</tr>
<tr>
<td>23</td>
<td>ABC23 Rpo26</td>
<td>Rpb6</td>
<td><em>RPB6/RPO26</em></td>
<td>lethal</td>
<td>No</td>
<td>Woychik <em>et. al.</em>, 1990</td>
</tr>
<tr>
<td>16</td>
<td>B16 Rpo27</td>
<td>Rpb7</td>
<td><em>RPB7</em></td>
<td>lethal</td>
<td>Yes</td>
<td>Archambault <em>et. al.</em>, 1990</td>
</tr>
<tr>
<td>14.5</td>
<td>ABC14 Rpo28</td>
<td>Rpb8</td>
<td><em>RPB8</em></td>
<td>lethal</td>
<td>No</td>
<td>Mckune <em>et. al.</em>, 1993</td>
</tr>
<tr>
<td>13</td>
<td>B13 Rpo211</td>
<td>Rpb11</td>
<td><em>RPB11</em></td>
<td>lethal</td>
<td>No</td>
<td>Woychik <em>et. al.</em>, 1990</td>
</tr>
<tr>
<td>12</td>
<td>B12 Rpo29</td>
<td>Rpb9</td>
<td><em>RPB9</em></td>
<td>conditional</td>
<td>No</td>
<td>Woychik <em>et. al.</em>, 1993</td>
</tr>
<tr>
<td>10α</td>
<td>ABC10α Rpo210α</td>
<td>Rpb10</td>
<td><em>RPC10</em></td>
<td>I, III</td>
<td>lethal</td>
<td>Treich <em>et. al.</em>, 1992</td>
</tr>
<tr>
<td>10β</td>
<td>ABC10β Rpo210β</td>
<td>Rpb12</td>
<td><em>RPB10</em></td>
<td>I, III</td>
<td>lethal</td>
<td>Woychik &amp; Young, 1990</td>
</tr>
</tbody>
</table>

1There are three separate systems of nomenclature for the subunits of yeast RNAPs
Table 3. Subunit structure of *S. cerevisiae* RNAPIII

<table>
<thead>
<tr>
<th>Size</th>
<th>Subunit name$^1$</th>
<th>Gene</th>
<th>RNAP</th>
<th>Disruption</th>
<th>Dissociable?</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>C160</td>
<td>Rpo31</td>
<td>Rpc1</td>
<td><em>RPO31</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>128</td>
<td>C128</td>
<td>Rpo32</td>
<td>Rpc2</td>
<td><em>RET1</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>82</td>
<td>C82</td>
<td>Rpo33</td>
<td>Rpc3</td>
<td><em>RPC82</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>53</td>
<td>C53</td>
<td>Rpo34</td>
<td>Rpc4</td>
<td><em>RPC53</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>40</td>
<td>AC40</td>
<td>Rpo35</td>
<td>Rpc5</td>
<td><em>RPC40</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>37</td>
<td>C37</td>
<td>Rpo36</td>
<td>Rpc6</td>
<td>Not cloned</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>34</td>
<td>C34</td>
<td>Rpo37</td>
<td>Rpc7</td>
<td><em>RPC34</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>31</td>
<td>C31</td>
<td>Rpo38</td>
<td>Rpc8</td>
<td><em>RPC31</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>27</td>
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<td>Rpo39</td>
<td>Rpc9</td>
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<td>I, II</td>
<td>No</td>
</tr>
<tr>
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<td>Rpc11</td>
<td>RPB6/RPO26</td>
<td>I, II</td>
<td>No</td>
</tr>
<tr>
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<td>AC19</td>
<td>Rpo312</td>
<td>Rpc12</td>
<td><em>RPC19</em></td>
<td>lethal</td>
<td>No</td>
</tr>
<tr>
<td>14.5</td>
<td>C14.5</td>
<td>Rpo313</td>
<td>Rpc13</td>
<td><em>RPB8</em></td>
<td>I, II</td>
<td>No</td>
</tr>
<tr>
<td>10α</td>
<td>ABC10α</td>
<td>Rpo314</td>
<td>Rpc14</td>
<td><em>RPC10</em></td>
<td>I, II</td>
<td>No</td>
</tr>
<tr>
<td>10β</td>
<td>ABC10β</td>
<td>Rpo315</td>
<td>Rpc15</td>
<td><em>RPB10</em></td>
<td>I, II</td>
<td>No</td>
</tr>
</tbody>
</table>

---

$^1$ There are three separate systems of nomenclature for the subunits of yeast RNAPs
structures of these enzymes are very similar; both contain a 25 Å wide channel formed by
the bending of a finger-like stalk over the molecule, a feature similar to that found in E. coli
RNAP (see above). However, in RNAPII Δ4/7 the finger-like stalk completely closes the
channel, in a manner reminiscent of the closed channel in E. coli core RNAP (compare Fig.
2A to 2B). This is consistent with the fact that both RNAPII Δ4/7 and E. coli core RNAP
are not capable of promoter-specific transcription without added factors: the
Rpo24p/Rpo27p dimer along with initiation factors for RNAPII (Edwards, et al., 1991)
and σ subunit for E. coli RNAP (Burgess, 1976). Therefore, as in the case for core E. coli
RNAP, RNAPIIΔ4/7 may adopt the conformation of an elongating RNAP (Darst, et al.,

In yeast RNAPI and RNAPII, the 25Å channel opens up into a 25 Å groove on the
surface of the molecule. The total length of the 25 Å channel and groove should
accommodate 20-25 bp of double-stranded DNA which would have to be bent during
binding to the polymerase. In fact, E. coli RNAP induces bending of the template DNA
during initiation of transcription and RNA chain elongation (Heumann, et al., 1988,
Kuhnke, et al., 1987). The higher resolution of the RNAPII structure allows the detection
of a 12-15 Å wide channel, wide enough for binding single-stranded RNA or DNA, which
branches off from the floor of the 25 Å channel and has been postulated to form the exit

II- EVOLUTIONARY RELATIONSHIP OF RNAP SUBUNITS

Molecular cloning of genes encoding the subunits of RNAPs from various species
has revealed sequence similarities among these polypeptides. In many cases, subunits with
sequence similarities have been shown to provide similar functions in their respective
RNAPs (see section III).
A- **Largest and second-largest subunits**

Depending on the stringency and the criteria used for the homology-search parameters either 8 (Jokerst, et al., 1989) or 6 (Allison, et al., 1985, Berghofer, et al., 1988, Memet, et al., 1988) colinear blocks of sequence homology have been reported for the largest subunits of all multisubunit RNAPs; I will use the former, since it is cited more frequently in the literature. These colinear regions of homology are termed regions A through H and are depicted in Fig. 3A. Five (Berghofer, et al., 1988), 6 (Yano and Nomura, 1991), 9 (Falkenburg, et al., 1987, Sweetser, et al., 1987) or 13 (James, et al., 1991) conserved regions have been reported for the second largest subunit. The nine regions of colinear homology, termed A through I, are most commonly cited and are depicted in Fig. 3B.

B- **E. coli α subunit and its homologues**

The E. coli α subunit shows sequence similarity to common subunits AC40p (Mann, et al., 1987, Martindale, 1990) and AC19p (Dequard-Chablat, et al., 1991) in yeast, subunits in yeast RNAPII Rpo23p (Kolodziej and Young, 1989) and Rpb1lp (or Rpo211p) (Woychik, et al., 1993) and Solfolobous subunits L and D (Langer, et al., 1995) (Fig. 4). This sequence similarity may reflect a common function since α, AC40p and Rpo23p are required for assembly of their respective RNAPs (see section II). The sequence in common between α and the archaeabacterial and eukaryotic subunits lies at the N-terminus of α and is termed the α motif (Fig. 4). Archaeabacterial subunit L and yeast subunits AC19p and Rpb1lp (or Rpo211p) share sequence similarity outside the α motif as do subunit D and yeast subunits AC40p and Rpb3p (or Rpo23p) (Langer, et al., 1995). AC40p and AC19p (Lalo, et al., 1993), and subunits L and D (Langer, et al., 1995) form heterodimers which correlates with the homodimerization of α (Burgess, 1976) in E. coli RNAP and Rpo23p in yeast RNAPII (Kolodziej and Young, 1991).
Fig. 3. Regions of homology between the largest and second largest subunits of eukaryotic and eubacterial RNAPs. Schematic representations of the largest subunits (A) and second-largest subunits (B) from *E. coli* and *Saccharomyces cerevisiae* RNAPs. The black boxes numbered alphabetically represent the colinear regions of sequence homology between eukaryotic and eubacterial RNAP subunits. CTD refers to a C-terminal domain specific to the largest subunit of eukaryotic RNAPII. It contains repeats of a heptapeptide sequence, PTSPSYS.
C- *E. coli* σ subunit and its homologues

σ^70^ and the dissociable yeast Rpo24 subunit have sequence similarity in a region of σ^70^ whose function is not known. Since both Rpo24p (Edwards, et al., 1991) and σ^70^ (Burgess, 1976) are required for promoter-specific transcription by their corresponding polymerases, the sequence homology might be related to a common function performed by these subunits.

D- Other subunits


The prominent feature of the conserved sequences is the presence of a zinc-binding consensus in these subunits. Component E of archaebacteria is homologous to dissociable subunits Rpb7p (or B16) (McKune, et al., 1993) in RNAPII and C25 (or Rpc10p) (Thuriaux and Sentenac, 1992) in RNAPIII.

III- FUNCTIONAL ROLES OF RNAP COMPONENTS

A- T7 RNAP

1. Determinants of promoter specificity. Although bacteriophage RNAPs are very similar, recognition of their respective promoters is carried out with high specificity. One of the promoter-specificity determinants in T7 RNA polymerase has been identified as
Fig. 4. *E. coli* RNAP α subunit and its homologous archaebacterial and eukaryotic subunits. The α subunit shares a short amino acid sequence, called the α-homology domain (black box), with the third (Rpo23p) and the 11th (Rpb11p) subunits of yeast RNAPII, the subunits common between yeast RNAPI and RNAPIII, AC40p and AC19p, and archaebacterial subunits L and D. The abbreviations used are *E. c.*, *Escherichia coli*; *S.c.*, *Saccharomyces cerevisiae*; and *S. a.*, *Solfolobous acidocaldarius*. 
amino acid N748 (McAllister, 1993). Substitution of N748 with a variety of other amino acids, changes the promoter specificity of T7 RNAP to that of other phages (T3, SP6, K11) (McAllister, 1993). N748 resides in the putative DNA-binding groove, at a position estimated to be a helical turn away from the palm subdomain, which is thought to contain the catalytic site (Sousa, et al., 1993). This positioning matches the spacing between the initiation site of transcription and the primary DNA-sequence determinants of promoter specificity located 10 and 11 bps upstream (McAllister, 1993). Other regions of the polymerase involved in promoter recognition were identified among a collection of linker-insertion mutations of the enzyme (Gross, et al., 1992). One such mutant contained an insertion near a region of T7 RNAP (144-159) that resides in the putative DNA-binding cleft and exhibits sequence homology to a region of the bacterial σ factor which interacts with the -10 region of E. coli promoters (Waldburger, et al., 1990).

2. Residues involved in catalysis. Sequence comparison of all single-subunit DNA-directed polymerases has identified five conserved amino acids; these correspond to K631, Y639, G640, D812 and D537 in T7 RNAP (Delarue, et al., 1990). All of these residues are components of the putative DNA-binding cleft. D812 and D537 reside in the palm subdomain; mutational analysis of these residues in T7 RNAP (Osumi-Davis, et al., 1992), and the equivalent residues in the Klenow fragment of E. coli DNA polymerase I, has shown that they are critical for catalytic activity (Bonner, et al., 1992, Kohlstaedt, et al., 1992, Osumi-Davis, et al., 1992, Polesky, et al., 1990, Sankar and Porter, 1992). Residues K631, Y639 and G640 are part of the finger subdomain. K631 cross-links to photoreactive analogs of the initiating nucleotide (Maksimova, et al., 1991, Schaffner, et al., 1987); mutations of this residue reduce the rate of catalysis (Vmax) and in some cases increase the Km for certain nucleotides (Maksimova, et al., 1991). Mutational alteration of Y639 in T7 RNA polymerase (Osumi-Davis, et al., 1992) and the corresponding residue in the Klenow fragment (Polesky, et al., 1990) also reduce the rate of catalysis and increase
the Km for nucleotide substrates, supporting a role for this amino acid in the catalytic activity of the enzyme. However, T7 RNAP with a conservative change of Y639 to F has kinetic properties similar to those of the wild-type enzyme, suggesting that the aromatic property of this side chain are important for its function (Osumi-Davis, et al., 1992). The Y639F mutant polymerase allows T7 RNAP to use dNTPs (deoxyNucleoside TriPhosphates) as well as rNTPs (riboNucleoside TriPhosphates) as substrates (Sousa and Padilla, 1995). Since T7 RNAP is known to use RNA as a template, albeit at low efficiency (Konarska and Sharp, 1989), a single point mutation (Y639F) allows it to function as a DNA-directed and RNA-directed DNA and RNA polymerase. Mutation of the corresponding tyrosine (Y766) in the Klenow fragment reduces fidelity; however, the ability of the mutant polymerase to utilize rNTP has not been tested (Carroll, et al., 1991, Polesky, et al., 1990). Y639F in T7 RNAP does not reduce fidelity (Sousa and Padilla, 1995). Recently, a point mutation (F155V) in the active site of Moloney murine leukemia virus (MMLV) RT has been identified that allows rNTP incorporation by this DNA polymerase, albeit at a 100-fold reduced rate (Gao, et al., 1997). F155 does not correspond to the Y639 in the primary sequence of the MMLV RT; however, it is predicted to occupy a position in the three dimensional structure of the enzyme similar to that of Y639 in T7 RNAP (Joyce, 1997). This position is close to the catalytic site, corresponding to the junction between the finger and palm domains (Joyce, 1997). Although it is not known how Y639 in T7 RNAP determines substrate specificity, modeling of the template-primer and nucleotide substrate into the three dimensional structure of MMLV RT (Georgiadis, et al., 1995) suggests that the bulky phenyl group of Phe-155 overlaps with the 2'-OH of an incoming rNTP, thus sterically inhibiting its binding to the polymerase (Georgiadis, et al., 1995).

The penultimate residue of T7 RNAP, F882, is involved in binding the elongating nucleotide; deletion or replacement of this residue with an amino acid with a non-aromatic side chain increases the Km for all rNTPs, with a much greater increase for purines than
pyrimidines (Patra, et al., 1992). Replacement of F882 with a different aromatic amino acid does not affect function (Patra, et al., 1992), suggesting a hydrophobic interaction between the side chain of F882 and the base of the elongating nucleotide. This suggestion is supported by the greater increase in Km for purines in the non-functional mutant forms of F882 and the fact that this residue is positioned very close to the putative active site in the three-dimensional structure of T7 RNAP (Sousa, et al., 1993).

3. **Residues involved in RNA binding.** Proteolytic digestion and mutational analysis have implicated the N-terminal one-third of T7 RNA polymerase in binding nascent RNA. In the absence of this domain, T7 RNA polymerase can effectively recognize the promoter DNA and mediate initiation with kinetic properties (Km and kcat) similar to the wild-type enzyme (Muller, et al., 1988). However, the truncated polymerase is unable to bind RNA in gel retardation assays and synthesizes only short abortive transcripts (Muller, et al., 1988). Furthermore, T7 RNAP that contains mutations in the N-terminal domain produces higher levels of abortive products that are of smaller size compared with those of the wild-type enzyme (Patra, et al., 1992). Therefore, the N-terminal domain of T7 RNAP stabilizes the ternary complex, perhaps by binding to nascent RNA, allowing the RNAP to enter the elongation phase of transcription.

**B- Multisubunit RNAPs**

1. **Components of the catalytic center.** Observations from a large number of biochemical and genetic experiments have shown that the two largest subunits form the catalytic center of the enzyme. The information available about the function of the two largest subunits has been obtained primarily from mutational and biochemical analysis of RNAPs from *E. coli* and *Saccharomyces cerevisiae*. Below, I summarize the functional properties of the two largest subunits:
1.1 THE LARGEST SUBUNIT.

1.1.A. ZINC-BINDING. The largest subunits of yeast nuclear RNAPs (Treich, et al., 1991, Werner, et al., 1992) and the β' subunit of E. coli RNAP (Wu, et al., 1977) bind zinc in vitro through homology region A. Mutational analysis has identified two different functions for the zinc-binding domain. Two observations suggest that one function of the zinc-binding domain is to contribute to the stability/assembly of the enzyme: 1) Temperature sensitive (ts) mutations in the zinc binding domain of the largest subunit of yeast RNAPIII (C160p or Rpo31p) jeopardize the stability of the enzyme, leading to the dissociation of three subunits (C82p, C34p and C31p) (Werner, et al., 1992), 2) ts zinc-binding domain mutations in the largest subunit of yeast RNAPI (A190p) reduce the steady-state level of this subunit, which can be rescued by overexpression of the mutant subunit (McCusker, et al., 1991, Wittekind, et al., 1988).

A second function for the zinc binding domains of the largest subunits was suggested by experiments with elongation complexes of E. coli RNAP (Nudler, et al., 1996). The zinc binding domain of β' is cross-linked to a photoreactive nucleotide on the double-stranded template immediately in front of the catalytic center of RNAP (Nudler, et al., 1996). This cross-linking is abolished in the presence of mutations in the zinc-binding motif (Nudler, et al., 1996); the same mutations reduce the processivity of the enzyme indicating that the zinc binding domain stabilizes the ternary elongation complex by binding to DNA (Nudler, et al., 1996).

1.1.B. DNA-BINDING. In addition to the data presented above, the ability of the largest subunit to bind DNA has been shown by: 1) DNA cross-linking experiments with yeast RNAPIII (Bartholomew, et al., 1993), 2) change in susceptibility of the largest subunit of mouse RNAPII to proteolysis upon binding of the enzyme to DNA (Horikoshi,
et al., 1985), 3) prevention of yeast RNAPII inhibition by antibodies to Rpo21p upon preincubation of the enzyme with DNA (Breant, et al., 1983).

1.1.C. RNA BINDING. The ability of the subunits of RNAP to bind nascent RNA has been analyzed by using either chemical or photo cross-linkable active groups, either at the 5'-end or the 3'-end of a nucleotide analog or nascent RNA. The 5'-side of the initiating nucleotide does not cross-link to the largest subunit during the formation of the first few phosphodiester bonds, but does so after limited chain elongation (Riva, et al., 1987). Cross-linking to the 3'-end of the nascent RNA has been studied extensively. Cross-linking to the 3'-terminus of nascent RNA has been demonstrated for the largest subunit of *E. coli* RNAP (Borukhov, et al., 1991, Hanna and Meares, 1983) and yeast RNAPI (Kelly, et al., 1990). The site of cross-linking in the *E. coli* largest subunit has been mapped and differs depending on whether the RNAP ternary complex is in an arrested or a productive elongation conformation (Borukhov, et al., 1991, Markovtsov, et al., 1996). In a productive elongation complex the 3'-end of nascent RNA cross-links to sequences between amino acids 400-465, which encompasses homology region D (Borukhov, et al., 1991, Markovtsov, et al., 1996), implicating these sequences in the catalytic activity of the enzyme. In an arrested complex, the 3'-end of the nascent RNA cross-links to sequences C-terminal to homology region G (Met932-Met1025) (Borukhov, et al., 1991, Markovtsov, et al., 1996). Based on models describing the conformation of the ternary complex in an arrested conformation (Borukhov, et al., 1993, Izbaz and Luse, 1992, Reines, 1992), the 3'-end of nascent RNA comes out of register from the active site preventing further elongation. Therefore, the cross-linking near region G probably does not represent residues which take part in the catalytic activity of the enzyme.

1.1.D. CATALYTICALLY ESSENTIAL RESIDUES. It is known that catalytically essential acidic residues in the Klenow fragment of *E. coli* DNAP (DNA-
dependent DNA polymerase) and reverse transcriptase (RNA-dependent DNA polymerase) chelate Mg$^{2+}$, which is directly involved in catalysis (Joyce and Steitz, 1994). The Mg$^{2+}$ binding site of *E. coli* RNAP was mapped by replacement of this ion with Fe$^{2+}$; the ability of Fe$^{2+}$ to produce highly reactive hydroxyl radicals allowed cleavage of the template DNA and protein at positions close to the site of ion binding (Zaychikov, et al., 1996). Hydroxyl-radicals generated by Fe$^{2+}$ cleaved the template at -1 and -2 (relative to the nucleotide hybridizing to the 3'-end of the RNA) in the open promoter complex, and the largest subunit of the enzyme within homology region D, close to a highly conserved motif NADFDGD (Zaychikov, et al., 1996). Replacement of the three acidic residues with alanine in this motif inhibits Fe$^{2+}$-mediated DNA and protein cleavage; this inactivates the enzyme but does not affect the ability of the RNAP to assemble, bind the promoter or form an open complex (Zaychikov, et al., 1996). Based on the above observations, along with the ability of the 3'-end of nascent RNA to cross-link to region D, Zaychikov et al. (1996) concluded that the largest subunit contains residues in motif NADFDGD of homology region D that are involved directly in catalysis. Mutational analysis of this motif in the largest subunit (C160p) of yeast RNAPIII (Dieci, et al., 1995) supports the above conclusion. All substitutions in NADFDGD are lethal to yeast. One ts mutation alters the Asn residue in NADFDGD and is suppressed by mutation of a nearby residue (Dieci, et al., 1995). RNAPIII purified from this mutant strain has altered catalytic properties which include a reduced elongation rate and an increased Km for UTP, but is normal in initiation of transcription and in the assembly/stability of the enzyme (Dieci, et al., 1995).

1.1.E. **BINDING TO ELONGATION FACTOR SII.** Elongation of transcription by RNAPII can be blocked either by DNA-sequence dependent arrest sites or DNA-bound proteins (Aso, et al., 1995). Transcription elongation factor SII assists in passage through transcription arrest sites (Reines and Mote, 1993, SivaRaman, et al., 1990) by binding directly to RNAPII (Agarwal, et al., 1991, Sopta, et al., 1985) and stimulating an intrinsic
endoribonuclease activity in this enzyme (Guo and Price, 1993, Izban and Luse, 1993). As described above, in an arrested elongation complex the 3'-end of the RNA molecule comes out of register from the active site, thus preventing further polymerization (Borukhov, et al., 1993, Izban and Luse, 1992, Reines, 1992). It is thought that SII-stimulated cleavage of the nascent RNA allows the RNAP to resume elongation from a newly generated mRNA 3'-end repositioned in the active site (Guo and Price, 1993, Izban and Luse, 1993).

Deletion of the gene encoding SII (PPR2) in Saccharomyces cerevisiae does not lead to lethality but it causes sensitivity to the drug 6-azauracil (6AU) (Exinger and Lacroute, 1992). When added to a yeast culture, 6AU depletes intracellular GTP and UTP pools (Exinger and Lacroute, 1992). Since reduction of NTP concentrations in vitro leads to a decrease in elongation rate and an increase in pausing by RNAP (Kadesch and Chamberlin, 1982, Kassavetis and Chamberlin, 1981, Kerppola and Kane, 1990, Reisbig and Hearst, 1981), it has been postulated that 6AU sensitivity of a PPR2 deletion is due to the inability of RNAPII to be rescued from transcription arrest sites (Archambault, et al., 1992, Exinger and Lacroute, 1992). In order to identify regions of RNAPII that interact with SII, Archambault et al (1992) obtained seven mutations in the largest subunit of RNAPII that mimic the phenotype of PPR2 deletions. All of the mutations mapped between conserved regions G and H. Two observations suggest that this region of Rpo21p forms an SII binding-site. First, the 6-AU sensitivity of these RPO21 mutant alleles is suppressed by over-expression of PPR2 (Archambault, et al., 1992). Second, the binding affinity of the mutant polymerases for SII is reduced by more than 50-fold compared to the wild-type enzyme (Wu, et al., 1996). Conserved region B has also been implicated in binding to SII based on the observation that a fusion protein containing region B or an antibody directed to this fusion protein inhibits SII-stimulated transcription (Rappaport, et al., 1988). However, there are no data demonstrating direct binding of SII to this region.
1.1.F. FUNCTIONS PROVIDED BY THE CTD  The largest subunit of eukaryotic
RNAPII (Rpo21p) contains a C-terminal domain (CTD) which is composed of tandem
repeats of a heptapeptide sequence PTSPSYS. There are 26-27 repeats in Saccharomyces
Deletion of the CTD in these organisms causes lethality, indicating that this domain
provides an essential function for RNAPII in vivo (Allison, et al., 1988, Bartolomei, et al.,

Yeast containing partial deletions of the CTD (leaving 10 to 13 repeats) are viable
but have a ts and cs growth phenotype and are auxotrophic for inositol (Nonet, et al.,
1987, Nonet and Young, 1989). The inositol auxotrophy of CTD deletion mutants is due
to the inability of RNAPII to induce transcription of the INO1 gene in the absence of
inositol (Scafe, et al., 1990). CTD deletion mutants are also unable to fully induce the
GAL10 promoter in the presence of galactose (Scafe, et al., 1990). The inability to induce
these promoters is due to failure of CTD deletion mutants to support activated transcription,
suggesting that the CTD is important for response to at least some transcriptional activators.
Allison and Ingles (1988) showed that longer or shorter CTD domains can suppress or
enhance, respectively, the activation defects of mutant Gal4 proteins containing deletions in
the activation domain. Furthermore, nuclear extracts of CTD-truncation mutants have been
tested for transcriptional activation by Gal4-VP16 or Gcn4p activators from a hybrid
promoter that contains binding-sites for these factors (Liao, et al., 1991). Progressive
deletion of the CTD leads to a progressive decrease in activation mediated by these
activators, further supporting the idea that the CTD is required for response to
transcriptional activators.

A requirement by transcriptional activators for the CTD has also been shown in
mammalian cells (Gerber, et al., 1995). α-amanitin resistant CTD-truncation alleles of
RPO21 were tested for activation by a variety of transcriptional enhancers in vivo; the CTD is required for activation by all enhancers tested. In addition to response to transcriptional activators, the CTD is also required for accurate initiation from the mouse DHFR promoter in vitro (Thompson, et al., 1989). However, the requirement for the CTD does not apply to all promoters or transcriptional activators. The CTD is not required for accurate initiation of transcription in vitro from the adenovirus major late promoter (AMLP) (Buratowski and Sharp, 1990) or the hsp70 promoter in vitro (Zehring, et al., 1988). Sp1-mediated transcriptional activation is independent of the CTD in vivo (Gerber, et al., 1995) and in vitro (Zehring and Greenleaf, 1990). Proteolytic removal of the CTD from calf thymus RNAPII does not inhibit activation by adenovirus major late transcriptional activator in vitro (Buratowski and Sharp, 1990). Finally, truncation of the CTD does not affect induction of the yeast HIS4 promoter in vivo (Scafe, et al., 1990). Therefore, the function provided by the CTD might be redundant in the context of certain promoters, making its presence dispensable.

1.1.1.I. SRB proteins and the mediator complex. Requirement for the CTD in activated transcription makes this domain a good candidate for interaction with transcription regulatory proteins. A number of CTD-interacting proteins have been identified genetically. Mutations in nine different Srb (suppressors of RNAP B) proteins have been identified as suppressors of the cs phenotype associated with partial deletions of CTD (Nonet and Young, 1989). These are encoded by SRB2 and SRB4 through SRB11 (Hengartner, et al., 1995, Liao, et al., 1995, Thompson, et al., 1993). None of the Srb proteins, except Srb4p, Srb6p and Srb7p, are required for viability, but deletions cause pleiotropic phenotypes similar to those of CTD deletion strains (Hengartner, et al., 1995, Koleske, et al., 1992, Liao, et al., 1995, Thompson, et al., 1993). For example, deletion of SRB2 and SRB10 reduces the level of transcriptional activation mediated by the INO1 and GAL10 UAS elements in vivo, respectively (Koleske, et al., 1992, Liao, et al., 1995). Furthermore, transcription initiation is defective in nuclear extracts prepared from SRB2,
SRB5 or SRB2/SRB5 deletion strains and initiation can be restored by addition of purified recombinant Srb2p and Srb5p to these extracts (Koleske, et al., 1992, Liao, et al., 1995). Some of the Srb proteins are part of a large multiprotein complex called the mediator complex, which also includes a number of positive and negative transcription regulatory proteins (Kim, et al., 1994b, Li, et al., 1995). The mediator forms a complex with RNAPII ("core RNAPII") along with a number of transcription initiation factors in vivo to form a "holoenzyme" (Kim, et al., 1994b, Koleske and Young, 1994). A monoclonal antibody to the CTD (8WG16) (Kim, et al., 1994b) or a GST-CTD fusion protein (Thompson, et al., 1993) dissociates the mediator complex from RNAPII, indicating that the CTD mediates the formation of the "holoenzyme". Unlike "core RNAPII" supplemented with purified initiation factors, the "holoenzyme" complex is capable of performing activated transcription in response to transcription activators such as Gal4-Vp16 (Kim, et al., 1994b, Koleske and Young, 1994) and Gcn4p (Kim, et al., 1994b) in vitro. The above observations indicate that components of the mediator complex transmit the signals of regulatory proteins to RNAPII through the CTD and provides an explanation for the requirement of the CTD for activated transcription.

1.1.F.II. An alternative view of the role of the CTD in transcription activation.

The conditional growth phenotype of CTD truncations can also be suppressed by an intragenic suppressor mutation in region H (FP1141, Nonet and Young, 1989). Xiao et al. (1994a & 1994b) showed that the CTD, an acidic region of Rpo2lp encompassing region H (subdomain I) and sequences between region H and the CTD (subdomain II) can activate transcription in vivo when fused to the DNA-binding domain of Gal4p. They noted that the proline-rich activation domain of the CAAT-binding transcription activator CTF/NF1 has an equivalent of three CTD repeats (Xiao, et al., 1994b), and that the acidic region of Rpo2lp mentioned above has sequence homology to the acidic activation domain of VP16p (Xiao, et al., 1994a). These sequence conservations are paralleled with a functional conservation: sequences in the CTD repeat can substitute functionally for the
CTF-activation domain (Xiao, et al., 1994b), and the activation domain of VP16 can substitute for the function of the acidic subdomain in the context of Rpo21p (Xiao, et al., 1994a). Furthermore, affinity chromatography showed that both the CTD and the activation domain of CTF/NF1 can bind to and compete for binding to the TATA box-binding transcription initiation factor TBP in vitro (Xiao, et al., 1994b). On the other hand, the acidic region of Rpo21p and the VP16 activation domain bind to and compete for binding to transcription initiation factors TBP and TFIIB in vitro (Kim, et al., 1994a, Lin, et al., 1991, Stringer, et al., 1990). The above observations suggest a role for both the CTD and the acidic domain of Rpo21p in activation of transcription (Xiao et al., 1994a & 1994b): Binding of domains of Rpo21p, such as the CTD and the acidic domain, to TBP and TFIIB may allow recruitment of RNAPII to the promoter. In order that RNAPII proceed with transcription it needs to break these interactions. Sharing of domains between RNAPII and activators allows these factors to displace RNAPII by binding to the same domains of TBP and TFIIB, and thereby activate transcription. Suppression by a mutation in region H of the phenotype associated with CTD deletions might compensate for weaker interaction of Rpo21p with TBP in the absence of a complete set of the CTD repeats (Xiao et al., 1994a & 1994b).

1.1.F.III. A negative regulator of transcription, Sin1p. Deletion of the gene encoding Sin1p suppresses the pleiotropic phenotypes that are associated with CTD deletions and allows induction of the INO1 in the absence of inositol (Peterson et al., 1991). Sin1p is a HMG-like protein which binds to DNA non-specifically and is a negative regulator of transcription (Kruger and Herskowitz, 1991, Silverman and Fink, 1984, Sternberg, et al., 1987). Based on the above observations, together with the demonstrated ability of CTD to non-specifically bind double-stranded DNA (Suzuki, 1990, Peterson et al., 1991), Peterson et al. (1991) suggested two alternative models for the interaction of the CTD and Sin1p. First, at least one function of the CTD is to bind to DNA and clear the promoter of negative regulators such as Sin1p. Second, rather than being a
consequence of CTD action, inhibition or removal of Sin1p is a prerequisite for the ability of CTD to promote binding of RNAPII to the promoter.

1.1.F.IV. A connection to mRNA processing. Another group of CTD-interacting proteins has been identified from a mouse cDNA-library that interacts with a mouse CTD peptide in a two-hybrid assay (Fields and Song, 1989, Yuryev, et al., 1996). These proteins contain domains rich in Ser and Arg residues (SR), reminiscent of a class of RNA-binding proteins termed SR proteins (Fu, 1995, Zahler, et al., 1992). Several lines of evidence suggest that interaction of the CTD with the SR proteins serves to couple transcription to splicing. First, antibodies to the CTD, or wild-type but not mutant CTD peptides, inhibit splicing reactions in vitro (Yuryev, et al., 1996). Second, SR proteins are detected in early spliceosome complexes by an antibody that interacts with the Ser-Arg-rich motif of these proteins (Neugebauer, et al., 1995). The same antibody inhibits splicing reactions in vitro (Yuryev, et al., 1996). Third, proteins that cross-react with this anti-SR antibody co-immunoprecipitate with RNAPII from HeLa nuclear extracts (Yuryev, et al., 1996). These in vitro biochemical results have been corroborated by the observation that full length CTD is required for mRNA splicing as well as for poly-adenylation and transcription termination in vivo (McCracken et al., 1997). Furthermore, the cleavage-polyadenylation factors CstF and CPSF bind to a GST-CTD fusion protein and they seem to be a component of the holoenzyme (Sachs and Wahle, 1993, McCracken et al., 1997).

It has been suggested that CTD provides a platform on which nascent RNA and splicing and processing factors are brought together, hence coupling transcription to splicing and polyadenylation (Yuryev, et al., 1996, McCracken et al., 1997).

1.1.F.V. Phosphorylation of the CTD. The largest subunit of RNAPII is phosphorylated on serine, threonine and tyrosine residues of the CTD (Baskaran, et al., 1993, Cadena and Dahmus, 1987). Several lines of evidence suggest that phosphorylation of the CTD occurs concomitantly with the transition from initiation to elongation of transcription. First, during the assembly of the preinitiation complex in vitro, the
unphosphorylated form of RNAPII (the IIA form) preferentially assembles into the complex, and the phosphorylated form (the IIO form) is unable to form a pre-initiation complex (Chesnut, et al., 1992, Kang and Dahmus, 1993, Lu, et al., 1991). Second, only the IIO form of RNAPII is cross-linked to nascent RNA in an α-amanitin sensitive manner in a run-on transcription with HeLa nuclei (Cadena and Dahmus, 1987). Exclusive cross-linking of the IIO form to nascent RNA is also seen in vitro when a transcription initiation complex assembled from purified initiation factors and the IIA form of RNAPII is chased into the elongation mode (Kang and Dahmus, 1993), suggesting that a component of the preinitiation complex might phosphorylate the CTD (see below). Third, an antibody specific to the IIA form of the enzyme preferentially inhibits initiation but not elongation of transcription (Laybourn and Dahmus, 1989). The appearance of CTD phosphorylation during transition to the elongation mode of transcription has also been shown in vivo upon heat-shock induction of Drosophila hsp70 and hsp26 promoters. In response to heat-shock, transcription of hsp70 and hsp26 is activated by inducing elongation of an RNAPII molecule that is normally paused at a site close to the promoter (O'Brien, et al., 1994). The form of RNAPII associated with paused and elongating polymerases was studied by heat induction of Drosophila Kc cells followed by UV cross-linking of DNA to the polymerase and immunoprecipitation of protein-DNA complexes by either IIA- or IIO-specific antibodies. Only the IIO-specific antibody reacts with template DNA after heat shock (O'Brien, et al., 1994). Paused RNAPII is also associated with the 5'-ends of the transcription units of Drosophila β1 tubulin and Gapdh-2 (O'Brien, et al., 1994). As for the hsp promoters, only the IIO form of RNAPII cross-links with DNA that is positioned downstream of the pause site (O'Brien, et al., 1994). Based on the previous observation that the CTD binds specifically to TBP in vitro only in a non-phosphorylated form (Usheva, et al., 1992), O'Brien et al. (1994) suggested that RNAPII binds to TBP in the initiation complex and that transition into elongation requires dissociation of this interaction, perhaps aided by phosphorylation of the CTD. However, although
phosphorylation of the CTD is concomitant with transition from initiation to elongation, it does not seem to be an obligatory step for this process when tested \textit{in vitro} with highly purified transcription initiation factors (Serizawa, et al., 1993). Perhaps, phosphorylation of the CTD is required when RNAPII is involved in complex interactions, for example in the context of the holoenzyme.

1.1.F.VI. CTD kinases. A number of CTD kinases have been identified. One of these kinases is a component of the TFIIH transcription initiation factor (Feavor \textit{et al.}, 1991, Lu, et al., 1992). The TFIIH-associated CTD-kinase might be the enzyme that is partly, if not completely, responsible for phosphorylation of the CTD which occurs concomitantly with the transition from initiation to elongation (see above). This kinase is a cyclin-activated kinase/cyclin pair which is composed of the Kin28p/Ccllp in yeast and the MO15/cyclinH complex in higher eukaryotes (Feavor, et al., 1994, Svejstrup, et al., 1996, Roy, et al., 1994, Serizawa, et al., 1995, Sheikhattar, et al., 1995). In addition to the CTD kinase and the CDK activities, MO15p, but not Kin28p, has a cyclin-dependent kinase-activating kinase (CAK) activity (Cismowski, et al., 1995, Fesquet, et al., 1993, Fisher and Morgan, 1994, Poon, et al., 1993, Tassan, et al., 1994). Yeast CAK is a cyclin-independent kinase encoded by \textit{CIVI} (Thuret, et al., 1996, Kaldis, et al., 1996). \textit{kin28} ts mutants show a rapid drop in the steady-state levels of mRNAs and a concomitant decrease in the ratio of phosphorylated to unphosphorylated CTD, upon a shift to the non-permissive temperature (Valay, et al., 1995, Cismowski, et al., 1995). This observation clearly demonstrates that CTD-phosphorylation by TFIIH is required for normal transcription \textit{in vivo}.

Another CTD kinase/cyclin pair encoded by \textit{SRB10} and \textit{SRB11}, respectively, (Liao, et al., 1995) is part of the holoenzyme. Purified holoenzyme contains a CTD kinase activity; this kinase activity is dramatically reduced in the presence of a non-functional mutant form of Srb10p (Liao, et al., 1995). A third kinase, termed Ctk1p, with homology to cyclin-dependent kinases was identified in yeast extracts (Lee and Greenleaf, 1989).
Ctklp forms the 58 kDa component of a heterotrimeric complex that copurifies with the CTD kinase activity (Lee and Greenleaf, 1989). Ctk2p, a 38 kDa component of this complex, has homology to cyclins which likely regulates the activity of Ctklp (Sterner, et al., 1995). Mutations in CTKI reduce the level of phosphorylation of the CTD in vivo (Lee and Greenleaf, 1991). The observation that cyclin-dependent kinases act as modifiers of CTD suggests that cell-cycle dependent transcription of genes could be implemented through this domain of RNAPII.

Cisek and Corden identified two different mammalian CTD kinases, both of which contain the mouse homologue of the major cell-cycle regulator, Cdc28p (in Saccharomyces cerevisiae) (Cisek and Corden, 1989, Zhang and Corden, 1991). A DNA-dependent CTD kinase has been purified from HeLa nuclei that is composed of a 34kDa catalytic subunit (component A) and a regulatory DNA-binding heterodimer (component B), which comprises 67 and 83 kDa components (Dvir, et al., 1993). Component B is identical to the previously known Ku human autoantigen (Dvir, et al., 1992). Payne and Dahmus (1993) isolated two distinct CTD kinases, Ctdk1p and Ctdk2p, from HeLa cell extracts. The kinases differ by their nucleotide specificity, maximum number of phosphates incorporated per RNAPII, and specificity for non-CTD substrates (Payne and Dahmus, 1993). Ctdk1 and Ctdk2 are not related to cyclin-dependent kinases since they do not cross-react with antibodies directed against the S. pombe cdc2 kinase (S. cerevisiae cdc28). Nor are their catalytic activities stimulated by DNA; therefore, they are different from other CTD kinases (Payne and Dahmus, 1993). P-TEFb is a Drosophila transcription elongation factor which stimulates early elongation by phosphorylating the CTD in vitro (Marshall, et al., 1996). Although the CTD-kinases purified by Cisek and Corden, Dvir et al., Payne and Dahmus, and Marshall et al. phosphorylate CTD peptides and the RNAPII enzyme in vitro, it is not clear if they play a role in modification of the CTD in vivo (Cisek and Corden, 1989, Dvir, et al., 1992, Zhang and Corden, 1991, Marshall et al., 1996).
All of the kinases described above are ser/thr kinases. The only known CTD tyr-
kinase is the product of the c-Abl proto-oncogene (Baskaran, et al., 1996, Baskaran, et al.,
1993). Phosphorylation of the CTD by c-Ablp depends on; 1) the phosphotyrosine-
binding SH2 domain of c-Ablp, which binds to phosphorylated CTD (Duyster, et al.,
1995), and 2) the C-terminal CTD-binding domain of this enzyme (Baskaran, et al., 1996).
Several observations suggest that c-Ablp is the enzyme responsible for tyrosine
phosphorylation of the CTD in vivo. First, a GST-CTD fusion protein is phosphorylated
in vivo when co-transfected with full length c-Abl into mammalian cells (Baskaran, et al.,
1996). c-Ablp can autophosphorylate in the absence of the CTD-binding domain but
cannot phosphorylate the GST-CTD fusion protein in vivo (Baskaran, et al., 1996).
Second, transient over-expression of full-length c-Abl, but not of the CTD-binding domain
deletion mutant, increases the level of tyrosine phosphorylation of RNAPII in vivo
(Baskaran, et al., 1996). Third, RNAPII is co-immunoprecipitated from cell extracts with
full-length but not deletion alleles of c-Abl lacking the CTD-binding domain, and is
phosphorylated by c-Ablp in this complex (Baskaran, et al., 1996). Binding to and
phosphorylation of the CTD by c-Ablp seems to be required for the function of c-Ablp in
vivo, since in the absence of the CTD binding domain c-Ablp is unable to activate
transcription from the c-fos promoter (Baskaran, et al., 1996).

1.1.F.VII. CTD phosphatases. As mentioned above, initiation of transcription
requires unphosphorylated RNAPII (Chesnut, et al., 1992, Kang and Dahmus, 1993, Lu,
et al., 1991). Since transition from initiation to elongation is concomitant with
phosphorylation of the CTD (Cadena and Dahmus, 1987, Kang and Dahmus, 1993,
Laybourn and Dahmus, 1989), reinitiation would require a CTD phosphatase. A CTD
phosphatase has been identified and partially purified from HeLa cell extracts (Chambers
and Dahmus, 1994). The activity of this phosphatase seems to be specific since it removes
phosphate from the CTD that has been phosphorylated by Ctk1p and Ctk2p, but does not
remove a phosphate added by casein kinase II (Chambers and Dahmus, 1994). Neither
does it dephosphorylate non-CTD substrates such as phosphorylase a or the α and β subunits of phosphorylase kinase (Chambers and Dahmus, 1994). Whether this phosphatase plays any role in CTD dephosphorylation in vivo is not known.

1.1.F.VIII. Glycosylation might regulate phosphorylation. Purified Calf thymus RNAPII has been shown to be glycosylated at ser and thr residues throughout the CTD (Kelly, et al., 1993). Glycosylation has been detected exclusively on the IIA from of RNAPII, leading to the suggestion that glycosylation might be a mechanism by which the level of phosphorylation of the CTD is regulated (Kelly, et al., 1993).

1.2. THE SECOND-LARGEST SUBUNIT.

1.2.A. ZINC BINDING. Like the largest subunit, the second-largest subunits from both E. coli (Wu, et al., 1977) and yeast RNAPs (Treitch, et al., 1991) bind zinc in vitro. The zinc-binding domain of the yeast enzymes has been mapped to a recognizable zinc-binding motif in the C-terminal end of the second-largest subunit (Treitch, et al., 1991). No such motif has been found for the bacterial subunit and the site of zinc binding is not known. Mutations in the zinc-binding domain of the second-largest subunit of yeast RNAPI suppress mutations in the zinc-binding domain of the largest subunit of this enzyme, suggesting that the two zinc-biding domains interact in the enzyme complex (Yano and Nomura, 1991). Furthermore, mutations in the zinc binding domain of the second-largest subunit of RNAPII in yeast can be suppressed by overexpression of the mutant allele suggesting that the zinc-binding sequences are required for the stability of the second-largest subunit (Treitch, et al., 1991).

1.2.B. DNA BINDING. Using photoreactive deoxynucleotide analogs placed on the non-template strand, Bartholomew et al. (1993) showed that the second-largest subunit of yeast RNAPIII (C128p Or Rpo32p) cross-links and is, therefore, closely associated
with DNA during both the initiation and elongation phases of transcription. Using a similar strategy, the region of *E. coli* RNAP β subunit that cross-links to the template strand in a ternary elongation complex has been mapped (Nudler, et al., 1996). Sequences in homology region I cross-link to deoxynucleotide residues that are positioned in the active site of the polymerase (i.e., that hybridize to the 3'-end of nascent RNA or are flanking this site) (Nudler, et al., 1996), suggesting that region I plays an important role in the stability of the ternary elongation complex (see below). South-western blot analysis of *Drosophila* RNAPII has shown that homology regions C and D of the second-largest subunit of this enzyme have non-specific DNA-binding ability (Gundelfinger, 1983, Kontermann and Bautz, 1994).

1.2.C. RNA BINDING. When the initiating nucleotide substrate is modified to carry a cross-linkable active group at the 5'-end of the nucleotide, only the second-largest subunit of *E. coli* RNAP (Armstrong, et al., 1976) and yeast RNAPI, II, and III (Riva, et al., 1987) is cross-linked. The regions that are cross-linked to the nucleotide analogs have been mapped to homology region H (Grachev, et al., 1989a, Grachev, et al., 1989b, Riva, et al., 1990) and homology region I (Grachev, et al., 1989a, Grachev, et al., 1989b, Treich, et al., 1992b). The cross-linking residues in regions H and I of the *E. coli* β subunit have been mapped to Lys1065 and His 1237, respectively (Mustaev, et al., 1991). These residues reside approximately 3 Å away from the α-phosphate of the priming nucleotide (Mustaev, et al., 1991). Therefore, they lie on the upstream side of and facing the priming substrate. Mutation of Lys1065 or His 1237 either abolishes or reduces the transition from initiation to the elongation phase of transcription (promoter clearance) by *E. coli* RNAP, suggesting that these residues stabilize the ternary complex by interactions with the 5'-end of the transcript (Mustaev, et al., 1991).

The second-largest subunits of *E. coli* (Borukhov, et al., 1991, Hanna and Meares, 1983) and yeast RNAPI (Kelly, et al., 1990) cross-link to the 3'-end of nascent RNA.
Cross-linking of the *E. coli* RNAP β subunit to the 3'-end of nascent RNA occurs only when the ternary complex is in a productive mode of transcription; β does not cross-link to the 3'-end of RNA in an arrested complex (Markovtsov, et al., 1996). The site of β cross-linking has been mapped between residues 515 and 600, which correspond to homology region D, and between residues 1091 and 1107 in region H (Markovtsov, et al., 1996, Severinov, et al., 1995). The latter residues are approximately 30 amino acids C-terminal to Lys 1065, which cross links to the 5'-end of the initiating nucleotide (Mustaev, et al., 1991), suggesting that these sequences in homology region H form a continuous part of the active site (Markovtsov, et al., 1996).

Sequences between 515 and 600 of β (homology region D) not only cross-link to the 3'-end of nascent RNA but are also the predominant sites of mutations that allow *E. coli* RNAP to become resistant to the drug Rifampicin (Jin and Gross, 1988, Severinov, et al., 1993). Rifampicin inhibits *E. coli* RNAP by blocking transition from initiation to elongation and is thought to block the movement of RNA out of the active site (Mustaev, et al., 1994). Indeed, the site of action for rifampicin maps to the vicinity of the active site.

Cross-linking of various regions of β with either the 5'-end or 3'-end of nascent RNA suggests that these sequences are closely associated with the active site; this is supported by the observation that mutations in these regions alter the catalytic activity of the enzyme. Mutations in region I (cross-links to the 5'-end of RNA) and region D (cross-links to 3'-end of RNA) alter the efficiency of pausing and termination by *E. coli* RNAP in vivo and in vitro (Landick, et al., 1990). In addition to regions D and I, other sequences in β, when mutated, alter the efficiency of elongation by *E. coli* RNAP. These include sequences just N-terminal to homology region C (amino acids 186-433). Certain mutations in, or complete deletion of, this region confer resistance to the T4 phage protein Alc (the phenotype is called paf for prevent Alc function), which induces premature termination of *E. coli* RNAP (Severinov, et al., 1994, and references therein). *paf* mutations do not affect normal functions of *E. coli* RNAP (Severinov, et al., 1994). However, some point
mutations in the region encompassing amino acids 186-433 alter the elongation properties of the enzyme without giving rise to a Paf phenotype (Severinov, et al., 1994). This region of β may have a dual function: interaction with termination regulatory factors and determination of the rate of elongation of the RNAP.

Sequences in the 186 to 433 region of β are not conserved in the eukaryotic second-largest subunits. However, sequences that correspond spatially to this region in the higher eukaryotic second-largest subunit, as well as homology regions D and I, seem to perform the same functions in higher eukaryotes. Using targeted mutagenesis, Shabban et al. (1995) mutagenized the corresponding regions of the second-largest subunit of RNAPIII (Rpo32p) and found altered efficiency of transcription termination by RNAPIII in the rpo32 mutants (James, et al., 1991, Shabban, et al., 1995). In some rpo32 mutant enzymes the increased termination efficiency correlated with a decreased rate of elongation (Shabban, et al., 1995). Such a correlation is also seen for some β mutant enzymes (Landick, et al., 1990). Mutant forms of yeast RNAPII, which show a reduced rate of transcription elongation and increased efficiency of pausing at natural pause sites, have been identified by isolating 6 Azauracil sensitive mutations in RPO22 (Powell and Reines, 1996). These mutations lie either in homology region H or A. Although identification of elongation-defective mutations in region H is consistent with its participation in the active site (see above), the manner in which sequences in region A contribute to normal elongation is not clear.

2. Subunits required for assembly of RNAPs.

2.1. THE α HOMODIMER IN E. COLI AND ITS FUNCTIONAL HOMOLOGUES. The components of E. coli RNAP can be expressed separately and used to assemble a fully functional enzyme in vitro (Ishihama, 1981). This has allowed determination of the pathway of assembly of the enzyme. The assembly of E. coli RNAP starts by homodimerization of the α subunit, which is followed by formation of a βα2
complex. Next, β' joins the complex to form the core enzyme, followed by association of the σ subunit, which gives rise to the holoenzyme complex (Ishihama, 1981). Using assembly-defective mutations in the three largest subunits of yeast RNApolII, Koloedziej et al. (1991) found a pathway of assembly that parallels that seen in E. coli RNAP. A dimer of the third-largest subunit of yeast RNApolII (Rpb3p) associates with the second-largest subunit (Rpb2p), and then joins in with the largest subunit of the enzyme (Rpb1p) (Koloedziej and Young, 1991).

Deletion analysis (Igarashi, et al., 1991, Igarashi and Ishihama, 1991) or limited proteolytic digestion of the E. coli α subunit (Blatter, et al., 1994) has determined that the N-terminal two-thirds of this subunit is necessary and sufficient for the assembly of a functional core RNAP (Fig. 4). Mutations in the α subunit that block the assembly of E. coli RNAP map to this N-terminal domain (Igarashi, et al., 1990, Ishihama, et al., 1980). As mentioned above, the α subunit shares sequence homology with Rpb3p (or Rpo23p), Rpb11p, AC40p and AC19p from yeast and subunits D and L from archaeabacteria (Fig. 4). The sequence homology region, termed the α-motif, lies in the N-terminal region of α that is required for the assembly of E. coli RNAP. Mutations in two of the subunits that share sequence homology with α, Rpo23p and AC40p, block the assembly of their respective RNAPs (Koloedziej and Young, 1991, Mann, et al., 1987). There is no evidence to indicate a role in the assembly of RNAP for AC19p or for the other subunits that share homology with the α subunit. However, it is known that AC40p heterodimerizes with AC19p (Lalo, et al., 1993). Furthermore, electron microscopic analysis of antibody-labeled enzyme with subunit-specific antibodies has been used to map the localization of the AC40p/AC19p dimer in yeast RNApolI (Klinger, et al., 1996) and the α subunit in E. coli RNAP (Tichelaar, et al., 1983). The AC40p/AC19p heterodimer and the α homodimer occupy similar positions relative to the putative DNA-binding cleft in their respective polymerases, supporting the suggestion that they perform similar functions. The fact that AC40p/AC19p is a heterodimer is not inconsistent with this suggestion, since it has been
shown that the two α subunits in the homodimer do not occupy the same environment in the E. coli RNAP. Heyduk et al. (1996) have mapped the regions of the α subunit that bind to β and β' by using hydroxyl-radical protein footprinting. Four regions of α are protected from hydroxyl-radical mediated fragmentation upon formation of the core enzyme; these lie in the N-terminal domain of α (Heyduk, et al., 1996). Reconstitution of the core enzyme with heteromeric α indicates that it is the same α protomer that contacts both β and β' (Heyduk, et al., 1996).

2.2. SMALL SUBUNITS REQUIRED FOR STABILITY/ASSEMBLY OF YEAST RNAPI. Subunit A12.2p is non-essential for growth of yeast; however, there is a reduced steady-state amount of the largest subunit of RNAPI (A190) and a slow growth rate at 37°C in a strain containing a deletion of the gene encoding this subunit (Nogi, et al., 1993). A12.2p binds zinc in vitro (Treich, et al., 1991) and contains an N-terminal and a C-terminal zinc-binding motif; the latter is dispensable for A12.2p function (Nogi, et al., 1993). Overexpression of A190 suppresses the ts phenotype caused by deletion of A12.2, indicating that A12.2p affects assembly of RNAPI by conferring stability to the largest subunit of this enzyme, perhaps by interacting with the zinc-binding domain of this protein (Nogi, et al., 1993). A14p is another non-essential subunit of RNAPI, which leads to a ts growth phenotype when deleted (Smid, et al., 1995). RNAPI purified from an A14 deletion strain is unstable; in the absence of A14p, subunits ABC23p (or Rpo26p) and A43p dissociate from the enzyme and give rise to a catalytically inactive polymerase (Smid, et al., 1995). Therefore, A14p is required for stable association of the ABC23p and A43p subunits in the RNAPI complex.

3. Other functions provided by the α subunit. On the basis of proteolytic digestion the α subunit can be divided into an N-terminal (amino acids 8 to 241) and a C-terminal domain (amino acids 249-329), connected by a short interdomain link (Fig. 4). The two domains can homodimerize independently in vitro (Blatter, et al., 1994).
mentioned above, the N-terminal domain of α is necessary and sufficient for the assembly of the enzyme in the core complex and for the formation of the holoenzyme (Blatter, et al., 1994, Igarashi, et al., 1991, Igarashi and Ishihama, 1991). In contrast, the C-terminal domain is not required for enzyme assembly but provides a variety of regulatory functions for the holoenzyme.


3.2. INTERACTION WITH PROMOTER DNA. Some E. coli promoters, such as the ribosomal rRNA P1 promoter, can support a high level of transcription without the need for transactivators (Deuschle, et al., 1986, Newlands, et al., 1992, Newlands, et al., 1991, Rao, et al., 1994, Ross, et al., 1993). This high activity is mediated by an A/T-rich sequence (termed the UP element) upstream of E. coli core promoter elements (-10 and -35 sequences) and the activation is dependent on the presence of the C-terminal end of the α subunit (Ross, et al., 1993). Deletion of the C-terminal domain of α or a point mutation (R265C) in this region prevents activation of transcription by the UP element (Ross, et al., 1993). The α subunit (Ross, et al., 1993) or a proteolytically generated C-terminal α fragment (Blatter, et al., 1994) can bind to the UP element and protect these sequences from DNase I digestion. The ability of the α subunit to bind to promoter DNA may allow a stronger interaction between RNAP and promoter DNA, thus increasing the level of transcription from promoters that contain the UP element (Blatter, et al., 1994).

3.3. INTERACTION WITH RNA AND REGULATION OF ELONGATION. Nascent RNA which contains a photoreactive nucleotide analog of UMP at least 24 bp
away from the 3’-end can be cross-linked to α (Liu and Hanna, 1995b, Liu, et al., 1996). The RNA cross-linking is dependent on the presence of the C-terminal domain of the α subunit (Liu and Hanna, 1995b, Liu, et al., 1996) and it relates to the role that this subunit plays in mediating the effects of the transcription elongation factor NusA. When RNAP enters the elongation phase of the transcription cycle, NusA replaces σ70 (Gill, et al., 1991, Greenblatt and Li, 1981) and interacts with the core enzyme as well as with nascent RNA in the ternary elongation complex (Liu and Hanna, 1995a). Through this interaction NusA slows the rate of elongation, enhances pausing and increases termination efficiency at intrinsic terminators (Richardson and Greenblatt, 1996). In addition, NusA is required for anti-termination by bacteriophage λ factors N and Q (Richardson and Greenblatt, 1996). When NusA binds to the RNAP, α no longer cross-links to RNA. However, the NusA-RNA interaction does not occur if the C-terminal domain of α is absent, unless NusA is supplied in five-fold excess (Liu and Hanna, 1995b, Liu, et al., 1996). Furthermore, both the NusA-mediated increase in pausing and termination and antitermination by the λ Q protein are severely reduced when the C-terminal domain of α is absent (Liu, et al., 1996). Interaction of NusA and RNAP is compromised at low-salt concentrations when the C-terminal domain of α is absent (Liu, et al., 1996). NusA binds to purified α, β, and β' independently, but binding of NusA to α is abolished in the absence of the C-terminal domain of α (Liu, et al., 1996). All of the above data suggest that α mediates regulation by NusA by stabilizing its interaction with RNAP in the ternary complex.

4. **Subunits required for accurate initiation of transcription.** Accurate and promoter specific initiation of transcription by multisubunit RNAPs requires transcription initiation factors which recruit RNAP to the promoter and mediate open complex formation. In eubacteria this function is performed by one polypeptide, the σ factor. One σ factor directs transcription in rapidly dividing cells while specialized σ factors are responsible for
transcription of specific genes under certain physiological and developmental conditions (reviewed in Helmann and Chamberlin, 1988). The prototypical eubacterial σ subunit, *E. coli* σ70, binds to bacterial promoters at specific DNA sequences (-10 and -35 upstream of the transcription initiation site) and recruits RNAP to the promoters (Helmann and Chamberlin, 1988). Promoter-specific initiation in eukaryotes requires a number of initiation factors, each of which performs a certain aspect of the function provided by σ for *E. coli* RNAP (for reviews about these factors see Conaway and Conaway, 1993, Geiduschek and Kassavetis, 1995, Willis, 1993). RNAP subunits in the eukaryotic core enzyme also play an important role in either the efficiency of initiation or in choosing the site of transcription initiation.

4.1. **RNAPII Subunits Involved in Initiation.** Subunits Rpb4p and Rpb7p are present in yeast RNAPII in a substoichiometric amount (Kolodziej, et al., 1990). They dissociate as a dimer from RNAPII in the presence of urea upon DEAE-Sephadex chromatography of the purified enzyme or during non-denaturing gel electrophoresis (Dezelee, et al., 1976, Ruet, et al., 1980). Cells containing a deletion of *RPB4* survive moderate temperatures and RNAPII purified from these cells (RNAPIIΔ4/7) lacks both Rpb4p and Rpb7p subunits (Woychik and Young, 1989). The so called RNAPIIΔ4/7 enzyme is able to elongate and terminate transcription as efficiently as the wild-type enzyme; however, it is defective in promoter-specific initiation *in vitro* (Edwards, et al., 1991). Addition of purified Rpb4p/Rpb7p heterodimer to the mutant extracts can rescue the initiation defect suggesting a function for these two subunits that is reminiscent of *E. coli* σ subunit (Edwards, et al., 1991). The mechanism by which the Rpb4p/Rpb7p heterodimer functions is not clear but it seems to increase the efficiency of initiation of transcription. In support of this idea, addition of the transcriptional activator Gal4-Vp16 to an *in vitro* transcription reaction can by-pass the requirement for Rpb4p/Rpb7p for transcription initiation on the appropriate template (Edwards, et al., 1991).
Initiation site selection differs in *Saccharomyces cerevisiae* and other eukaryotic cells. In *S. cerevisiae*, transcription is initiated at multiple sites that lie between 30 to 120 bp downstream of the TATA box. However, in *Schizosaccharomyces pombe* and in higher eukaryotes transcription initiation usually occurs at a single site approximately 30bp downstream of the TBP binding site (Russell, 1983). Using an *in vitro* transcription assay and purified initiation components from *S. cerevisiae* and *S. pombe*, Li et al. (1994) substituted the factors from one system for the other and tested the position of the transcription initiation sites. These experiments showed that the determinants of start site selection are the initiation factor TFIIIB and RNAPII (Li, et al., 1994). These results are consistent with genetic identification of mutations in *Saccharomyces* TFIIIB and RNAPII subunits Rpo21p and Rpb9p (or Rpo29p) that shift the initiation site of transcription *in vivo* (Berroteran, et al., 1994, Furter-Graves, et al., 1994, Hull, et al., 1995, Pinto, et al., 1994). Rpb9p is non-essential for growth of yeast but its absence leads to ts and cs growth defects (Woychik, et al., 1991). Rpb9 is a zinc-binding protein (Treich, et al., 1991) which contains two zinc-binding sequence motifs (Woychik, et al., 1991). The mechanism of start-site selection by Rpb9 is not clear but it may position the initiation sites by binding to DNA or to other components of the transcription initiation complex through its zinc-binding domains (Hull, et al., 1995).

### 4.2. **SUBUNITS OF RNAPIII INVOLVED IN TRANSCRIPTION INITIATION**

Transcription initiation by RNAPIII requires the initiation factor TFIIIB which, along with other initiation factors, mediates the recruitment of the enzyme to the promoter (Willis, 1993). After RNAPIII is recruited to the promoter, TFIIIB can also mediate multiple rounds of transcription in the absence of other initiation factors (Willis, 1993). TFIIIB is made up of TBP and 70kDa and 90kDa subunits (Willis, 1993). RNAPIII subunits C34p, C31p and C82p form a heterotrimeric complex (Werner et al., 1992 & 1993) which appear to be required for initiation of transcription based on the
following observations. First, C34p interacts with the 70 kDa subunit of TFIIB in a two-hybrid assay (Werner, et al., 1993). Second, DNA-RNAPIII cross-linking experiments in the initiation complex have shown that C31p, C34p and C84p are in close proximity to the DNA template, primarily on the 5'-side of the initiating RNAPIII molecule (Bartholomew, et al., 1993). Furthermore, among the RNAPIII subunits C34p extends furthest upstream on the promoter, which is consistent with its ability to bind TFIIB (Bartholomew, et al., 1993). Third, genetic and biochemical evidence suggests that C31p is required for initiation of transcription by RNAPIII (Thuillier, et al., 1995). C31p contains a C-terminal 30 amino acid stretch of acidic residues; deletion of the C-terminal 16 residues of this domain gives rise to a ts and cs growth defect but longer deletions are lethal (Thuillier, et al., 1995). A strain carrying a cs deletion allele of C31 (rpc31-236) shows a specific defect in tRNA transcription *in vivo* and *in vitro* (Thuillier, et al., 1995). The purified mutant polymerase initiates at a considerably slower rate than the wild-type enzyme in a defined transcription initiation assay. However, it has properties similar to wild-type when tested for rate of elongation and termination of transcription (Thuillier, et al., 1995). The ts phenotype of rpc31-236 is suppressed by overexpression of the largest subunit of RNAPIII (C160p) (Thuillier, et al., 1995). The above observations have been interpreted as follows: first, rpc31-236p is defective in assembly of RNAPIII at high temperature in addition to the initiation defect; second, Rpc31p mediates the association of C84p and C34p with the remainder of the RNAPIII subunits through interaction with C160p (Thuillier, et al., 1995).

4.3. **SUBUNITS OF RNAP INVOLVED IN INITIATION OF TRANSCRIPTION.** Components of RNAP transcription initiation have been studied most extensively in mouse (Schnapp, et al., 1994 and references therein). Two DNA-binding proteins, UBF and TIF-IB (which contains TBP), bind to rDNA promoters and mediate the recruitment of RNAP and associated factors TIF-IA and TIF-IC to form a
productive initiation complex (Schnapp and Grummt, 1991). Using protein-affinity chromatography and glycerol gradient centrifugation, Schnapp et al. (1994) showed that UBF binds directly to mouse RNAPI. Furthermore, far western blotting experiments show that UBF also binds to yeast RNAPI and RNAPII (Schnapp, et al., 1994). The subunits of yeast RNAPI and RNAPII that are the target of UBF interaction are A34.5p and C53p, respectively. In mouse RNAPI, UBF interacts with a 62 kDa component (Schnapp, et al., 1994). This component has been resolved into three polypeptides of 53, 51 and 49 kDa (Hanada, et al., 1996). Far-western blotting and protein-affinity chromatography have shown that the 53 kDa component, termed PAF53 (for polymerase associated factor), is the target of UBF interaction (Hanada, et al., 1996). The gene encoding PAF53 has been cloned; the gene product shows considerable sequence similarity to the yeast RNAPI 49 kDa subunit A49p (Hanada, et al., 1996).

A modified form of yeast RNAPI I, termed A*, that lacks subunits A34.5p and A49p is not capable of binding UBF (Schnapp, et al., 1994). A* is capable of non-specific transcription from synthetic poly [d(A-T)] but has reduced transcription activity from double-stranded calf thymus DNA (Huet, et al., 1975). Similarly, there are two forms of catalytically active RNAPI in mammalian cells, termed Ia and Ib, which differ by the lack of the 62 kDa multimeric component in form Ia (Gissinger and Chambon, 1975, Matsui, et al., 1976, Schwartz and Roeder, 1974). The observation that subunits A34.5p and A49p are dispensable for RNAPI catalytic activity and their ability to interact with the initiation factor UBF suggests that they serve a regulatory role during initiation of transcription by RNAPI. A regulatory role for A34.5p and A49p is supported by the following observations; 1) deletion of the gene encoding yeast A49p is not lethal but gives rise to reduced growth-rate and reduced 5.8S rRNA synthesis in vivo (Liljelund, et al., 1992), 2) antibodies to PAF53 (mouse homologue of yeast A49p, see above) inhibit rDNA transcription but not non-specific transcription from a poly [d(A-T)]-poly [d(A-T)] template in mouse cell extracts (Hanada, et al., 1996), 3) Inhibition of rDNA transcription by anti-
PAF53 antibodies can be rescued by addition of recombinant PAF53, indicating that this subunit is required for promoter-specific initiation (Hanada, et al., 1996).

As mentioned above, UBF also binds to the yeast RNAPIII subunit C53p in far-western blot assays (Schnapp, et al., 1994). It has been postulated that UBF might also play a role in initiation of RNAPIII transcription, similar to TBP which directs initiation of all three RNAPs but originally was thought to be specific to RNAPII transcription initiation (Schnapp, et al., 1994).

IV- TRANSCRIPTIONAL REGULATION OF GENES ENCODING RNAP SUBUNITS

Since RNAPs play a central role in the expression of cellular genes, maintaining the correct amount of the functional enzymes is crucial for normal growth. This is especially important for multisubunit polymerases where the amount of all the subunits should be stoichiometrically correct for the assembly of a functional enzyme. There is evidence for transcriptional and post-transcriptional feed-back regulatory mechanisms that respond to changes in the amount of active RNAP in both E. coli and mammalian cells. For example, a decrease in the amount of cellular transcription either due to incubation of cells in the presence of Rifampicin (Fukuda and Nagasawa-Fujimori, 1983, Morgan and Hayward, 1987, Newman, et al., 1982) or due to underproduction of β (Dennis, et al., 1985) leads to an increase in the synthesis of β and β'.

A similar compensatory mechanism is operative in mammalian cells (Guialis, et al., 1977, Guialis, et al., 1979, Somers, et al., 1975). Heterozygous cell lines containing both α-amanitin-resistant (AMA^r) and α-amanitin-sensitive (AMA^s) alleles of RPO21 express similar proportions of AMA^r and AMA^s RNAPII when grown in the absence of α-amanitin. However, when cells are grown in the presence of the drug, the inactivated form of RNAPII (AMA^s) is degraded preferentially (Guialis, et al., 1977, Guialis, et al., 1979, Somers, et al., 1975). The total amount of active RNAPII remains constant under these conditions due to increased synthesis of the AMA^r form of RNAPII, which compensates
for the deficit of the AMAS form (Guialis, et al., 1977, Guialis, et al., 1979, Somers, et al., 1975). Deletion and mutational analysis of the promoters of genes encoding yeast RNAP subunits have also revealed regulatory mechanisms controlling the synthesis of RNAP subunits (Della, et al., 1990, Jansma, et al., 1996). Below, I present a brief account of what is known about transcriptional control of genes encoding RNAP subunits.

A- Regulation of expression of $\beta$ and $\beta'$

Genes $rpoB$ and $rpoC$ encode $E. coli$ RNAP subunits $\beta$ and $\beta'$, respectively, and like many bacterial genes they are transcribed as part of an operon. They are located at the distal end of the large $rplKAJlrpoBC$ gene cluster, which includes four ribosomal protein genes (reviewed in Yura and Ishihama, 1979). Transcription of this operon is directed by two promoters, one located in front of $rplJ$ that directs transcription of $rplJlrpoBC$ and a second preceding $rplK$ that leads to transcription of the genes in the entire operon (Barry, et al., 1980, Barry, et al., 1979, Ralling and Linn, 1984, Steward and Linn, 1991). An attenuator upstream of $rpoB$ ($rpoBa$), in the intercistronic region between $rplL$ and $rpoB$, maintains the amount of transcription for $rpoBC$ at about 20% of the level for upstream $rplJL$ genes (Steward and Linn, 1991). The number of RNAP molecules that read-through $rpoBa$ depends on the number of polymerase molecules that initiate at the promoter and encounter this attenuator (Steward and Linn, 1991, Steward and Linn, 1992). Decreasing the number of initiating RNAPs by using weak heterologous promoters decreases attenuation and increases the amount of read-through from $rpoBa$ (Steward and Linn, 1992). Initiation from a strong heterologous promoter has little affect on read-through from $rpoBa$, indicating that $rpoBa$ functions to ensure synthesis of sufficient $rpoBC$ mRNA for biosynthesis of the amount of RNAP necessary for normal cellular function (Steward and Linn, 1992). In fact, a rifampicin-induced decrease in the amount of active RNAP in vivo, increases the amount of read-through of $rpoBa$ indicating that the compensatory feed-back response to this drug occurs at the level of transcription (Morgan
and Hayward, 1987). Increasing the total amount of cellular RNAP by simultaneous overexpression of all of the subunits leads to negative regulation of \textit{rpoBC} transcription (Dykxhoorn, et al., 1996). Excess holoenzyme both reduces initiation at the \textit{rplJ} and \textit{rplk} promoters, and decreases readthrough of the rpoBa attenuator (Dykxhoorn, et al., 1996). The exact molecular mechanisms by which the above mentioned feedback transcriptional responses occur is not understood.

**B- Regulation of expression of yeast RNAP subunits**

Analysis of the promoter sequences of genes encoding RNAP subunits (Della, et al., 1990, Dequard-Chablat, et al., 1991, Jansma, et al., 1996) has identified consensus binding-sequences for two yeast transcriptional activators, Abf1p and Grf2 (Reb1p). Abf1p and Grf2p also bind to and regulate a number of housekeeping genes (reviewed in Dhawale and Lane, 1993). Electrophoretic mobility shift analysis with purified Abf1p shows binding of Abf1p to the promoters of \textit{RPC40, RPC160, RPO21} and \textit{RPO22 in vitro} (Della, et al., 1990, Jansma, et al., 1996). Deletion analysis of these promoters (except for \textit{RPC160}) and mutation of the Abf1p-binding sites has shown that this factor contributes to normal expression of these genes (Della, et al., 1990, Jansma, et al., 1996). Similar analyses have shown that Grf2p binds to the promoters of \textit{RPO21} and \textit{RPO22} and that it contributes to expression of these genes (Jansma, et al., 1996). In the context of these promoters, the Abf1p and Grf2p binding sites are redundant; either is sufficient for normal levels of transcription (Jansma, et al., 1996). A T-rich element, which appears downstream of the binding sites for Abf1p and Grf2p, is also required (and non-redundant) for normal expression of \textit{RPO21} and \textit{RPO22} (Jansma, et al., 1996). Such T-rich elements function as upstream activating sequences in a variety of yeast promoters (Goncalves, et al., 1995, Iyer and Struhl, 1995, Lue, et al., 1989, Schultes and Szostak, 1991).
V- A BRIEF THESIS OUTLINE

The work described in this thesis is aimed at understanding the functional role and the regulation of expression of the common subunit, Rpo26p. In Chapter II, I describe a genetic screen which allowed identification of a collection of functionally defective mutant alleles of *RPO26*. Results from biochemical analysis of the assembly/stability of RNAPI and RNAPII for a ts mutant allele, *rpo26-31*, are also presented in Chapter II. These results show that the rpo26-31p is unstable at the non-permissive temperature, a condition that leads to instability of the largest subunits of RNAPI and RNAPII and a defect in the assembly of these enzymes. Therefore, at least one function provided by a common subunit is to confer stability to the other subunits in the enzyme. The work in Chapter III, describes genetic identification of a suppressor mutation that causes accumulation of rpo26-31p to near wild-type levels and partially suppresses the ts phenotype associated with this mutant subunit. The suppressor mutation (*pup3-1*) resides in a gene encoding a catalytic subunit of the yeast 20S proteasome. The *pup3-1* mutation fails to suppress ts mutations in the largest subunit of RNAPII (Rpo21p) or to cause accumulation of unstable forms of the Rpo21p subunit. This apparent subunit-specificity of the proteasome suggests that the steady-state levels of different polymerase subunits are regulated independently. Chapter IV describes the identification and characterization of a number of *RPO26* promoter mutations. These mutations were identified through the genetic screen described in Chapter II. As described in Chapter IV, these mutations modifies the consensus sequence for binding of the yeast transcription factor Abf1p, inhibits its binding to the *RPO26* promoter in *vitro*, and reduces the level of transcription from this promoter. Furthermore, Abf1p binding-site mutations shift the position of initiation sites of transcription upstream in the promoter, revealing a possible previously unknown function for Abf1p. The final chapter in this thesis (Chapter V) provides a summary of the work presented in chapters II through IV, and describes future experiments that extend from the results of the work shown in this thesis.
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MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell 79: 1093-1101.


CHAPTER II

Rpo26p, A SUBUNIT COMMON TO YEAST RNA POLYMERASES, IS ESSENTIAL FOR THE ASSEMBLY OF RNA POLYMERASES I AND II AND FOR THE STABILITY OF THE LARGEST SUBUNITS OF THESE ENZYMES


J. Archambault contributed to this work by designing the genetic screen, and constructing strains and plasmids necessary to initiate the project.
ABSTRACT
Eukaryotic nuclear RNA polymerases (RNAPs) are composed of two large subunits and a
number of small polypeptides, some of which are common among these enzymes. Rpo26p
is one of the five subunits common to yeast RNAPs. In order to understand the function of
Rpo26p, I isolated 34 different mutations in RPO26 that cause cell death in a strain carrying
a temperature-sensitive (ts) mutation in the gene (RPO21) encoding the largest subunit of
RNAPII. These mutant alleles were grouped into three phenotypic classes (null, ts, and
neutral) on the basis of the phenotype they imposed in combination with wild-type RPO21.
I addressed the function of Rpo26p by biochemical analysis of the ts rpo26-31 allele. The
steady-state level of rpo26-31p was reduced at high temperature; this was accompanied by
a concomitant decrease in the level of at least two other subunits, the largest subunits of
RNAPI (A190p) and RNAPII (Rpo21p). Pulse-chase metabolic labeling and
immunoprecipitation of RNAPII showed that at high temperature rpo26-31 did not lead to
dissociation of Rpo26p from the polymerase, but rather prevented the assembly of
RNAPII. Over-expression of rpo26-31 partially suppressed the ts phenotype and led to
accumulation of the mutant subunit. However, over-expression only marginally
suppressed the assembly defect of RNAPII. Furthermore, A190p and Rpo21p continued
to accumulate at low levels under these conditions. I suggest that Rpo26p is essential for
the assembly of RNAPI and RNAPII and for the stability of the largest subunits of these
enzymes.
INTRODUCTION

As described in Chapter I, the existence of common subunits among eukaryotic RNA polymerases has been known for some time (Buhler, et al., 1976a, Carles, et al., 1991, Valenzuela, et al., 1976), and the genes encoding the common subunits of yeast RNAPs (Archambault and Friesen, 1993) and their human counterparts (Shpakovski, et al., 1995, and references therein) have been cloned. The functions provided by these subunits are essential for the RNAPs, since deletion of any of the genes encoding these subunits leads to cell death in yeast (Archambault and Friesen, 1993). Furthermore, these essential functions are highly conserved throughout evolution since four of the yeast common subunits can be replaced by their human homologues (Shpakovski, et al., 1995 and references therein). This functional conservation is reflected in the high degree of sequence similarity (40 to 75% amino acid identity) that is observed between homologous subunits from these organisms (Reviewed in Shpakovski, et al., 1995). RNAP subunits homologous to the yeast common subunits have been found in Archaebacteria (reviewed in Langer, et al., 1995), but not in Eubacteria (E. coli). This finding suggests that the common subunits do not play a direct role in the catalytic function of the polymerase. The exact nature of the essential and conserved function provided by the common subunits remains largely unknown.

I have undertaken a genetic analysis of the function of Rpo26p, one of the subunits common among the three yeast RNAPs. Rpo26p is an acidic subunit with an apparent and a predicted molecular weight of 23 and 18 (Archambault, et al., 1990, Woychik, et al., 1990), respectively. It is phosphorylated in all three RNAP complexes mainly on serine and threonine residues (Bell, et al., 1976, Bell, et al., 1977, Buhler, et al., 1976b, Kolodziej, et al., 1990). Work from the Friesen lab has shown that RPO26 interacts genetically in two ways with RPO21, the gene encoding the largest subunit of RNAPII (Archambault, et al., 1990). First, an increased gene dosage of RPO26 suppresses, in an allele-specific manner, the temperature-sensitive (ts) phenotype conferred by a linker-
insertion mutant allele of \textit{RPO21} (\textit{rpo21}-4). Second, combination of non-lethal mutations in \textit{RPO26} with the \textit{ts} \textit{rpo21-4} allele gives rise to cell death at all temperatures (Archambault, et al., 1990). This genetic phenomenon, called synthetic lethality, is often observed among genes that encode functionally interacting proteins (Guarente, 1993).

In an effort to understand the function of the \textit{RPO26} gene product, I isolated a collection of mutant alleles of \textit{RPO26}. Taking advantage of the genetic interaction between \textit{RPO26} and \textit{RPO21}, a sensitive genetic screen was devised for the isolation of \textit{RPO26} mutant alleles. In this chapter, I describe this collection of mutant alleles of \textit{RPO26}. Using a \textit{ts} allele of \textit{RPO26} (\textit{rpo26-31}), I provide biochemical evidence that Rpo26p is required for the assembly of both RNAPI and RNAPII and for the stability of the largest subunits of these enzymes.
MATERIALS AND METHODS

Bacterial strains and plasmids: The bacterial strain JF1754 (hsdR, Lac- Gal- metB leuB hisB) was used as a cloning host. The plasmids used in this study are listed in Table 1. Plasmid pRPO26HAT (TRP1 CEN6 ARS RPO26) was constructed by inserting a 34 bp double-stranded oligonucleotide at a BclI site (nucleotide position 1022, all nucleotide numbers according to reference (Archambault, et al., 1990) immediately upstream of the RPO26 open-reading frame (ORF). The sequence of the top strand of this oligonucleotide is 5'-GATCATG TACCCATACGATGTCTCCAGATTACGCG-3' and encodes a nine amino acid epitope (underlined) from the Haemagglutinin (HA) antigen of the influenza virus (Field, et al., 1988) which is tagged on the N-terminus of the RPO26 protein (Rpo26p). Plasmid pSN260 contains a HindIII(282)/Ssp1(1756) fragment of the RPO26 genomic clone inserted into the Smal site of pFL39 (Bonneaud, et al., 1991, see Table 1). Plasmid pSN261 was derived from pSN260; pSN260 was cut with BclI (1022), in RPO26 sequences, and HindIII in the polylinker downstream of the Ssp1 (1756) site in RPO26. This resulted in the removal of sequences downstream of the initiation codon of HA-RPO26, which were then replaced with a BclI (1022)/HindIII (1780) fragment of HA-RPO26 from pRPO26HAT. Plasmid pSN308 is a derivative of pSN261 (Table 1). The latter plasmid was modified by insertion of a double-stranded 39bp oligonucleotide (5'-GATCATGCACCAAAACACACCAAAACAGCAGCGC-3', top strand) at a BclI site upstream of the HA sequence. The sequence on this oligonucleotide encodes a Met followed by 8 His residues (underlined), which is tagged on the HA-RPO26 ORF, giving rise to a His-HA-tagged Rpo26p. Both HA-tagged and His-HA-tagged RPO26 encode functional subunits. Plasmid pSN316 was constructed by subcloning the entire RPO26 genomic insert from pRPO26HAT plasmid, on a PstI/EcoRI fragment, into pRS424 (2μm, TRP1) (Christianson, et al., 1992). The integrative plasmid pJAY120 contains rpo26-A42 expressed from the RPO26 promoter. This plasmid was derived from pJAY108 (Table 1) by deleting the 2μm replicating sequences and LEU2-d (on a
### TABLE 1. List of plasmids used in this study

<table>
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<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
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<tr>
<td>pRPO26</td>
<td><em>URA3, CEN3, ARS1</em>, 2.1kb Xba1/Cla1 RPO26 genomic fragment</td>
<td>Archambalut. et. al., 1990</td>
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<tr>
<td>pRPO26HAT</td>
<td><em>TRP1, CEN6, ARS1</em>, same <em>RPO26</em> fragment as pRPO26 but tagged with HA-epitope at the ATG²</td>
<td>This study</td>
</tr>
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<td>pFL39</td>
<td><em>TRP1, CEN6, ARS1</em></td>
<td>Bonneau et. al., 1991</td>
</tr>
<tr>
<td>pSN260</td>
<td>HindIII (282)/Ssp1 (1756) fragment of RPO26 insert from pRPO26HAT cloned into the SmaI site of pFL39</td>
<td>This study</td>
</tr>
<tr>
<td>pSN261</td>
<td>Derivative of pSN260, HindIII (282)/HindIII(1780) RPO26 fragment from pRPO26HAT²</td>
<td>This study</td>
</tr>
<tr>
<td>pSN266</td>
<td>Same as pSN261 but sequences from Bci1 (1022) to HindIII (1780) correspond to rpo26-31 mutant plasmid²</td>
<td>This study</td>
</tr>
<tr>
<td>pSN308</td>
<td>Derivative of pSN260, but with double tagged (His)g-HA-RPO26²</td>
<td>This study</td>
</tr>
<tr>
<td>pJAY112</td>
<td><em>TRP1, CEN6, ARS1, RPO26</em> (Bci1 1022 to HindIII 1780) expressed from GAL10 promoter</td>
<td>This study</td>
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<td>pJAY116</td>
<td>Same as pJAY112 but with RPO26-Δ42</td>
<td>This study</td>
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<td>pJAY113</td>
<td>Same as pJAY112 but with RPO26-Δ84</td>
<td>This study</td>
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<td>This study</td>
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<td>URA3 Integrative plasmid, RPO26-Δ42 expressed from the RPO26 promoter²</td>
<td>This study</td>
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<td><em>URA3, CEN1, ARS1, RPO21</em></td>
<td>Archambalut. et. al., 1992</td>
</tr>
<tr>
<td>prpo21-4 to -8, -17 to -19</td>
<td>Same as pJS121 but containing rpo21-4 to rpo21-8, -17 to -19</td>
<td>Archambalut. et. al., 1992</td>
</tr>
<tr>
<td>pYCD11nr</td>
<td>Same as pJS121 but containing rpo21RII CTD deletion allele</td>
<td>Xiao et. al., 1994</td>
</tr>
<tr>
<td>pSN316</td>
<td>RPO26 genomic insert from pRPO26HAT cloned into pRS424²</td>
<td>This study</td>
</tr>
<tr>
<td>pSN317</td>
<td>Same as pSN316 but with rpo26-31</td>
<td>This study</td>
</tr>
</tbody>
</table>

¹Nucleotide numbers are given in paranthesis and are according to Archambault et. al., 1990

²For details of construction see Materials and Methods
HindIII/EcoRI fragment), and replacing the galactose inducible promoter (BclI fragment) with the RPO26 promoter sequences (BclI 140/BclI 1022).

Yeast strains and growth conditions: The names and relevant genotypes of all yeast strains used in this study are listed in Table 2. Strains JAY472 and JAY476 contain a partial duplication of the chromosomal RPO26 locus (designated in Table 2 as pJAY97) such that a truncated and non-functional copy of RPO26 is expressed from the RPO26 promoter, and a full-length copy is transcribed from the galactose-inducible GAL1 promoter (Archambault, et al., 1990). JAY472 contains the rpo21-4 ts allele, while JAY476 contains wild-type RPO21. Yeast strain JAY567 was constructed as follows: rpo26-A42, expressed from the RPO26 promoter, was integrated into the chromosome by two-step gene replacement. The integrative plasmid pJAY120 (rpo26-A42 URA3) was linearized at a HindIII (281) site within the RPO26 genomic fragment (747 bp upstream of RPO26 ORF) and was introduced into strain W303-1A. Ura+ -transformants were spread on 5-Fluoroorotic Acid (5-FOA)-containing medium and DNA from Ura- (5-FOA) colonies were tested by DNA-blot analysis for the replacement of RPO26 with rpo26-A42. The same strategy was used to modify RPO26 in the strain JAY47 (pGAL-RPO21) for the construction of JAY570. Cells were grown in rich medium or in defined medium supplemented with required amino acids as described (Sherman, et al., 1986). Minimal medium lacking inositol was prepared according to Culbertson and Henry (Culbertson and Henry, 1975). For metabolic-labeling experiments cells were grown in low-sulfate medium (LSM) as described in Kolodziej and Young (Kolodziej and Young, 1991b).

Construction and screening of a bank of randomly mutagenized RPO26 alleles: pRPO26HAT was mutagenized with hydroxyamine according to Budd and Campbell (1987). The bank of mutagenized plasmids was introduced into E.coli strain JF1754. Plasmid DNA from approximately 68,000 independent E. coli transformants was purified.
Table 2. List of yeast strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAY472</td>
<td>MATa rpo21-4 rpo26::pJAY97</td>
<td>(Archambault, et al., 1990)</td>
</tr>
<tr>
<td>JAY476</td>
<td>MATa rpo26::pJAY97</td>
<td>(Archambault, et al., 1990)</td>
</tr>
<tr>
<td>JAY444</td>
<td>MATa rpo26::LEU2 [pRPO26]</td>
<td>(Archambault, et al., 1990)</td>
</tr>
<tr>
<td>JAY47</td>
<td>MATa pGAL-RPO21</td>
<td>(Archambault, et al., 1990)</td>
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<td>MATa RPO26-Δ42^2</td>
<td>This study</td>
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<td>JAY570</td>
<td>MATa pGAL-RPO21 RPO26-Δ42^2</td>
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<td>MATa rpo26::LEU2 [pSN266]</td>
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<td>MATa rpo26::LEU2 [pSN317]</td>
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<td>MATa [pSN266]</td>
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</tr>
<tr>
<td>SNY193</td>
<td>MATa [pFL39]</td>
<td>This study</td>
</tr>
</tbody>
</table>

---

^1 All strains are derived from W303-1A (MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1) or W303-1B (MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1). Both strains were obtained from R. Rothstein.

^2 The construction of these strains is described in Materials and Methods.
and used to transform yeast strain JAY472. JAY472 transformants (Trp+) were selected on galactose solid medium at 30°C. Ten thousand individual colonies were patched onto galactose (-Trp) and glucose (-Trp) solid medium and were incubated for 2 days at 30°C. Colonies that failed to grow in the presence of glucose (condition under which the expression of wild-type RPO26 is repressed) but were able to grow using galactose as a carbon source (when transcription of wild-type RPO26 is induced), were selected for further study because they potentially contained non-functional alleles of RPO26. Plasmid DNA from each mutant strain was recovered in E. coli cells, reintroduced into JAY472 and tested a second time for ability to support growth on glucose medium.

Mapping of RPO26 mutations: Fragment BclI (1022)/HindIII (1780) from the mutant plasmids was used to replace a BclI (1022)/HindIII (in the polylinker downstream of SspI[1756] in RPO26) fragment from plasmid pSN260. This allowed expression of the mutant rpo26 alleles from a wild-type RPO26 promoter. The phenotype imposed by each newly generated construct in strain JAY472 (pGAL-RPO26 rpo21-4) was determined as described above.

Phenotype of RPO26 mutant alleles in combination with RPO21: This was tested in two different yeast strains: 1) JAY476, in which wild-type chromosomal RPO26 is expressed from the GAL1 promoter, and 2) JAY444, in which the entire chromosomal RPO26 ORF is replaced with LEU2 and wild-type RPO26 is expressed from a plasmid.

JAY476: The mutant alleles of RPO26 were introduced into the cell and Trp+ transformants were selected in the presence of galactose (wild-type RPO26 expressed) at 30°C. Drop tests (a suspension of cells in the form of a drop) were performed on galactose (-Trp) and glucose (-Trp) solid media at 15°C, 23°C, 30°C and 37°C.

JAY444 (rpo26::LEU2 [pRPO26]): Mutant alleles of RPO26 were introduced into JAY444 and Trp+ transformants were streaked on solid medium containing 5-FOA at 23°C.
to select for loss of the pRPO26 plasmid. Mutant alleles of RPO26 that failed to support growth on 5-FOA were classified as null mutants. Those that gave rise to 5-FOA\(^{+}\) colonies were tested for growth defects at various temperatures as described above.

N-terminal deletion alleles of RPO26: rpo26-Δ42 was constructed by site-directed mutagenesis according to Kunkel et al. (1987). rpo26-Δ84 was made by deleting sequences upstream of a HaeIII site at position 1319 in RPO26. An internal methionine is used as the site for translation initiation. The ability of rpo26-Δ42 to confer lethality in combination with various rpo21 ts alleles was tested in yeast strain JAY570 (pGAL-RPO21, rpo26-Δ42). Plasmids pJS121 (RPO21), prpo21-4 to prpo21-8, prpo21-17 to 19 and pYCD1rnr (see Table 1) were introduced into JAY570 and Ura\(^{+}\) transformants were tested for growth in the presence of glucose (RPO21 expression repressed). To test the growth phenotypes of rpo26-Δ42 and rpo26-Δ84, plasmids pJAY116 (pGAL-rpo26-Δ42), pJAY113 (pGAL-rpo26-Δ84) and pJAY112 (pGAL-RPO26) were used to transform JAY444 (rpo26::LEU2 [pRPO26]). Trp\(^{+}\)-transformants were grown in the presence of galactose and then were spread on galactose-5-FOA-containing medium. 5-FOA\(^{+}\) colonies (if any) were tested for growth at various temperatures in the presence of galactose.

DNA manipulations: All DNA manipulations were performed essentially according to Maniatis et al. (1982). Nucleotide sequence determination was performed by the dideoxy chain-termination method (Sanger, et al., 1977).

Cell-labeling: Strains SNY102 (HA-RPO26), SNY103 (HA-rpo26-31), SNY172 (His-HA-RPO26) and SNY190 (HA-rpo26-31 on 2 \(\mu\)m plasmid) were used for labeling experiments. Conditions for metabolic labeling of cells with \(^{35}\)S-methionine (NEN research products, 1175 Ci/mmol) were according to published protocols (Kolodziej and
Young, 1991a). In summary, cells were labeled in LSM in two ways: to test stability of RNAPII, SNY102, 103 and 172 were grown overnight at 23°C to an Optical Density (O.D.) at 600 nm of 0.5. An equivalent of 5x10^7 cells was labeled with 1 mCi of 35S-methionine at 23°C for 2 h, chased with 10 mM unlabeled methionine (final concentration) at the same temperature for 30 min and shifted to 37°C for 1 h. A second culture of SNY103 was both labeled and chased at 23°C. In order to assess the ability of newly synthesized polymerase subunits to assemble at 37°C, SNY102, 103, 172 and 190 were grown overnight to O.D.600 of 0.5 at 23°C and 5x10^7 cells were removed for labeling. The cells were first incubated with 1 mCi of 35S-methionine for 10 min at 23°C, and then transferred to 37°C for 110 min.

Cell lysis and immunoprecipitation of RNAPII: Cells were broken with glass beads and RNAPII was immunoprecipitated according to Kolodziej and Young (1991a). In order to ensure that labeling was equally efficient for all strains, the amount of radioactivity incorporated per mg protein was measured for each strain as follows: protein concentration was determined by the method of Bradford (1976) using BIO-RAD protein assay dye reagent concentrate. The amount of radioactivity incorporated into cellular proteins was measured by removing 3 and 6 µl aliquots from each cell-extract and precipitating with 400 µl of 25% TCA. Following a 1 h incubation on ice, the precipitates were collected on 0.45 µM Millipore nitrocellulose filters that had been presoaked in 1% methionine. The filters were washed with 5% TCA, rinsed with ethanol and air dried. The amount of radioactivity associated with each filter was counted in Ecoscint O (DiaMed) using a Beckman LS 6500 Scintillation counter. The amount of radioactivity incorporated per µg protein was essentially the same for all strains. An approximately equal amount of radioactively labeled protein isolated from each strain was used for immunoprecipitation of RNAPII using the monoclonal antibody 8WG16 (Thompson, et al., 1990) which recognizes the C-terminal domain (CTD) of Rpo21p. The 8WG16 ascites fluid was a gift from Jack Greenblatt. In
order to test the specificity of α-CTD immunoprecipitated polypeptides, an extra aliquot of SNY102 cells was labeled and the extract was used for immunoprecipitation with a monoclonal antibody to the human retinoblastoma protein (PHARMINGEN). The immunoprecipitated samples were separated on a 12% SDS-polyacrylamide gel along with a purified preparation of RNAPII containing His-HA-Rpo26p (a gift from Aled Edwards). The gel was stained using the colloidal Coomassie staining kit (Novex), dried and exposed to Kodak BioMax MR film at -80°C.

Immunoprecipitation of Rpo26p: Strains JAY444 (RPO26 on CEN/ARS plasmid), SNY102 (HA-RPO26 on CEN/ARS plasmid), and SNY190 (HA-rpo26-31 on 2µ plasmid) were used for this experiment. Cells were grown exponentially in YPD at 23°C, diluted in YPD and shifted to 37°C for 16 h up to O.D. at 600 nm of 0.6; this allowed the cells to undergo a sufficient number of cell divisions (at least 6) at 37°C and to dilute the RNAP molecules synthesized before the temperature shift. Cells were harvested and extracts were prepared according to the protocol described in Harlow and Lane (1988). Equal amounts of protein from each cell extract were used for immunoprecipitation of HA-Rpo26p and associated subunits using the 12CA5 monoclonal antibody (a gift from Deming Xu) according to Kolodziej and Young (1991a), except that no milk powder was added to the immunoprecipitation buffer. The immunoprecipitated complexes were eluted from protein A beads (Sigma) in SDS-PAGE loading buffer and by heating at 65°C for 10 min. Samples were separated on a 4-20% SDS-polyacrylamide gradient gel (BIO-RAD), transferred to Immobilon membrane from Millipore, and the membrane was probed with rabbit polyclonal antisera raised against: Rpo26p (prepared in this lab), Rpo21p (α-B185, a gift of Christopher Carles and Michel Riva), A190p (α-A190, a gift of Masayasu Nomura), or with the 8WG16 monoclonal antibody that recognizes CTD. A separate gel was prepared for probing with each antiserum. Horseradish-peroxidase labeled goat anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies. Signals were
detected by treating the membrane with the ECL reagent LumiGLO from Kirkegaard and Perry Laboratories and exposing the membrane to Kodak BioMax MR film.

Immunoblot analysis of Rpo26p stability: Cells were grown for desired lengths of time at either the permissive or non-permissive conditions and an equivalent of 1.2x10^8 cells was harvested. Cells were broken with glass beads according to Kolodziej and Young (1991a). Protein concentration was measured by the method of Bradford (Bradford, 1976), and an equal amount of protein from each strain was loaded onto a 10% SDS-PAGE gel. Proteins were separated by gel electrophoresis and were transferred to ImmobilonP membrane. The membrane was probed for Rpo26p using the polyclonal α-Rpo26p I generated to this subunit, and for the 69 kD subunit of the yeast vacuolar H^+-ATPase (Kane, et al., 1989) using monoclonal antibody 8B1-F3 obtained from Molecular Probes. The latter was used as a control for loading equal amounts of protein.

Generation of rabbit α-Rpo26p antibody: A recombinant bacterially expressed GST-Rpo26p fusion protein was purified on glutathione-agarose beads (Sigma), and was injected into rabbits. The α-Rpo26p antiseraum was partially purified by ammonium sulfate precipitation prior to use.

Over-expression of rpo26-31: The growth properties of strains SNY102 (RPO26 on CEN/ARS plasmid), SNY103 (rpo26-31 on CEN/ARS plasmid), SNY189 (RPO26 on 2 μm plasmid) and SNY190 (rpo26-31 on 2 μm plasmid) were tested on YPD at 23°C and at 37°C. To analyze the steady state level of Rpo26p, the above strains were grown at 23°C and shifted to 37°C for 6 h. Cell extracts were prepared and the level of Rpo26p was monitored as described above.
RESULTS

Screening for mutant alleles of \textit{RPO26} using synthetic lethality

An invariable feature of eukaryotic RNAPs is the presence of common subunits, yet information concerning their role in the functions of these enzymes is minimal. The aim of my work is to understand the functional role of the \textit{RPO26} gene product, one of the subunits common to yeast RNAPs. It has been observed previously (Archambault, et al., 1990) that several \textit{rpo26} mutations which do not affect the growth of otherwise wild-type yeast cells, confer lethality when combined with a \textit{ts} mutation in \textit{RPO21} (\textit{rpo21-4}). Based on this observation, a sensitive screen was devised for the isolation of functionally defective mutant alleles of \textit{RPO26}. In this screen, I used a plasmid-borne library of randomly mutagenized \textit{RPO26} to isolate individual mutant alleles that confer synthetic lethality on the yeast strain JAY472. Two features of the genotype of JAY472 are relevant to the genetic scheme: 1) JAY472 contains the \textit{rpo21-4} allele (Archambault, et al., 1990), and 2) in JAY472, expression of the chromosomal \textit{RPO26} is under the control of the \textit{GAL1} promoter. Cell growth in the presence of glucose results in repressed transcription of the chromosomal \textit{RPO26} gene and, therefore, requires that a functional \textit{RPO26} gene be provided on a plasmid (Archambault, et al., 1990). Starting with 10,000 JAY472 transformants, I identified 63 plasmids that conferred a reproducible lethal phenotype on JAY472 in the presence of glucose, indicating that they carried a mutant allele of \textit{RPO26}. Examples of the lethal phenotype conferred by \textit{RPO26} mutant alleles are shown in Figure 1A.

Localization of mutations

Mutations that confer the synthetic-lethal phenotype on JAY472 might reside in the promoter or in the ORF of \textit{RPO26}. In order to distinguish between these possibilities, a 758 bp fragment that contained the \textit{RPO26} ORF and approximately 200 bp of downstream untranslated sequences was isolated from each mutant plasmid. This fragment was used to
Fig. 1- Growth phenotype conferred by mutant alleles of RPO26.

A) Phenotype conferred by three alleles of RPO26 in yeast strain JAY472 (rpo21-4, pGAL-RPO26). The lethal phenotype conferred by mutant alleles of RPO26 on JAY472 in the presence of glucose (RPO26 not expressed, right column), normal growth in the presence of galactose (RPO26 expressed, left column). B) Phenotype conferred by three alleles of RPO26 in yeast strain JAY476 (RPO21, pGAL-RPO26) at various temperatures. pFL39 in both panels A and B refers to empty vector. C) Growth phenotype of various combinations of the deletion allele of RPO26 (RPO26-Δ42) and different alleles of RPO21 in strain JAY570 (RPO26-Δ42, pGAL-RPO21). Note that the rpo21-4 allele conferred a slow-growth phenotype in JAY570 (RPO26 on chromosome, panel C) compared to JAY472 (RPO26 on low-copy plasmid, panel A), when tested at 30°C. This is because the phenotype of rpo21-4 is sensitive to the copy number of the RPO26 gene; the ts phenotype of rpo21-4 was suppressed by increased RPO26 gene dosage, when carried on a low-copy plasmid (Archambault, et al., 1990).
replace the same fragment in an analogous unmutagenized plasmid (see Materials and Methods), allowing expression of the former fragment from a wild-type RPO26 promoter. The phenotype conferred by subcloned RPO26 mutant alleles was retested in JAY472 (pGAL-RPO26, rpo21-4), as described above. All but five of the mutant alleles continued to confer synthetic lethality when expressed from a wild-type RPO26 promoter. Sequencing of the promoter of RPO26 for the remaining five mutants showed that they carried mutations in the promoter region. More detailed analysis of these mutants is described in Chapter IV (Nouraini, et al., 1996a).

Sequence analysis of rpo26 mutant alleles

I determined the sequence of the RPO26 ORF and 200 bp of downstream untranslated sequences for each of the 63 plasmids recovered in the screen. Except for the promoter mutations mentioned above, all had mutations in the RPO26 ORF. Thus, 29 different functionally defective mutant alleles of RPO26 were identified (Table 3), 16 of which were isolated more than once. These data suggest that the majority of possible hydroxylamine-induced mutations in RPO26 that can confer a synthetic-lethal phenotype with rpo21-4 have been recovered. All missense and nonsense mutations (except one, M11) affected amino acids that are clustered in the C-terminal two-thirds of the protein (amino acids 50 to 155, Fig. 2). In contrast, no mutations were isolated that changed amino acids at the N-terminus of Rpo26p (amino acids 1 to 50), despite the fact that 34 of the 50 codons encoding this region could be mutated by hydroxylamine. This finding suggests that the C-terminal portion of the Rpo26p is sufficient to support cell growth (see below). I constructed two N-terminal deletion alleles of RPO26 in order to test this hypothesis.

rpo26-Δ42 and rpo26-Δ84 encode truncated Rpo26p that lacks the first 42 and 84 amino acids, respectively. When expressed on a plasmid from the GAL10 promoter, rpo26-Δ42 was able to complement a chromosomal deletion of RPO26 in a plasmid-
### TABLE 3. Detailed description of *RPO26* mutant alleles isolated in this study

<table>
<thead>
<tr>
<th>Allele number</th>
<th>Phenotype</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rpo26-1</em></td>
<td>Null</td>
<td>Q59 to stop</td>
</tr>
<tr>
<td><em>rpo26-2</em></td>
<td>Null</td>
<td>Q63 to stop</td>
</tr>
<tr>
<td><em>rpo26-3</em></td>
<td>Null</td>
<td>Q60 to stop</td>
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<tr>
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<td>Q100 to stop</td>
</tr>
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<tr>
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<td>M1 to I</td>
</tr>
<tr>
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<td>1)M85 to I</td>
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<td>2)G to A, 5'-splice site</td>
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<td>G95 to D</td>
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<td>1)G1115 to A in IVS3</td>
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<td></td>
<td>2)G1121 to A, 3'-splice site</td>
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<td>3)P139 to L</td>
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<td></td>
<td>2)P83 to S</td>
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1 Phenotype analyzed in the presence of *RPO21*
2 Nucleotide number according to Archambault et al. (1990)
3 IVS= intervening sequence
4 Described elsewhere (Chapter IV; Nouraini, et al., 1996a)
Fig. 2- Schematic representation of \textit{RPO26} mutant alleles and associated amino-acid changes. Boxes labeled with - and + signs designate regions of Rpo26p with a preponderance of negatively and positively charged amino acids, respectively. Each amino acid change is designated with a number in parentheses which represents the mutant \textit{rpo26} allele number. The mutant alleles are divided based on the phenotype they confer on yeast in the presence of wild-type \textit{RPO21}. Shown also are two deletion mutant alleles (\textit{RPO26-Δ42} and \textit{RPO26-Δ84}), constructed by site-directed mutagenesis, and their associated phenotypes.
shuffling assay (see Materials and Methods). In contrast, \textit{rpo26-Δ84} failed to support growth under these conditions (data not shown). Furthermore, the growth phenotype of strain \textit{JAY567}, in which full length chromosomal \textit{RPO26} was replaced with \textit{rpo26-Δ42}, was indistinguishable from wild-type at all temperatures tested (data not shown). Finally, \textit{rpo26-Δ42} was unable to confer synthetic lethality when tested in combination with various ts (\textit{rpo21-4} to -8, and \textit{RPO21RII}) and neutral (\textit{rpo21-17} to -19) mutant alleles of \textit{RPO21} (Fig. 1C). Therefore, I conclude that amino acids 1-42 of Rpo26p are not required for growth under the conditions that were tested.

\textbf{Phenotype of \textit{RPO26} mutant alleles in an \textit{RPO21} background}

I determined the phenotype of the newly identified \textit{rpo26} mutant alleles in the presence of wild-type \textit{RPO21} (instead of \textit{rpo21-4}), by introducing the plasmids harboring these alleles into yeast strain \textit{JAY476} (\textit{pGAL-RPO26}). \textit{JAY476} transformants were selected on galactose medium and then were tested for growth on glucose medium at various temperatures (15°C, 23°C, 30°C, and 37°C). The \textit{rpo26} mutant alleles were grouped into three classes (Table 3) on the basis of the phenotype they imposed in the presence of wild-type Rpo21p when grown on glucose medium: 1) null, those alleles that did not support growth at any temperature, 2) ts, alleles that exhibited a growth defect at 37°C but could support normal growth at 23°C, 3) neutral, alleles that were capable of supporting growth at all temperatures tested (Fig. 1B and Fig. 2).

Only one allele, *rpo26-31*, showed a severe growth defect in the absence of inositol (not shown). The phenotypes imposed by the *rpo26* mutant alleles were virtually identical when tested in a strain that carries a complete deletion of chromosomal *RPO26* (data not shown).

**Analysis of the steady-state level of Rpo26p**

I compared the steady-state level of Rpo26p in the ts mutants and in a wild-type strain at the permissive and non-permissive temperatures in order to assess the functional defect imposed by the ts mutations. The growth rate of the *rpo26-31* strain decreased rapidly, beginning approximately 30 min after shift to 37°C. The remainder of the ts strains showed a decrease in the growth rate only 3 to 4 h after shift (data not shown).

Extracts were prepared from cells that were shifted to 37°C for either 6 h (for *rpo26-31*) or 10 h (for the remainder of the ts strains); these were analyzed for the steady-state level of Rpo26p, which was reduced in all of the mutant strains at the non-permissive temperature. For the *rpo26-31* mutation, the steady-state level of Rpo26p was reduced by more than four-fold (compare lanes 7-9 to 13-15 in Fig. 3A, also lanes 7-9 and 16-18 in Fig. 3B). In the remainder of the ts mutants the amount of Rpo26p was reduced by only two-fold or less (data not shown). I chose *rpo26-31* from among the *RPO26* ts mutant alleles for further analysis, since it conferred the most severe temperature-dependent growth defect.

**Suppression of the ts phenotype by over-expression of *rpo26-31***

The reduction in the amount of *rpo26-31* p at high temperature might be due to a decrease in the thermodynamic stability of this subunit or to an inability to assemble into the polymerase complex, or both. In either case Rpo26p might become more susceptible to degradation, which might be remedied by over-expression of the mutant subunit. As shown in Figure 4A, over-expression of *rpo26-31* suppressed, albeit not completely, the ts phenotype due to this mutation. Furthermore, over-expression of *rpo26-31* also
Fig. 3- Temperature- and time-dependence of the steady-state amount of Rpo26p in the presence of the rpo26-31 mutation. A) Strains SNY102 (HA-RPO26) and SNY103 (HA-rpo26-31) were grown at 23°C to early log phase. The cells were divided into two portions, one was kept at 23°C while the other portion was shifted to 37°C for 6 h. Cells were harvested after 6 h of growth, and extracts were prepared. Four µg (lanes 4, 7, 10 and 13) 8 µg (lanes 5, 8, 11 and 14) and 16 µg (lanes 6, 9, 12 and 15) of protein were loaded on a 10% SDS-PAGE gel, and used for blotting. Lane 1 contains a partially purified preparation of RNAPII from the W303-1A yeast strain (see Table 2), and lanes 2 and 3 contain 10 µg of extract prepared from SNY102 (HA-RPO26) and SNY172(HIS-HA-RPO26), respectively. B) SNY102 and SNY103 were grown as described above, except aliquots of cells were removed before, and 2, 4, and 6 h after the temperature shift. An equivalent of 4 µg (lanes 1, 4, 7, 10, 13 and 16), 8 µg (lanes 2, 5, 8, 11, 14 and 17) and 16 µg (lanes 3, 6, 9, 12, 15 and 18) of protein was used. Lane 19 contains partially purified RNAPII. Protein-blots were probed with polyclonal antibody against Rpo26p, and a monoclonal antibody directed against the 69kDa subunit of yeast vacuolar H^+-ATPase.
Fig. 4- Over-expression of *rpo26-31*, suppression of the ts phenotype and accumulation of *rpo26-31p*. A) Growth phenotype of yeast when *rpo26-31* is expressed from a low-copy (top left panel) or a high-copy (bottom left panel) plasmid, at the permissive (23°C) and the non-permissive temperature (37°C). B) Protein-blot showing the steady state level of Rpo26p in the four strains shown in A. Cells were grown at 23°C to mid-log phase and shifted to 37°C for 6 h. Eight µg (lanes 1, 2, 5 and 7) and 16 µg (lanes 2, 4, 6 and 8) of cell extract were used for protein-blot analysis of the steady-state level of Rpo26p, and of Vma1p as loading control. Lane 9 contains a sample of a partially purified fraction of RNAPII.
suppressed the Ino\(^{-}\) phenotype associated with this mutation (not shown). Analysis of the steady-state level of Rpo26p (Fig. 4B) showed that over-expression of \(rpo26-31\) (lanes 7 and 8) resulted in a level of \(rpo26-31p\) that was more than twice the amount obtained when wild-type \(RPO26\) was expressed from a low-copy plasmid (lanes 1 and 2).

**Analysis of the stability/assembly of RNAPII**

Partial rescue of the \(rpo26-31\) ts phenotype by over-expression of \(rpo26-31p\) suggests that the mutation may weaken the interaction of Rpo26p with other polymerase subunit(s), thus affecting either the stability or the assembly of one or more of the three RNA polymerases. If the stability of association of \(rpo26-31p\) with the assembled RNA polymerase is reduced at high temperature, one would expect this subunit to dissociate from the polymerase complex upon a shift to the non-permissive temperature. To test this idea, I labelled strains with \(HA-RPO26\), \(HA-rpo26-31\) and \(His-HA-RPO26\) with \(^{35}\)S-methionine, in order to test this possibility. Labeling was carried out at 23\(^{\circ}\)C (pulse) followed by a shift to 37\(^{\circ}\)C for 1 h in the presence of an excess of non-radioactive methionine (chase). As a control, a portion of the strain with \(HA-rpo26-31\) was both labeled and chased at 23\(^{\circ}\)C. RNAPII complexes were immunoprecipitated from cell extracts using a monoclonal antibody (8WG16) that is specific to the C-terminal domain (CTD) which is unique to the largest subunit of RNAPII, and were analyzed by SDS-PAGE. Figure 5 shows the composition of the immunoprecipitated complexes along with that of a purified preparation of RNAPII which contains His-HA-Rpo26p. The clear difference in mobility of HA-Rpo26p (marked by a star in Fig. 5 lanes 1, 3 and 4) and His-HA-Rpo26p (marked by an arrow in Figure 5, also compare lanes 2 and 3 in Fig 3A) allows unambiguous identification of the subunit and determination of its presence or absence in the polymerase complex (note that HA-\(rpo26-31p\) has greater mobility than its HA-tagged wild-type counterpart). As shown in Fig. 5, \(rpo26-31p\) was present in the RNAPII complex both before (lane 3) and after (lane 4) the shift to 37\(^{\circ}\)C. This result
Fig. 5- Association of rpo26-31p with RNAPII. Strains SNY102 (lane 1), SNY172 (lane 2), SNY103 (lanes 3 and 4) were metabolically labeled with $^{35}$S-methionine at 23°C for 2h and chased with unlabeled methionine either at 37°C (lanes 1, 2 and 4) or at 23°C (lane 3). Cell extracts were prepared and used for immunoprecipitation with the 8WG16 monoclonal antibody directed against the C-terminal domain of Rpo21p. The position of migration of HA-Rpo26p is shown by a star (lanes 1, 3 and 4) and His-HA-Rpo26p is pointed to by an arrow (lane 2). A sample of a purified preparation of RNAPII containing His-HA-Rpo26p was run on the same gel and the position of migration of the subunits were determined by staining the gel with colloidal Coomassie blue before autoradiography. The position of migration of the subunits are shown by solid bars labeled with the name of the subunit on the left side of the figure.
indicates that RNAPII complexes assembled at the permissive temperature are stable at 37°C and that the rpo26-31 mutation does not affect the stability of RNAPII.

The ability of newly synthesized rpo26-31p to be incorporated into the RNAPII complex was analyzed at 37°C in order to investigate whether rpo26-31 affected the assembly of this enzyme. Since rpo26-31p accumulated only in low amounts at 37°C, a strain was used in which the HA-rpo26-31 allele was over-expressed. This strain, along with cells expressing HA-RPO26, HA-rpo26-31 and His-HA-RPO26 on low copy plasmids were labeled at 37°C for 2 h. Cell extracts were prepared and RNAPII complexes were immunoprecipitated with the 8WG16 antibody (Fig. 6A). As a negative control in this experiment, the immunoprecipitation analysis of extracts from cells that express HA-RPO26 was also carried out using a monoclonal antibody that recognizes the human retinoblastoma protein (not present in yeast). As before, the difference in mobility of His-HA-Rpo26p (Figure 6A, lane 1, arrow) and HA-Rpo26p (Figure 6A, lanes 3 and 5, star) was used to identify the subunit and to determine that it was able to assemble into the RNAPII complex at high temperature. However, despite the fact that rpo26-31p was overexpressed relative to wild-type Rpo26p, the total amount of RNAPII immunoprecipitated from the mutant strain was substantially lower (Fig. 6A, lane 5) than the amount precipitated from a wild-type strain (lanes 1 and 3).

The difference in the amount of RNAPII (Fig. 6A) was not due to a reduced overall rate of protein synthesis in the mutant strain. This was shown by repeating the metabolic-labeling experiment and using a monoclonal antibody to the 69 kDa subunit (Vma1p) of the multisubunit vacuolar H+-ATPase (Kane, et al., 1989) to immunoprecipitate the ATPase complex, an enzyme with no known functional relevance to RNAPII. As shown in Fig. 6B, the level of newly-synthesized subunits of the ATPase complex was comparable among the wild-type (lanes 1 and 3) and the mutant (lanes 4 and 5) strains. Therefore, the reduction in the amount of RNAPII detected in the mutant strain is likely to be the result of a specific deficiency in assembled RNAPII at high temperature. Furthermore, since the
Fig. 6- Assembly of RNAPII in the presence of rpo26-31 at 37°C. Strains SNY172 (lane 1), SNY102 (lanes 2 and 3), SNY103 (lane 4) and SNY190 (lane 5) were metabolically labeled with 35S-methionine at 37°C for 2 h and cell extracts were prepared. A) RNAPII complexes were immunoprecipitated with 8WG16. A control immunoprecipitation was performed using a monoclonal antibody to the human retinoblastoma protein. The band representing HA-Rpo26p is marked with a star (lanes 3 and 5) and His-HA-Rpo26p is designated by an arrow (lane 1). The migration positions of the subunits of purified RNAPII containing His-HA-Rpo26p are shown on the left. B) The vacuolar H+-ATPase complex was immunoprecipitated using a monoclonal antibody to the 69 kDa subunit. The monoclonal anti-RBp antibody was also used for a control immunoprecipitation. The expected position of migration of the H+-ATPase subunits is shown on the right, and the position of migration of molecular weight markers is shown on the left.
antibody used for immunoprecipitation of RNAPII was directed to Rpo21p; this subunit must fail to accumulate at 37°C in the rpo26-31 strain.

**Immunoprecipitation of Rpo26p and associated subunits**

Results from immunoprecipitation of RNAPII with an antibody directed against Rpo21p (see above) are consistent with the idea that the *rpo26-31* mutation reduces the affinity of rpo26-31p either for Rpo21p or for other subunits in the polymerase, a situation that leads to a reduction in the steady-state level of the Rpo21 subunit. To reinforce this interpretation I immunoprecipitated HA-Rpo26p from extracts of wild-type and mutant cells grown at the non-permissive temperature. Strains that express either HA-Rpo26p or untagged Rpo26p (as negative control) on low-copy plasmids, and a third strain that overexpresses HA-rpo26-31, were grown exponentially at 23°C and then were shifted to 37°C for 16 h; this allowed the cells to undergo a sufficient number of divisions (at least 6) to dilute out polymerase molecules that had been assembled at the permissive temperature. Cell extracts were prepared and HA-Rpo26p and associated subunits were immunoprecipitated using monoclonal antibody 12CA5, that recognizes the HA epitope. Protein blotting was used to monitor the presence of the Rpo21 subunit with the CTD-specific 8WG16 antibody (Fig. 7A) and with a polyclonal antibody (α-B185) that was raised against the whole subunit (Fig. 7B). The amount of Rpo21p detected in cell extracts (lane 2 versus lanes 3 and 4 in Fig. 7A and B), or found in association with Rpo26p (lane 6 versus lanes 7 and 8 in Fig. 7A and B) was reduced in the presence of the *rpo26-31* mutation, even though an excess amount of rpo26-31p was produced in the mutant strain (Fig. 7D, compare lane 2 to lanes 3 and 4, and lane 6 with lanes 7 and 8). Hence, the results of this experiment support the conclusion drawn from immunoprecipitation of RNAPII using 8WG16 antibody (see above).
Fig. 7- Immunoprecipitation of HA-Rpo26p and associated subunits. Strains JAY444 (RPO26 on CEN/ARS plasmid), SNY102 (HA-RPO26 on CEN/ARS plasmid), and SNY190 (HA-rpo26-31 on 2μm plasmid) were grown at 23°C and shifted to 37°C for 16 h. Cell extracts were prepared and, starting from equal amounts of protein from each extract, HA-Rpo26p and associated complexes were immunoprecipitated using monoclonal antibody 12CA5, which recognizes the HA epitope. Shown is the results of protein-blot analysis of crude extracts (lanes 1 to 4) and the immunoprecipitated complexes (lanes 5 to 8), probed with the following antibodies: A) the monoclonal 8WG16 antibody raised against the C-terminal domain of Rpo21p, B) a polyclonal rabbit antibody raised against the whole Rpo21p (or B185) subunit, C) a polyclonal rabbit antibody raised against the largest subunit of RNAPI (A190), and D) the polyclonal rabbit antibody raised against Rpo26p. Lane 9 in panels A, B and D contain a sample of a partially purified fraction of RNAPII containing HA-Rpo26p. In panel C, lane 9 contains a sample of a partially purified fraction of RNAPI.
Analysis of the assembly of RNAPI

Since the Rpo26p subunit is common to RNAPI, II, and III, it is possible that the rpo26-31 mutation, in addition to affecting the stability of Rpo2lp, may also affect the accumulation of subunits of either of the other two polymerases. To this end, I determined the amount of the largest subunit of RNAPI (A190) present in cell extracts and in α-HA immunoprecipitates using a polyclonal antibody (α-A190) raised against this protein. As shown in Fig. 7C, the steady-state level of A190 in the cell extracts (compare lane 2 to lanes 3 and 4) and the amount of this subunit co-immunoprecipitated with HA-Rpo26p (compare lane 6 to lanes 7 and 8) was reduced in the presence of the rpo26-31 mutation. These observations indicate that the defect caused by the rpo26-31 mutation affects RNAPI as well as RNAPII.

An independent test for assembly of Rpo26p into RNAP

The above observations suggest that the failure of rpo26-31p to accumulate is due partly, if not completely, to the inability of this subunit to assemble into RNAPII (and RNAPI) at high temperature. In a yeast strain that expresses both rpo26-31p and Rpo26p, the wild-type subunit should be able to compete with the mutant subunit for assembly into RNAPII and RNAPI. As a consequence, one would expect a reduced steady-state amount of rpo26-31p in the presence of Rpo26p compared to its absence. This hypothesis was tested by comparing the steady-state level of a plasmid-encoded, HA-tagged Rpo26p (either wild-type or mutant) in the presence of an untagged chromosomally expressed Rpo26p, in relation to when the chromosomal RPO26 was replaced with LEU2. The difference in mobility of the two forms of Rpo26p (HA-tagged and untagged) in an SDS-PAGE gel allowed a determination of the individual contribution of each allele to the overall steady-state level of Rpo26p. As shown in Figure 8, when both the plasmid- and chromosome-encoded RPO26 were wild-type (Fig. 8, lanes 5-7), an equal amount of both species of Rpo26p was present. In contrast, the steady-state amount of plasmid-encoded subunit was
reduced when \textit{rpo26-31} was carried on the plasmid (lanes 8-10). This reduction in the steady-state level of \textit{rpo26-31}p was observed only in the presence of wild-type Rpo26p; in the absence of wild-type \textit{RPO26} expression, the amount of \textit{rpo26-31}p was similar to that of wild-type Rpo26p at the permissive temperature (compare lanes 2-4 to 11-13 in Fig. 8, and also lanes 4-6 to 7-9 in Fig. 3A). These results support the suggestion that accumulation of \textit{rpo26-31}p depends on association with other RNAP subunits, and that the \textit{rpo26-31} mutation compromises its ability to assemble into an RNAP complex.
Fig. 8-Stability of rpo26-31p as a function of association with RNA polymerase. Strains SNY193 (RPO26, pFL39), SNY191 (RPO26, HA-RPO26), SNY192 (RPO26, HA-rpo26-31), and SNY103 (rpo26::LEU2, HA-rpo26-31) were grown at 23°C and cell extracts were prepared. The steady-state amount of Rpo26p, Rpo21p and Vma1p was analyzed for each strain by protein-blotting with polyclonal a-Rpo26p, and monoclonal α-CTD and α-Vma1p antibodies, respectively. Lane 1 contains a partially purified preparation of RNAPIII prepared from W303-1A. Note that all the lanes in this figure are from the same gel.
Discussion

Essential regions of Rpo26p

Starting with a library of randomly mutagenized RPO26, I have isolated a collection of functionally defective mutant alleles of this gene that are synthetic lethal with rpo21-4. Clustering of the amino acid changes in the C-terminal end of Rpo26p, and the ability of rpo26-Δ42 to support normal growth and sporulation suggests that the N-terminal region of Rpo26p is dispensable for all functions of this subunit. The finding that the N-terminal region of Rpo26p is dispensable for growth provides an explanation for the lack of conservation of this region in the homologous subunit of Archaebacteria (Fig. 9). It also explains the low degree of sequence similarity observed for this region of Rpo26p among various eukaryotes (Fig. 9). It has been suggested that the acidic N-terminal region is needed for an unknown function that is required only in eukaryotes (Shpakovski, et al., 1995). Perhaps the function of the acidic N-terminus of Rpo26p is redundant in the eukaryotic transcriptional machinery, and therefore its absence escapes detection.

A region of 13 amino acids in the C-terminal domain of Rpo26p is conserved highly in eukaryotes and in archaebacteria (Fig. 9), suggesting that this region of Rpo26p provides an essential function. This is supported by the finding that the only two lethal amino-acid substitutions in Rpo26p isolated in my study affect residues in this region (E89K in rpo26-9, and G95D in rpo26-10, underlined in Fig. 9).

Biochemical analysis of the defect caused by rpo26-31

I have shown that rpo26-31 leads to a significant reduction in the steady-state level of rpo26-31p and in the amount of assembled RNAPI and RNAPII. I suggest that the reduction in the steady-state level of rpo26-31p is due to degradation of the mutant subunit since the ts phenotype of rpo26-31 can be partially suppressed in two ways: by over-expression of the mutant subunit (this study), and by mutation of a gene encoding a putative catalytic subunit of the cellular proteasome (Nouraini, et al., 1996b).
Fig. 9- Sequence conservation of Rpo26p across various eukaryotic and Archaebacterial species. Shown is a schematic representation of Rpo26p from various species. The N-terminal box of homology that is observed only in eukaryotic subunits contains a high proportion of acidic amino acids. The degree of homology between the subunits was assessed in a DNA Strider program by searching for identical amino acids using a window of 15 residues. The gray-shaded C-terminal region in the subunit from all of the species represents a region with a high-degree of amino-acid identity (7 out of 15 amino acids) between the yeast subunit and each heterologous subunit. The black box represents a region of high homology (7 identical amino acids out of 13) conserved in Rpo26p subunits from all the species. The amino acids that when mutated give rise to a null phenotype, as determined by our genetic screen, are underlined.
*Saccharomyces cerevisiae*  

*Homo sapiens*  

*Drosophila melanogaster*  

*Schizosaccharomyces pombe*  

*H. marismortui*  

*S. acidocaldarius*  

S. c. 89 ERARILGTRALQI 101  
H. s. 61 ERARVLGTRALQI 73  
D. m. 91 ERARVLGTRALQI 104  
S. p. 79 ERARILGTRALQI 91  
H. m. 22 EIARISARALQY 34  
S. a. 9 EKARKLGARALQL 21  

_**E.A.R.1g.RALQ.**_
However, over-expression of the mutant subunit is only marginally effective in restoring the amount of assembled RNAPII (Fig. 6A, compare lanes 4 and 5), suggesting that the mutant subunit is unable to assemble into the polymerase complex as efficiently as the wild-type subunit. This is supported by the observation that rpo26-31p becomes unstable in the presence of a source of wild-type Rpo26p (Fig. 8) under conditions in which the mutant subunit normally accumulates to wild-type levels (at 23°C). Presumably, the wild-type subunit can bind more efficiently than rpo26-31p to the polymerase complex, rendering the mutant subunit susceptible to degradation.

These observations are consistent with the idea that the rpo26-31 allele encodes an unstable polypeptide that becomes stabilized following assembly into the RNAP complex. Two possibilities, not mutually exclusive, may account for the temperature-dependent instability of the rpo26-31 protein. First, instability may be an intrinsic property of rpo26-31p due to misfolding, and assembly with RNAP may stabilize the subunit in its proper conformation. At high temperature, the instability of rpo26-31p may be more pronounced; over-production of rpo26-31p may compensate for this by raising the steady-state level of the mutant subunit. Second, the instability of rpo26-31p may be a consequence of its reduced ability to assemble with the rest of the RNAP complex. In this case, an increase in temperature would reduce further the affinity of the subunit for the RNAP complex; over-production of rpo26-31p may compensate for this by pushing the equilibrium toward complex formation. According to this idea, unassembled Rpo26p, either mutant or wild-type, is prone to degradation.

As indicated above, only a marginal increase in the amount of RNAP at high temperature is sufficient to alleviate the growth defect imposed by rpo26-31. This observation suggests that the amount of RNAPs in a yeast cell exceeds the requirement for normal growth, at least at moderate temperatures. Several studies lend support to the observation that yeast is able to grow in the absence of a normal complement of RNAPs. First, Archambault et al (1996) placed the expression of RPO21 under the control of the
repressible LEU2 promoter. When the expression of RPO21 was repressed by the addition of leucine to the growth medium, the steady-state level of Rpo21p was reduced by at least ten-fold; nevertheless, the cells acquired a lethal phenotype only at 37°C. Second, Mosrin et al. (1990) showed that partial suppression of a nonsense mutation in RPC31, which encodes an RNAPIII subunit, using a tRNA suppressor reduced the level of Rpc31p; however, the cells grew slowly only on minimal medium (Mosrin, et al., 1990). Third, deletion of the gene encoding the RNAPII subunit A12.2 led to a reduction in the steady-state level of the largest subunit of RNAPI and a ts growth phenotype (Nogi, et al., 1993). Temperature-sensitive mutations that create an assembly defect have been identified previously (Kolodziej and Young, 1991a, Mann, et al., 1987) in genes that encode other subunits of RNAPs, and can be divided into two classes. The first includes mutations that lead to the accumulation of sub-complexes at the non-permissive temperature. Mutations that affect the three largest subunits of RNAPII are examples of this class (Kolodziej and Young, 1991a). The second class includes those that do not lead to the accumulation of subcomplexes at the non-permissive temperature. The latter group is exemplified by a ts mutation in AC40, a subunit that is common to RNAPI and RNAPIII (Mann, et al., 1987). The mutation in rpo26-31 falls in the latter class since it reduces the total amount of RNAPI and RNAPII without an accumulation of subcomplexes.

The requirement of a small subunit of an RNA polymerase for the stability of the large subunits is not unprecedented. As indicated above, the absence of the A12.2 subunit of RNAPI leads to a reduction in the steady-state level of the largest subunit of this enzyme (A190). Similarly, it is possible that Rpo26p is required for the stability of unassembled Rpo21p and A190p. If the stability of Rpo21p is dependent on its ability to assemble into the polymerase complex, why does it accumulate when the assembly of RNAPII is blocked by mutations in RPO21, RPO22 and RPO23? In the assembly experiments performed by Kolodziej and Young (Kolodziej and Young, 1991a) association of the smaller subunits with the three largest subunits was not analyzed. Therefore, it is possible that Rpo21p that
is not assembled with Rpo22p and Rpo23p remains associated with Rpo26p or a subcomplex of subunits containing Rpo26p and therefore is able to accumulate.

The postulated role of Rpo26p as an assembly factor is consistent with the fact that the appearance of common subunits coincides with the evolution of complex RNAPs in Archaebacteria as compared to the simple four-subunit RNAP in prokaryotes (Reviewed in Langer, et al., 1995). It is possible that the common and other small subunits evolved so as to acquire a stabilizing effect on the larger subunits. By stabilizing the larger subunits, the small subunits may also serve to drive the assembly of the multisubunit RNAP towards complex formation. The common subunits might also have acquired other functions. For example, Human Rpo25p has been shown to interact with the human hepatitis B virus X (HBx) transcriptional activator (Cheong, et al., 1995) and has been implicated in mediating the transactivation by HBx of RNAPI, RNAPII, and RNAPIII transcription (Cheong, et al., 1995). The \textit{RPO26} mutant alleles in my collection might include those that affect a possible secondary role of Rpo26p. Furthermore, my genetic screen may have specifically targeted those mutant alleles of \textit{RPO26} that impair the role of this subunit in the assembly of the RNAPs and the stability of their largest subunits. An alternative strategy for isolating mutant alleles of \textit{RPO26} might reveal other functional roles for this subunit in eukaryotic transcription.
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CHAPTER III

GENETIC EVIDENCE FOR SELECTIVE DEGRADATION OF RNA POLYMERASE SUBUNITS BY THE 20S PROTEASOME IN SACCHAROMYCES CEREVISIAE

The contents of this chapter have been submitted to Nucl. Acids. Res. for publication.

Sue Nelson and Marcus Li were students who, under my guidance, contributed to the initial genetic experiments and cloning of PUP3, respectively.
ABSTRACT

I describe the isolation of *sces32* as an extragenic suppressor of a temperature sensitive (ts) mutation (*rpo26-31*) in the gene encoding Rpo26p, a subunit common to yeast nuclear RNA polymerases (RNAP). *rpo26-31* also confers inositol auxotrophy, inhibits the assembly of RNAPI and RNAPII, and reduces the steady-state level of Rpo26p and the largest subunit of RNAPI (Rpo11p or A190p) and RNAPII (Rpo21p). *rpo26-31* accumulated to wild-type levels in the *sces32* strain, but the amount of assembled RNAPII remained notably reduced at high temperature. Hence, *sces32* only partially suppressed the ts phenotype and was unable to suppress the Ino⁻ phenotype of *rpo26-31*. *SCS32* is identical to *PUP3* which encodes a putative catalytic subunit of the yeast proteasome.

*sces32* was able to suppress the phenotype of other ts alleles of *RPO26*, all of which reduce the steady-state level of this subunit. However, it was unable to suppress the ts phenotype of mutant alleles of *RPO21*, or cause accumulation of the unstable rpo21-4p. These observations suggest either that different subsets of the cellular proteasomes are responsible for degradation of non-functional forms of Rpo21p and Rpo26p, or that non-functional forms of Rpo21p are recognized more efficiently than mutant Rpo26p by the cellular proteasome.
INTRODUCTION

As described in Chapters I and II, biochemical analyses of extracts from yeast cells containing mutant forms of the 45 kDa subunit of RNAPII (Rpo23p), the 40 kDa subunit common between RNAPI and RNAPIII (AC40p), the 23 kDa subunit (Rpo26p) common among three nuclear RNAPs, and the 12 kDa subunit of RNAPI (A12.2p) have shown that these subunits are required for assembly or stability of their respective RNAPs (Archambault and Friesen, 1993, Nogi, et al., 1993, Nouraini, et al., 1996, Chapter II).

The manner in which Rpo26p and A12.2p contribute to the assembly of their respective RNAPs is by stabilizing the largest subunits of these enzymes. Deletion of the gene encoding A12.2p confers a temperature sensitive (ts) growth defect on yeast and it leads to a reduction in the steady-state level of the largest subunit of RNAPI (Nogi, et al., 1993). As I described in Chapter II, a ts mutant allele of RPO26, rpo26-31, that inhibits the assembly of Rpo26p into RNAP complexes and results in decreased amount of this subunit at high temperature, also leads to reduced steady-state level of the largest subunit of RNAPI and RNAPII (Nouraini, et al., 1996).

The observation that the steady-state level of free subunits is reduced might reflect a cellular regulatory response which serves to prevent accumulation of non-functional subcomplexes of RNAP components. Such a regulatory mechanism has been reported for mammalian cells (Guilalis, et al., 1977, Guilalis, et al., 1979, Somers, et al., 1975). Heterozygous α-amanitin-resistant/α-amanitin-sensitive (AMAf/AMAs) cell lines express similar proportions of AMAf and AMAs RNAPII when grown in the absence of α-amanitin; however, when grown in the presence of this drug the inactivated AMAs RNAPII is preferentially degraded (Guilalis, et al., 1977, Guilalis, et al., 1979, Somers, et al., 1975).

In order to identify components of the regulatory mechanism that mediate removal of inactive RNAP subcomplexes in yeast, I isolated spontaneous suppressors of the ts rpo26-31 mutant allele. This chapter focuses on the characterization and cloning of one
such suppressor mutation which lies in PUP3, the gene that encodes a putative catalytic subunit of the yeast 20S proteasome. The 20S proteasome is the catalytic component of the eukaryotic 26S proteasome, which is the major proteolytic machinery of the cell (Goldberg, 1995). The suppressor mutation (pup3-l) partially suppresses the ts phenotype associated with rpo26-31 and enables the unstable rpo26-31p to accumulate at the non-permissive temperature. However, the pup3-l mutation does not suppress the ts phenotype of mutations in the largest subunit of RNAPII. Nor does it enable an unstable Rpo21p mutant subunit to accumulate at the non-permissive temperature. These results suggest that non-functional forms of Rpo26p and Rpo21p are degraded by different populations of proteasomal complexes or that they are recognized by the same proteasomal complex but at different rates.
MATERIALS AND METHODS

Yeast strains and growth media

Yeast strains used in this study are listed in Table 1. Strain SNY103 was used for isolation of spontaneous suppressors of the rpo26-31 ts phenotype. Strains SHY101 and SHY105 are identical to JAY476 (pGAL-RPO26) (Archambault, et al., 1990) and JAY444 (RPO26Δ::LEU2 [pRP026]) (Archambault, et al., 1990), respectively, except they have the opposite mating type. Strains SHY212 and SHY213 were isolated as follows: first, the scs32 suppressor strain was mated with SHY108 to create the diploid strain SHY211 (Table 1), which was sporulated and meiotic segregants were isolated by tetrad dissection. SHY212 and SHY213 are haploid progeny showing suppression of the rpo26-31 ts phenotype (pup3-1). SHY183 was constructed as follows: strain SHY212 was mated with W303-1A, the resultant diploid was sporulated and tetrads were dissected on YPD solid medium. Since the pup3-1 allele did not confer a discernible phenotype on yeast in the presence of RPO26, the ability of rpo26-31 to confer a growth defect at 35°C in the absence of pup3-1 was used to decipher the allele present at the PUP3 locus. Tetrads in which the ts phenotype of rpo26-31 was no longer suppressed were judged to have segregation of pup3-1 with RPO26; haploids with RPO26 pup3-1 combination were chosen and the identity of their PUP3 allele was confirmed by PCR amplification of this locus from genomic DNA, followed by sequence analysis. SHY183 was constructed by mating two of the haploids isolated in this way.

Cells were grown in rich medium or in defined medium supplemented with required amino acids as described (Sherman, et al., 1986). Minimal medium lacking inositol was prepared according to Culbertson and Henry (1975). For the purpose of metabolic labeling with 35S-methionine, cells were grown in Low Sulfate Medium (LSM) as described in Chapter II.
Table 1- List of strains used in this study

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<th>Strain</th>
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III- 6
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*All strains in this study are derived from *Saccharomyces cerevisiae* strain W303 (can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1) obtained from R. Rothstein.

†In this strain the expression of *RPO26* is driven by the inducible *GAL1* promoter. For a detailed description of the *RPO26* locus refer to Archambault et al., 1990.
**Plasmids**

Plasmids pSN261 and pSN266 were described in Chapter II. Plasmid pSN2 is a derivative of pUN80 (URA3 CEN4 ARS1) (Elledge and Davis, 1988), missing the sequences between XbaI/EcoRI in the polylinker. pSHB1 contains an approximately 7.0 kb fragment of chromosome V (see Fig. 2), which was isolated from a YCp50-based yeast genomic library (see below) based on the ability to complement the suppression of the rpo26-31 ts phenotype by *pup3-1*. pSHB2 contains an approximately 3.0 kb HindIII (position 32 in YCp50 upstream of insert)/EcoRI (in the insert) fragment isolated from pSHB1 and cloned into pRS316 (Sikorski and Hieter, 1989) (Fig. 2). pSHB3 contains an approximately 4.0 kb EcoRI (in the insert)/SalI (position 654 downstream of insert in YCp50) fragment isolated from pSHB1 and cloned into pRS316 (Fig. 2). pSHB4 was derived from pSHB3 by digestion of the latter with BamHI and re-ligation of the plasmid (Fig. 2). pSHB5 was constructed by cloning a 1.2 kb BamHI/EcoRI fragment from pSHB3 into pRS316 (Fig. 2). pSHB7 contains a 2.7 kb SpeI (28 bp upstream of *PUP3* translation initiation codon)/SalI fragment from pSHB3 cloned into pYGAL (a gift from Frank Jones). The same fragment has also been cloned into SmaI/SalI sites of pEMBLyex4 (*pGAL1 URA3 2µm*) to construct pSHB8 (Fig. 2). The expression of *PUP3* is driven by the repressible GAL10 and GAL1 promoters in pSHB7 and pSHB8, respectively. pYGAL contains the *PGK* transcription termination sequence on a *BglII/HindIII* fragment cloned into the *SphII/HindIII* sites of pJAY99 (constructed by J. Archambault). pJAY99 contains the *pGAL10* promoter on an *EcoRI/SmaI* fragment cloned into the *EcoRI/SmaI* sites of pFL39. To construct pSHB9, the 4.0 kb *EcoRI/SalI* insert of pSHB3 was cloned into a derivative of pRS316 in which the *SpeI* site in the polylinker has been destroyed by digestion and end-filling. pSHB11 was derived from pSHB3 by removing the polylinker sequences between *EcoRI* and *SstII*. Plasmid pSHB16 is identical to pSHB11, except it contains the *pup3-1* mutant allele. It was constructed as follows: *pup3-1* was rescued from the chromosome (see below) and a *BamHI/XbaI* fragment (see
Fig. 2) containing the *pup3-1* mutation was used to replace the analogous fragment in pSHB11. pJA452 (constructed by J. Archambault), pJA457 (Archambault, et al., 1992), and pYF1641 contain a 5.7 kb EcoRI/HindIII fragment containing *RPO21*, *rpo21-23* and *rpo21-4*, respectively, cloned into pFL39. pDJ40 (constructed by D. Jansma) contains a 7.0 kb HindIII fragment carrying *rpo21-1* on pFL39.

Plasmid pSHB15 was created for the purpose of constructing a *PUP3* chromosomal deletion, as follows: A 601 bp (fragment #1) and an approximately 2.2 kb (fragment #2) fragment were PCR amplified from pSHB1. Primers used to amplify fragment #1 were K/O1 (5'-AATAGAACCTGGATCCGAC-3'), which contains a *BamHI* site (underlined) and K/O2 (5'-CCCGAATTCC CGCTACACTC-3'), which contains an *EcoRI* site (underlined). K/O1 hybridizes to the coding strand of *PUP3* overlapping 21bp downstream of *PUP3* translation-initiation codon, and K/O2 hybridizes to the non-coding strand located at 580bp upstream of *PUP3* ORF (Bold in the sequence). Primers used to amplify fragment #2 were K/O3 (5'-CGCGGATCCTCGGTTCATGG-3'), which contains a *BamHI* site (underlined) and hybridizes to sequences in the non-coding strand located immediately downstream of the *PUP3* ORF (Bold), and the reverse primer that hybridizes to *LACZ* sequence in the plasmid. Fragment #1 was digested with *BamHI/EcoRI* and cloned into pBluescript to obtain plasmid pSHB12. Fragment #2 was digested with *BamHI* and *NotI* (397bp downstream of *PUP3* ORF), and this 397bp fragment was cloned into the *BamHI/NotI* sites of pSHB12 to obtain pSHB13. A 1.8kb *BamHI* fragment containing *HIS3* from pJJ215 (Jones and Prakash, 1990) was subcloned into the *BamHI* site of pSHB13, with *HIS3* inserted in the same orientation as *PUP3*, to obtain the plasmid pSHB15.
Isolation of suppressors of \textit{rpo26-31}

Forty independent colonies of strain SNY103 (\textit{rpo26Δ::LEU2} [pSN266]) were grown exponentially in liquid culture for two days at 23°C. An equivalent of 10^6 cells from each culture was spread on 40 Glucose (-Trp, -Leu) plates and incubated at 37°C for two days. Each plate contained an average of 9 colonies growing at the non-permissive temperature. To avoid true revertants, 40 colonies that grew at 37°C but at a considerably slower rate than wild-type (one representative from each plate) were chosen for further consideration.

Characterization of the suppressors

The following experiments were performed in order to determine whether the suppression phenotype was due to an extragenic mutation rather than a second-site mutation in \textit{rpo26-31}. First, the pSN266 plasmid carrying the \textit{rpo26-31} allele was purified from the suppressor strains and, after passage through \textit{E. coli}, was introduced into strain JAY444 (\textit{rpo26Δ::LEU2} [pRPO26]). Trp\textsuperscript{+} transformants were relieved of pRPO26 by plasmid shuffling, and the growth phenotype of cells was compared with that of the \textit{rpo26-31} strain (SNY103) grown at 35°C. Second, the plasmid carrying \textit{rpo26-31} was replaced with pRPO26 (\textit{RPO26 URA3 CEN ARS}) in the suppressor strains. An independent preparation of plasmid carrying \textit{rpo26-31} (pSN266) was used to replace wild-type \textit{RPO26} by plasmid shuffling (Sikorski and Boeke, 1991), and the growth phenotype of cells was tested at 35°C.

In order to determine whether the suppressor mutations were dominant or recessive, the suppressor strains were mated with strain SHY101 in which the expression of \textit{RPO26} is under the control of the repressible \textit{GALI} promoter. The ability of the resultant diploid strains to grow at 35°C was tested in the presence of glucose (expression of chromosomal \textit{RPO26} is repressed).
Strain SHY213 was mated to the 20 recessive suppressor strains, as well as to SNY103 as control. The growth phenotype of the diploid strains was tested at 35°C in order to test if any of the recessive suppressors are allelic to scs32.

Cloning of SCS32

Since the scs32 suppressor strain did not exhibit a scorable phenotype in the presence of RPO26, the SCS32 gene was cloned by complementation of suppression of the rpo26-31 ts phenotype by scs32. Prior to cloning of SCS32, experiments were performed to ascertain that the suppression by scs32 was due to mutation of a single gene. Strain SHY212 (scs32) was mated with SHY109 (SCS32), the diploid was sporulated and used for tetrad dissection. A total of 13 tetrads was dissected, all of which showed a 2:2 segregation of the suppression phenotype, indicating that the suppressor mutation resides in a single gene. Strain SHY212 was transformed with a plasmid library (Rose, et al., 1987) containing 10-15kb Sau3AI partially digested fragments of yeast genomic DNA cloned into the BamHI site of YCp50 (URA3 CEN4 ARS1). A total of 10,087 Ura+ transformants were patched on glucose (¬Trp -Ura -Leu) solid medium at 35°C and 30°C. Through this primary screen, 23 colonies were identified that no longer were able to grow at 35°C. In a secondary screen, the 23 putative positive transformants were relieved of the plasmid library using a plasmid shuffling assay (Sikorski and Boeke, 1991), and the ability of 5-FOA (5-FluoroOrotic Acid) resistant cells to grow at 35°C was determined. Only three of the 23 putative positive colonies were able to grow at 35°C (suppress the ts phenotype of rpo26-31) in the absence of the library plasmid. Plasmid DNA was isolated from these three, passed through E. coli, re-introduced into yeast strain SHY212, and the ability of Ura+ transformants to grow at 35°C was determined. Only one plasmid (pSHB1) was able to complement the suppression phenotype of scs32 following a second transformation.
Rescue of the \textit{pup3-1} mutation from the chromosome

Plasmid pSHB9 was digested with \textit{SpeI} (581 bp upstream of the \textit{PUP3} ORF) and \textit{BgII} (132 bp downstream of the \textit{PUP3} ORF). The plasmid was gel-purified and was introduced into strain SHY212 (\textit{rpo26-31 pup3-1}). Plasmid DNA was prepared from \textit{Ura+} transformants that were able to grow at 35°C (i.e., did not show complementation of suppression by \textit{pup3-1}). Following passage through \textit{E. coli}, plasmids were used for sequencing of the \textit{PUP3} ORF.

Chromosomal deletion of \textit{PUP3}

The entire insert of plasmid pSHB15 was released on a \textit{SalI/SstI} 2.9kb fragment and was introduced into the diploid yeast strain LP112 (W303-1A/B). \textit{His+} transformants were sporulated and used for tetrad dissection.

Test of allele specificity of suppression by \textit{scs32}

Plasmids pSN271, 273, 278, and 287 containing \textit{rpo26-32}, -30, -33, and -34 ts alleles, respectively, were used to replace pRPO26 (\textit{RPO26 URA3}) in strains SHY216 and JAY444 (as control), by plasmid shuffling. The ability of \textit{scs32} to suppress the ts growth defect of these mutant alleles of \textit{RPO26} was analyzed at 37°C.

Construction of an \textit{RPO21} deletion in \textit{scs32} and \textit{SCS32} backgrounds

Plasmid pRP196 (a gift from R. Young) contains a deletion allele of \textit{RPO21} in which a \textit{BgII} fragment containing a portion of the \textit{RPO21} open reading frame (ORF) was replaced with \textit{HIS3} (Nonet, et al., 1987). pRP196 was digested with \textit{EcoRI} (to release the insert) and was used to transform diploid yeast strains SHY183 (\textit{pup3-1/pup3-1}) and LP112 (\textit{PUP3/PUP3}). \textit{His+} transformants were sporulated and were used for tetrad dissection. At least 10 tetraads were dissected for each strain, all of which showed co-
segregation of the His\(^+\) phenotype with lethality. Plasmid pJAY101 (*RPO21 URA3 CEN ARS*) (Archambault, et al., 1992) was introduced into the His\(^+\) diploids described above, and Ura\(^+\) His\(^+\) transformants were used for tetrad dissection. pJAY101 was able to rescue the lethality of His\(^-\) haploid progeny and allowed growth of complete tetrads, confirming that the lethality of His\(^+\) transformants was due to deletion of *RPO21* sequences in the chromosome. His\(^+\) Ura\(^+\) haploids isolated from the above mentioned tetrads (strains SHY204 and SHY205) were used to test the ability of *scs32* to suppress the ts mutations in *RPO21*.

**Western-blot analysis**

The steady-state level of Rpo26p (in strains SNY102, SNY103, SHY212 and SHY158) and Rpo21p (in strains SHY206 through SHY209) was determined essentially as described (Nouraini, et al., 1996). Briefly, cells were grown exponentially at 23\(^{\circ}\) C and shifted to 35\(^{\circ}\) C or 37\(^{\circ}\) C for 6h. An equivalent of 1.2\(^{-}\)10\(^8\) cells was harvested, cell extracts were prepared and an equivalent amount of protein from each strain was used for protein-blot analysis. The amount of Rpo26p and Rpo21p was monitored using polyclonal antisera \(\alpha\)-Rpo26 (Nouraini, et al., 1996) and \(\alpha\)-B185 (a gift from Michelle Riva and Cristopher Carles), respectively.

**DNA manipulations**

All DNA manipulations were performed essentially as described by Maniatis et al. (1982). Nucleotide sequence determination was performed by the dideoxy chain-termination method (Sanger, et al., 1977)

**Immunoprecipitation of RNAPII from \(^{35}\)S-Methionine-labeled cell extracts**

Strains SNY102 (*HA-RPO26 PUP3*) and SHY212 (*HA-rpo26-31 pup3-1*) were metabolically labeled with \(^{35}\)S-methionine at 37\(^{\circ}\)C, and RNAPII complexes were
immunoprecipitated from crude extracts essentially as described (Nouraini, et al., 1996). The monoclonal antibody 8WG16 (Thompson, et al., 1990), which recognizes the C-terminal domain (CTD) unique to the largest subunit of RNAPII (Rpo21p), was used for the immunoprecipitation experiments. Immunoprecipitated complexes were separated on a SDS-PAGE gel (12% acrylamide) along with a purified preparation of RNAPII. The gel was stained for protein with a colloidal Comassie blue staining kit (Novex), and processed for autoradiography as described previously (Nouraini, et al., 1996).
RESULTS

Suppressors of a ts mutation in RPO26

I isolated spontaneous suppressors of a ts allele of RPO26 (rpo26-31), the gene encoding a subunit common to yeast RNAPs. Cells containing the rpo26-31 allele are auxotrophic for inositol, grow slowly at 30°C and are unable to grow at or above 35°C (Nouraini, et al., 1996). Suppressors were obtained by spreading forty independent cultures of a rpo26-31 strain at 37°C and isolating one colony from each plate. On average, 9 in every 10^6 colonies were able to grow at 37°C for each plate. The suppressor strains were called scs1 through scs40 (for suppressor of mutation in common subunit). Except for scs5, all of the suppressors supported similar growth rates at the non-permissive temperature; the growth rate of cells in the presence of scs5 was considerably lower under these conditions. All suppressors provided better suppression of the ts phenotype at 35°C than at 37°C; hence, the growth rate of cells was monitored at 35°C for the remainder of this study.

Characterization of suppressor strains

Two observations indicated that the suppressor strains contain extragenic mutations, rather than second-site mutations in rpo26-31. First, rpo26-31-containing plasmids purified from the suppressor strains continued to confer a ts phenotype, indistinguishable from rpo26-31, when they were introduced into an isogenic strain that lacked a suppressor mutation. Second, when the plasmid carrying rpo26-31 in the suppressor strains was replaced with an independent preparation of the same plasmid (see Materials and Methods), the growth rate of these newly transformed strains was identical to the original suppressor strains.

The growth phenotypes of suppressor strains in the presence of wild-type RPO26 were tested at various temperatures (15°C, 23°C, 30°C, and 37°C) in order to determine whether the suppressor mutations generated a secondary phenotype. The growth rate of
RP026 cells in the presence of the suppressor mutations was indistinguishable from wild-type under these conditions (not shown). Since the suppressor mutations themselves did not confer a discernible growth-defect, suppression of the ts phenotype of rpo26-31 was used for further characterization of the suppressor strains.

Suppression of the rpo26-31 ts phenotype was tested in diploid strains heterozygous for the suppressor mutations in order to identify recessive suppressors (see Experimental Procedures). Of the 40 diploids tested, 20 failed to grow at 35°C (failed to suppress the rpo26-31 ts phenotype), thus identifying these as recessive suppressors. The remainder of the diploid strains showed an intermediate growth phenotype at 35°C, suggesting that the suppressors in this group are due to semi-dominant mutations. The recessive suppressors were used for further study, since they provided a more easily scored growth phenotype.

rpo26-31 mutants require inositol for growth (Chapter II; Nouraini, et al., 1996), a phenotype which often is associated with mutations in genes that encode components of RNAPII and which stems from poor induction of the INO1 gene in the absence of inositol (Archambault, et al., 1992, Archambault, et al., 1996, Archambault, et al., 1990, Arndt, et al., 1995, Arndt, et al., 1989, Scafe, et al., 1990a, Scafe, et al., 1990b). Of the 20 recessive suppressors, only scs32 and scs5 were unable to support growth of the rpo26-31 strain in the absence of inositol (Fig. 1A). When tested in the presence of wild-type RPO26, neither scs32 nor scs5 conferred an Ino− phenotype on yeast (not shown). The
Fig. 1. Suppression of the growth phenotype and the reduction in the amount of Rpo26p imposed by *rpo26-31*. A) *scs32* suppresses the ts phenotype of *rpo26-31* but not the Ino\(^{-}\) phenotype imposed by this mutant allele. Similar numbers of cells were spotted on solid growth media in the absence (top panel) or the presence (bottom panel) of inositol, and incubated at the indicated temperatures. B) The steady-state amount of rpo26-31p is returned to wild-type levels in the presence of *scs32*. Strains SNY102 (*RPO26 SCS32*), SNY103 (*rpo26-31 SCS32*), SHY212 (*rpo26-31 scs32*) and SHY158 (*RPO26 scs32*), were grown exponentially at 23\( ^\circ \)C, shifted to 35 and 37\( ^\circ \)C for 6 h and cell extracts were prepared. Indicated amounts of protein from each extract were used for protein-blot analysis using a polyclonal antibody to Rpo26p.
failure of scs32 and scs5 to suppress the inositol auxotrophy, which is an RNAPII-specific defect, suggested the intriguing possibility that they might contain compensatory mutations in cellular components that specifically rescue the assembly defect of RNAPI (and perhaps RNAPIII). Under these circumstances, the functional defect imposed on RNAPII by rpo26-31 would not be corrected and the cells would remain auxotrophic for inositol. In order to explore this possibility, scs32 and scs5 were further characterized.

In order to identify suppressor mutations that are allelic to scs32, the mutant strain was crossed to the panel of 20 recessive suppressor strains, as well as to the original rpo26-31 strain as control, and the growth phenotype of the diploids at 35°C (suppression of rpo26-31 ts phenotype) was tested. The scs32 suppressor was chosen for this test because it provided better suppression than scs5 of the ts phenotype conferred by rpo26-31. None of the diploid strains (except for homozygous scs32) was able to grow at 35°C, indicating that the scs32 mutation is not allelic to any of the recessive suppressors in our collection.

**Cloning of SCS32**

The SCS32 gene was cloned by complementation of the suppression of the rpo26-31 ts phenotype by scs32 (see Experiment Procedures). A library of yeast genomic DNA was used to transform the scs32 suppressor strain. Among 10,087 transformants one, containing a 7.0kb insert in the plasmid pSHB1(Fig. 2), prevented suppression of the rpo26-31 ts phenotype by scs32 (prevented growth at 35°C). Restriction digestion and subcloning experiments were used to locate the complementing region on a 4.0kb fragment (Fig. 2). Sequencing analysis of the ends of the insert followed by a search in the DNA database showed that it contains a portion of the RAD51 ORF and upstream sequences located on chromosome V. Analysis of the upstream sequence identified two divergently transcribed and overlapping open reading frames (Fig. 2; ORF1 and ORF2). Further subcloning of the insert showed that at least one of these ORFs is required for
Fig. 2. Molecular cloning of PUP3 (SCS32) and identification of the pup3-l (scs32) mutation. PUP3 was cloned by complementation of suppression of rpo26-31. A YCp50-based genomic library, made from Sau3A-digested yeast DNA, was introduced into strain SHY212 (rpo26-31 scs32) and transformants were tested for growth at 35°C. Plasmid pSHB1, which was able to inhibit cell-growth at 35°C (complemented the suppression by scs32), contained a 7.0 kb insert from chromosome V. To identify the complementing region of the insert, pSHB1 was digested with various restriction enzymes as indicated. The fragments shown were subcloned into pRS316 and tested for complementation of the suppression phenotype. The solid line represents the yeast genomic insert and the wavy lines indicate YCp50 sequences. Plasmid pSHB16 contains the pup3-l (scs32) suppressor allele of PUP3 (SCS32) which was rescued from strain SHY212 as outlined in Materials and Methods. In plasmid pSHB8 expression of PUP3 is under control of the inducible GAL1 promoter. This plasmid was used to show that expression of PUP3 (in the presence of galactose) is necessary and sufficient for complementation of suppression of the rpo26-31 ts phenotype by scs32.
complementation of the suppression phenotype (Fig. 2). A search of the database for homologous protein sequences identified the ORF transcribed divergently from RAD51 (ORF2) as PUP3 (putative proteasomal subunit 3), which has been identified previously (Heinemeyer, et al., 1994) based on homology with the rat (Nishimura, et al., 1993) and bovine (Dick, et al., 1992) proteasomal subunits RC10-IIp and 0, respectively. ORF1 did not show significant homology to any other proteins in the database.

Several lines of evidence indicate that SCS32 is PUP3. First, when expression of PUP3 was placed under control of the GAL1 promoter (plasmid pSHB8) and introduced into the scs32 strain (SHY212), the transformants were able to grow at 35°C only when the expression of PUP3 was repressed (in the presence of glucose). This indicated that the expression of PUP3, but not ORF1, was necessary and sufficient to complement the suppression of rpo26-31 by scs32 (Fig. 2). Second, rescue of chromosomal PUP3 from the scs32 strain followed by sequence analysis identified a mutation (C25F) in the PUP3 ORF. When tested in the scs32 strain, the mutant form of PUP3 (pup3-1) was not able to complement suppression of the rpo26-31 ts phenotype by scs32. Third, it has been shown previously that the steady-state level of rpo26-31p is significantly reduced at 37°C (Nouraini, et al., 1996). Analysis of the steady-state level of rpo26-31p showed that this subunit accumulates to wild-type levels in the presence of scs32 both at 35°C and 37°C (Fig. 1B), consistent with the observation that the scs32 strain has a mutation in a putative catalytic subunit of the cellular proteasome that targets rpo26-31p.

**Analysis of the assembly of RNAPII**

As mentioned above, rpo26-31p accumulated to wild-type levels at 37°C in the presence of pup3-1, yet the ts growth defect was only partially suppressed and the cells remained auxotrophic for inositol (Fig. 1). In Chapter II, I showed that the amount of assembled RNAPII is notably reduced at 37°C in the rpo26-31 mutant strain (Nouraini, et al., 1996). Furthermore, although over-expression of this mutant allele from a high-copy
plasmid partially suppresses the Ino- and ts phenotypes, it cannot completely rescue the RNAPII assembly defect (Nouraini, et al., 1996). I monitored the amount of assembled RNAPII in the scs32 strain at 37°C in order to investigate whether partial suppression of the ts phenotype and the lack of suppression of the inositol auxotrophy was due to the inability of this enzyme to assemble to normal levels. Yeast strains expressing rpo26-3l in the presence of scs32 (Fig 3, lane 2), and an isogenic wild-type strain (RPO26 SCS32) (Fig. 3, lane 1) were metabolically labeled with 35S-methionine at 37°C; RNAPII complexes were immunoprecipitated from crude extracts, using a monoclonal antibody to the Rpo21 C terminal domain (CTD) (Thompson, et al., 1990), and were separated on an SDS-PAGE gel. As shown in figure 3, the amount of newly assembled RNAPII was notably reduced in the presence of rpo26-3l (lane 2) compared to wild-type (compare intensity of bands corresponding to Rpo21p, Rpo22p and Rpo25p in lane 1 to those in lane 2). This assembly defect was not rescued in the presence of scs32 (Fig. 3, lane 2), although rpo26-31p accumulated to wild-type levels under these conditions (Fig. 1B). Therefore, the failure of RNAPII to assemble to normal levels in the presence of scs32 provides an explanation for partial suppression of the ts phenotype and lack of suppression of the Ino- phenotype associated with rpo26-3l.

Chromosomal deletion of PUP3

In order to determine whether the product of PUP3 is essential for growth, chromosomal PUP3 (along with the overlapping ORF, see above) was replaced with HIS3 (Fig. 4A) in the diploid strain LP112 (see Experimental Procedures). His+ diploids were sporulated and used for tetrad dissection. All diploids analyzed (in total 20) showed only two viable spores (Fig. 4B), all of which were His-. The lethality of His+ spores could be rescued by plasmid pSHB3 (Fig. 4B), which contains the 4.0kb EcoRI/SalI complementing fragment (Fig. 2), suggesting that PUP3, the overlapping ORF, or both are required for viability. In order to determine convincingly which ORF was required, His+
Fig. 3. Assembly of RNAPII in the presence and absence of pup3-1 (scs32). Strains SNY102 (RPO26 PUP3) and SHY212 (rpo26-31 pup3-1) were metabolically labeled with [35S]methionine at 37 °C for 2 h, and RNAPII complexes were immunoprecipitated from crude extracts using a monoclonal antibody (8WG16) to the C-terminal domain of Rpo21p. The immunoprecipitated complexes were subjected to electrophoresis in a 10% polyacrylamide-SDS gel. The positions of migration of the RNAPII subunits are shown on the left.
Fig. 4. Construction of a *PUP3* deletion strain. A) Schematic representation of the *PUP3* deletion allele indicating the region of the ORF that was replaced by *HIS3*. B) phenotype of *PUP3* deletion. A yeast strain (SHY188) heterozygous for the *PUP3* deletion allele was sporulated (left column); this gave rise to only two viable spores from each tetrad, both of which were His⁺ (wild-type *PUP3*). The lethal phenotype of the *PUP3* deletion strain was rescued by plasmids pSHB11 (*PUP3*; middle) and pSHB16 (*pup3-1*; right) as described in Material and Methods. C) Expression of *PUP3* is necessary and sufficient for the viability of *PUP3* deletion strains. Plasmid pSHB7 (*pGAL10-PUP3*) was introduced into strain SHY194 (*pup3Δ::HIS3 [pSHB11]*) and the ability of cells to lose pSHB11 (become resistant to 5-FOA) was tested when the expression of *PUP3* was repressed in the presence of glucose or induced in the presence of galactose. Shown is the result of this experiment with four independent His⁺ spores from the sporulation shown in B (middle column).
A) Schematic representation of gene locations and enzymes:

- Sau3A
- PUP3
- BamH1
- EcoR1

B) Images of gene expression:

- pup3Δ2 ::His3
- pup3Δ2 ::His3 PUP3
- pup3Δ2 ::His3 pup3-1

C) Images of media conditions:

- Galactose+5-FOA
- Glucose+5-FOA
haploids containing pSHB3 were transformed with plasmid pSHB7, which contains PUP3 expressed conditionally from the GAL10 promoter. The ability of these cells to lose pSHB3 was tested by plasmid shuffling (ability to grow on 5-FOA), in the presence (on galactose) or absence (on glucose) of PUP3 expression. As shown in Fig. 4C, cells were able to lose pSHB3 only when PUP3 was expressed (in the presence of galactose), indicating that PUP3, and not the overlapping ORF, is essential for viability.

A plasmid carrying pup3-l was able to support growth of the PUP3 deletion strain (Fig. 4B). This result indicated that the pup3-l mutation did not completely abolish activity of the Pup3 proteasomal subunit and is consistent with the observation that scs32 does not confer a growth defect in the presence of RPO26.

**Specificity of suppression by scs32**

The ability of scs32 to suppress the ts phenotype of other ts mutations in RPO26 was tested in order to determine if the suppression was allele-specific. scs32 was able to partially suppress the ts phenotypes of rpo26-30, -32, -33, and -34 at 37°C (Fig. 5A); this is consistent with the fact that these mutant alleles also reduce the steady-state level of Rpo26p (Nouraini, et al., 1996). Next, I tested the ability of scs32 to suppress ts mutations in another polymerase subunit. The gene encoding the largest subunit of RNAPII was replaced with HIS3 (see Experimental Procedures) in two isogenic strains of either SCS32 or scs32 genotype. The growth of these strains was supported with plasmid-encoded RPO21, which then was replaced with plasmids carrying various ts alleles of RPO21 (rpo21-1, rpo21-4, -23) by plasmid shuffling (Sikorski and Boeke, 1991). These RPO21 ts alleles were chosen for two reasons; first, they show different degrees of growth defect at the non-permissive temperature, with rpo21-4 having the most and rpo21-23 the least severe growth defect (Fig. 5B). Second, rpo21-1 and rpo21-4 are known to be defective in the assembly/stability of RNAPII since the growth defect of rpo21-1 can be suppressed by overexpression of the mutant subunit (Jansma and Friesen, unpublished),
and the ts phenotype of \textit{rpo21-4} is associated with a reduced steady-state levels of Rpo21p (Fig. 5C) and can be suppressed by an increase in the gene dosage of \textit{RPO26} (Archambault, et al., 1990). \textit{rpo21-23} is defective in elongation of transcription due to its reduced binding-affinity for the transcription elongation factor SII (Archambault, et al., 1992, Wu, et al., 1996). \textit{scs32} was unable to suppress the ts phenotype of any of these mutant alleles at all temperatures tested (23°C, 30°C, 33°C and 37°C) (Fig. 5B). Cells harboring \textit{rpo21-4} are auxotrophic for inositol, have a slow growth phenotype at 30°C and are unable to grow at or above 35°C (Archambault, et al., 1992), a growth characteristic similar to cells containing \textit{rpo26-31}. Western-blot analysis showed that, similar to \textit{rpo26-31p}, the steady-state level of \textit{rpo21-4p} was reduced at 37°C (Fig. 5C, compare lanes 6 and 7 to lanes 8 and 9), but unlike \textit{rpo26-31p}, \textit{rpo21-4p} did not accumulate to wild-type levels in the presence of \textit{scs32} (Fig. 5C, compare lanes 4 and 5 to lanes 6 and 7). Thus, suppression by \textit{scs32} does not extend to ts mutations in at least one other polymerase subunit.
Fig. 5. Specificity of suppression by scs32. The growth phenotype conferred by various ts mutant alleles of \textit{RPO26} (A) or \textit{RPO21} (B) in the absence or presence of the \textit{pup3-1} (scs32) mutation. A suspension of cells in the form of a drop was applied on solid medium and incubated at the indicated temperatures. For each strain, 10 $\mu$l of a cell suspension at 2$\times$10$^5$ or 2$\times$10$^4$ cells/ml was used for the drop tests. C) Western-blot analysis of the steady-state level of rpo21-4p in the presence and absence of \textit{pup3-1}. Strains SHY206 (\textit{RPO21 PUP3}), SHY207 (\textit{rpo21-4 PUP3}), SHY208 (\textit{rpo21-4 pup3-1}); and SHY209 (\textit{RPO21 pup3-1}) were grown exponentially at 23°C and shifted to 37°C for 6 h. Cell extracts were prepared and the indicated amounts of protein were monitored for levels of Rpo21p using the polyclonal antibody $\alpha$-B185 which recognizes this subunit. The band below Rpo21p represents cross-reactivity of $\alpha$-B185 with an unknown protein which was used as an internal loading control.
DISCUSSION

In Chapter II, I showed that the ts rpo26-31 mutation reduces the steady-state level of Rpo26p at high temperature (Nouraini, et al., 1996). In this Chapter, I show that this reduction is due to degradation of Rpo26p since an extragenic suppressor (scs32) of the rpo26-31 ts phenotype that allows accumulation of this subunit to normal levels contains a mutation in one of the catalytic subunits (Pup3p) of the yeast 20S proteasome. The 20S proteasome is the catalytic core of the 26S proteasome, which is the major proteolytic machine of eukaryotes present both in the nucleus and cytoplasm (Goldberg, 1995). This complex enzyme is involved in a variety of cellular events (Reviewed in Hilt and Wolf, 1996) and is responsible for degradation of proteins that are misfolded due to heat stress or incorporation of the arginine analog, canavanine (Heinemeyer, et al., 1993, Hilt, et al., 1993, Hilt and Wolf, 1992). The archaebacterial 20S proteasome has been well characterized; it is made up of the products of only two genes which encode the regulatory α and the catalytic β subunits (Lowe, et al., 1995). The 20S complex is made up of a stack of four rings each containing seven subunits (Hilt and Wolf, 1996); β subunits form the two inner rings whereas α subunits form the two outer rings of the proteasome complex (Lowe, et al., 1995).

Yeast 20S proteasomal complex purified from vegetative cells has 14 distinct subunits (Chen and Hochstrasser, 1995) which, like other eukaryotic subunits, have been classified into either α-type or β-type subunits based on sequence similarity with the archaebacterial homologues (Fig. 6) (Hilt and Wolf, 1996, Peters, 1994). Genes encoding all 14 subunits have been identified and cloned; all but one of these subunits are essential for growth (Chen and Hochstrasser, 1995, Chen, et al., 1993, Emori, et al., 1991, Heinemeyer, et al., 1993, Heinemeyer, et al., 1991, Heinemeyer, et al., 1994, Hilt, et al., 1993, Lee, et al., 1992). Pup3p is a β-type subunit which has been implicated in the trypsin-like activity of the yeast proteasome since the bovine homologue of this subunit

As reported in this chapter, cells containing a deletion of PUP3 are not viable. A similar deletion of the PUP3 gene has been made previously, which led to loss of cell viability (Basile, et al., 1992). However, it was not determined whether the lethal phenotype was due to the absence of PUP3 function, the lack of ORF overlapping this gene, or both. I have answered this question by showing that the ability of a PUP3/ORF1 deletion strain to grow depends only on the expression of PUP3.

The sequences flanking the mutation in pup3-1 (C26F) are highly conserved among all available proteasomal subunits (See Fig. 6). In the archaeabacterial subunits these sequences form one strand (strand S2, underlined in Fig. 6) of a β-pleated sheet that makes up part of the substrate binding-pocket and structures surrounding the active site in the β subunit (Lowe, et al., 1995). The cysteiny1 residue which is mutated in pup3-1p is replaced by threonine in the archaeabacterial subunit (Fig. 6); the X-ray crystal structure of the archaeabacterial subunit does not suggest a role for the side chain of this residue in the catalytic activity of the enzyme. This observation, in combination with the fact that C26 is not an evolutionarily conserved amino acid (Boxed residues in Fig. 6), argues against a catalytic role for this residue in the yeast proteasome. Furthermore, this residue is never substituted with an amino acid containing a bulky side chain (Fig. 6), suggesting that the presence of phenylalanine at the position of C26 is not favorable and might generate a minor structural change, which, as a consequence, may lead to a reduced amount of the trypsin-like activity. The degree to which the activity of the proteasome is reduced in vitro due to mutations in other catalytic subunits of the yeast proteasome generally shows a positive correlation with the growth phenotype that the proteasomal mutations impose on the cell (Chen and Hochstrasser, 1995, Heinemeyer, et al., 1993, Heinemeyer, et al., 1991, Hiitt, et al., 1993). Generally, mutations that only marginally reduce the activity of the proteasome do not confer a detectable cell-growth or sporulation phenotype; however,
Fig. 6. Partial sequence alignment of α- and β-type proteasomal subunits. The sequence alignment was performed using the PileUp programme. The amino acid mutated in pup3-1 (scs32) and the equivalent residues in other proteasomal subunits are enclosed in a rectangle. S1 through S4 show regions of the α and β subunits from Thermoplasma acidophilum that have been shown by X-ray crystallography to form four strands of a β-pleated sheet surrounding the substrate-binding and active sites of the catalytic β subunit. The following are the abbreviations used: Sc, Saccharomyces cerevisiae; Rn, Rattus norvegicus; Hs, Homo sapiens; Dm, Drosophila melanogaster; At, Arabidopsis thaliana; Dd, Dictyostelium discoideum; Xl, Xenopus laevis; Bt, Bos taurus; Ta, Thermoplasma acidophilum.
those that severely reduce the activity of this enzyme \textit{in vitro} do lead to defective sporulation and a cell-growth defect at 38°C. Given that the \textit{pup3-1} strain has no detectable growth and sporulation defects, the 20S proteasome containing \textit{pup3-1p} may be only marginally inactivated for the trypsin-like activity of this enzyme.

A secondary phenotype associated with the \textit{ts rpo26-31} allele is reduction in the steady-state level of the largest subunit of RNAPII (Rpo21p) (Fig. 3, Chapter II and Nouraini, et al., 1996) and of RNAPI (A190p) (Chapter II and Nouraini, et al., 1996). Restoration of the steady-state level of \textit{rpo26-31p} to wild-type by mutating \textit{PUP3} or overexpression of \textit{rpo26-31p} does not lead to accumulation of Rpo21p (Fig. 3) or A190p to wild-type levels (Chapter II and Nouraini, et al., 1996). This observation suggests that the mechanism by which the steady-state level of Rpo26p is maintained is probably independent from that responsible for maintaining the levels of Rpo21p and A190p. This conjecture is supported by the observation that \textit{scs32} fails to suppress the \textit{ts} phenotype of \textit{rpo21} mutant alleles or to allow \textit{rpo21-4p} to accumulate in a \textit{RPO26} wild-type background. The apparent subunit-specificity of \textit{scs32} action can be interpreted in at least two ways. First, it is possible that the steady-state level of Rpo26p is regulated by a proteolytic complex distinct from that responsible for the degradation of Rpo21p (and perhaps A190p). The distinct proteolytic complexes might represent different functional forms of the 26S proteasomal complex. I do not favor this possibility for two reasons: first, work on the structure and function of the 20S proteasome argue against the presence of heterogeneous populations of this complex in yeast, unlike in higher eukaryotes where the presence of heterogeneous 20S complexes is well established (Coux et al., 1996, Hochstrasser, 1996b). Second, assignment of different proteasome complexes for independent regulation of the steady-state level of different proteins seems like an inefficient and wasteful strategy. I prefer an alternative interpretation of the specificity of suppression by \textit{scs32} which suggests that the same proteolytic complex might be responsible for the degradation of all RNAP subunits. The apparent specificity of \textit{scs32} to
Rpo26p might simply be due to the ability of unassembled or mutant Rpo21p to be recognized more efficiently by the proteasome than mutant or unassembled Rpo26p. Under these circumstances, a marginal decrease in the activity of the proteasome would be sufficient for Rpo26p but not for Rpo21p (or for A190p) to accumulate to wild-type levels.

One mechanism by which Rpo21p could be recognized more efficiently by the proteasome is by rapid ubiquitin modification. Except for isolated examples where ubiquitination seems to serve a signaling role (Chen, et al., 1996, Hochstrasser, 1996a, Wang, et al., 1996), ubiquitin modification of cellular proteins marks them for rapid degradation by the 26S proteasome (Hilt and Wolf, 1996). Indeed, it is known that the largest subunit of RNAPII (Rpo21p) is ubiquitinated in both yeast and mammalian cells (Bregman, et al., 1996 and Huibregtse, pers. communication). Rpo21p is ubiquitinated in yeast by the ubiquitin ligase Rsp5p whose interaction with Rpo21p is mediated by the CTD (Huibregtse, pers. communication). Knowing that CTD is a domain unique to Rpo21p suggests that Rsp5p specifically targets Rpo21p (among the subunits of RNAPII) that is free of association with the other polymerase subunits, thus mediating selective removal of the unwanted Rpo21p. It is equally possible that there are A190p or Rpo26p-specific ubiquitin ligases whose identification awaits further investigation.
REFERENCES


CHAPTER IV

MUTATIONS IN AN ABF1P BINDING SITE IN THE PROMOTER OF YEAST RPO26 SHIFT THE TRANSCRIPTION START SITES AND REDUCE THE LEVEL OF RPO26 mRNA

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ABSTRACT

A binding site for the transcription factor Abf1p was identified as an important promoter element of the gene that encodes Rpo26, a subunit common to all three yeast nuclear RNA polymerases (RNAP). Mutations in the Abf1p binding site were identified among a pool of rpo26 mutant alleles that were identified through a genetic screen. The genetic screen was based on the ability of rpo26 mutant alleles to confer synthetic lethality in combination with a temperature-sensitive mutation (rpo21-4) in the gene that encodes the largest subunit of RNAPII (Rpo21p). In the presence of the wild-type allele of RPO21, the rpo26 promoter mutations caused a cold-sensitive growth defect. Purified Abf1p bound to the wild-type RPO26 promoter in an electrophoretic mobility-shift assay but did not bind the mutated promoters. Mutations in the Abf1p binding-site reduced the expression of RPO26 by approximately 60% and shifted the RPO26 transcriptional start sites to positions further upstream than normal. These results suggest that binding of the Abf1p transcription factor to the RPO26 promoter is important in establishing the level of transcription for this gene, and in positioning the sites of transcription initiation.
INTRODUCTION

Regulation of transcription is an important aspect of the cellular responses to environmental cues. Transcriptional regulation can occur at various stages: initiation (Drapkin, et al., 1993), elongation (Kerpola and Kane, 1991), and/or termination (Richardson, 1993), or conceivably by controlling the intracellular amount of RNA polymerases (RNAPs). Since the common subunits are present in all three polymerases, regulation of the steady-state levels of these subunits could be an effective means for coordinate regulation of the amount of cellular RNAPs and, consequently, the level of cellular transcription. One way in which the level of common subunits can be regulated is through transcriptional regulatory sequences in their promoters. Identification of such regulatory sequences will be a valuable step in understanding mechanism(s) of transcriptional responses to environmental cues. In this chapter, I describe the identification of a promoter element, the binding site for the transcription factor Abf1p, which is important for normal transcription of RPO26. I provide evidence which suggests that Abf1p not only stimulates transcription of RPO26, but also sets the sites of transcription initiation on the RPO26 promoter.
MATERIALS AND METHODS

Plasmids and Strains

pRP026HAT (TRP1, CEN/ARS) contains a 2.1kb Sau3A RPO26 restriction fragment (Nouraini, et al., 1996) subcloned into the plasmid pFL39 (Bonneaud, et al., 1991). pRPO26cDNA (Archambault, et al., 1990) was isolated from a cDNA library described by McKnight and McConaughy (1983). Plasmid pSN261 contains a HindIII(-748)/HindIII(+754) RPO26 genomic fragment in pFL39 (see Fig. 1 for position of restriction sites numbered by choosing A of the RPO26 ATG as +1). Plasmids pSN302, pSN303 and pSN304 contain an AccI (-534)/HindIII(+754) RPO26 fragment in pFL39; they bear, respectively, the wild-type, double-mutant (rpo26-24), and single-mutant (rpo26-27) forms of the Abf1p-binding site in the promoter. Construction of pSN303 and pSN304 was as follows: sequences corresponding to -597 to +97 were PCR-amplified from hydroxylamine mutagenized plasmids corresponding to rpo26-24 and rpo26-27, respectively. These fragments were then digested with AccI(-534) and BclI(-5) and used to replace the corresponding sequences in pSN261. The plasmids thus constructed contain a wild-type RPO26 coding sequence downstream of a mutagenized promoter. pSN302 was constructed similarly, except that the promoter fragment originated from unmutagenized pRP026HAT. The PCR primers used in the construction were A34, containing sequences -597 to -582 (5'-GCTGGCGAGTCGTCAC-3') in the TFC2 ORF, and PE2, corresponding to sequences +97 to +77 (5'-CCTGTATCACAATGATAGAAG-3') in the RPO26 intron (See Fig. 1). All PCR-generated fragments used in this study were sequenced following amplification and cloning to ensure that no extraneous mutations were introduced during amplification. In order to construct plasmids pSN305 and pSN306, a 214bp fragment of the RPO26 promoter (bp -19 to -232) was PCR-amplified from pSN302 and pSN304, respectively, and subcloned into the BamHI site of pUC19. The primers used were Bandshift-1 (5'-GGGGGATCCCTiTACACTGTTACC-3') and Bandshift-2 (5'-GGGGGATCCGACAGCGATAGAGCAAG-3'). Yeast strain JAY472
(Archambault, et al., 1990) has a temperature-sensitive (ts) allele of RPO21 (rpo21-4). In strains JAY472 and JAY476, the chromosomal RPO26 gene is partially duplicated such that a truncated and non-functional RPO26 is expressed from the RPO26/TFC2 promoter and a full-length RPO26 is expressed conditionally from the GALI promoter (Archambault, et al., 1990). Note that in these strains the RPO26/TFC2 divergent promoter is intact. Therefore, expression of TFC2 is not affected. Strains SNYH167 through 169 are derivatives of JAY567 (Nouraini, et al., 1996) and contain plasmids pSN302 through pSN304, respectively. In JAY567 the chromosomal RPO26 is missing the RPO26 intron and nucleotides encoding the first 42 amino acids of the subunit (Nouraini, et al., 1996). This mutant RPO26 allele (RPO26-Δ42) encodes a truncated but functional protein, which can support normal cell growth under a variety of conditions (Nouraini, et al., 1996).

Genetic screen for synthetic lethality

Plasmid pRPO26HAT was mutagenized with hydroxylamine in vitro (Nouraini, et al., 1996) and, following passage through E. coli strain JF1754 (Himmelfarb, et al., 1987), the bank of mutagenized plasmids was introduced into the yeast strain JAY472. JAY472 transformants were selected on solid medium in the presence of galactose (chromosomal RPO26 expressed) at 30°C. Then, individual transformants were tested for growth defects on solid medium containing glucose (chromosomal RPO26 repressed) in order to identify plasmids that confer synthetic lethality in combination with rpo21-4. Plasmids that conferred a stable and reproducible synthetic-lethal phenotype to JAY472 were chosen as candidates for mutant alleles of RPO26. A more detailed description of this genetic screen is described in chapter II.
Mobility-shift analysis

The mobility-shift assays were done as described by Buchman and Kornberg (1990) except that Abf1p was preincubated with the binding reaction mixture (with or without unlabeled competitor oligonucleotides) for 10 min at room temperature prior to the addition of 6.7 fmoles of 32P-labeled probe. The probe was the BamHI insert of plasmids pSN305 (RPO26) or pSN306 (rpo26-27). Purified Abf1p (Buchman and Kornberg, 1990) was a gift from Andrew Buchman. Two double-stranded oligonucleotides (1-32, and 33-63) were used for competition assays; their sequences were derived from a 63bp BglII/AuI fragment of MATa (nucleotides 2001 to 2063, numbering according to Astell et al [1981]). Oligo 1-32 (5'-GATCTAAAATAATT CGTTTTCAATGATTAAAA-3', sequence of top strand) contains nucleotides 2001 to 2032 and an Abf1p-binding site (BOLD TYPE). Oligo 33-63 (5'-TAGCATAGTCGGGTITTTCITTAGTTTCAG-3', sequence of top strand), contains nucleotides 2033 to 2063, does not have an Abf1p recognition sequence, and was used as a non-specific competitor (McBroom, 1993)

Primer-extension analysis

Primers PE1 and PE3 were used for primer-extension experiments. PE3 and PE1 hybridize to the RPO26 mRNA and span sequences +273 to +251 (5'-CTTCTTTATTTG CTCATGCTGTTG), and +169 to +149 (5'-TTCTTCATAAGTCTCCTCATC -3'), respectively, in the RPO26 ORF. The position of each primer is shown in Fig.4A. Primer actin E2 (5'-CGTCACCGGCAAACCGGC-3') hybridizes to the second exon of ACT1 and allows measurement of ACT1 mRNA as an internal control. Analysis of transcription-initiation sites in the ACT1 promoter has shown one major and a number of minor start sites (Munholland, et al., 1990). In order to simplify quantitation of the ACT1 mRNA, primer actin E2 was labeled with 32P and diluted 20-fold with unlabeled actin E2 for the primer-extension experiments. Under these conditions, the only notably detectable initiation site for ACT1 is the major start site, which was used for quantitation.

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Primer-extension assays were performed as described (Hu, et al., 1994) with 2 μg of polyA+ mRNA. Quantitation of the amount of RPO26 message was performed using a PhosphorImager (Molecular Dynamics, model 425E) and the ImageQuant quantitation software. The amount of transcript produced from the plasmid-encoded RPO26 (driven by either a wild-type or a mutant promoter) was measured as the total amount of transcription initiated from upstream and downstream initiation sites. The amount of plasmid-encoded RPO26 was normalized to the amount of mRNA produced from the truncated chromosomal allele of RPO26 (expressed from a wild-type promoter), and also separately to the amount of message produced from the ACT1 gene.
RESULTS

As I described in Chapter II, using a synthetic lethality screen in combination with \textit{rpo21-4}, I isolated a total of 34 \textit{rpo26} mutant alleles. Twenty nine of these mutants contained mutations in the \textit{RPO26} ORF (Chapter II). Below, I describe the characterization of the remaining five mutant alleles which contain mutations in the \textit{RPO26} promoter.

\textbf{Identification of promoter mutations}

\textit{RPO26} and \textit{TFC2}, which is the gene encoding the RNAPIII transcription initiation factor TFIIIA, are transcribed divergently (Archambault, et al., 1992). Sequences that are both necessary and sufficient for expression of \textit{RPO26} lie in this intergenic region (233 bps) (Mckune and Woychik, 1994). In order to locate \textit{rpo26} mutations, a fragment was isolated from the mutagenized plasmids (\textit{BclI}-5 to \textit{HindIII} +754, see Fig. 1) that contained the \textit{RPO26} ORF and 200bp of untranslated downstream sequence. This fragment was then used to replace the wild-type counterpart in an unmutilagenized plasmid. As a result of this construction, expression of the \textit{RPO26} coding region derived from the mutagenized plasmids is driven by a wild-type promoter. If the hydroxylamine-generated mutations lie outside of the \textit{RPO26} ORF and 200 bp of downstream untranslated sequences, this newly generated plasmid should not be able to confer a synthetic-lethal phenotype on \textit{JAY472} (\textit{rpo21-4}). Five \textit{RPO26} mutant alleles were no longer synthetically lethal in combination with \textit{rpo21-4}. Sequence analysis determined that these mutant alleles were wild-type in the \textit{RPO26} ORF, but had mutations in the 5'-upstream region. The \textit{RPO26} promoter was searched for matches to consensus sequences for binding of a variety of yeast transcriptional activators. The sequence 5'-ATCATACTATACG-3' (-129 to -140, bottom strand) (Fig. 1) matches the binding-site consensus sequence (5'-RTCNYNNN NNACG-3') (Buchman and Kornberg, 1990) for the transcription factor Abf1p. Two of the mutant alleles, \textit{rpo26-24} and \textit{rpo26-27}, had mutations in this consensus sequence (Fig. 1). \textit{rpo26-}

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26 and rpo26-28 contained multiple base changes that included the Abf1p binding-site (Fig. 1), and rpo26-25 had two mutations that did not include the Abf1p recognition sequence (not shown).

Mutant alleles rpo26-24 and rpo26-27 were studied further in order to investigate the role of Abf1p in regulation of RPO26 transcription. The growth phenotype conferred by these mutant alleles was tested at various temperatures in the presence of the wild-type allele of RPO21 in yeast strain JAY476 (pGAL-RPO26, RPO21). These mutations generated a slow-growth defect at 15°C in a wild-type RPO21 background (Fig. 2B).

Although mutations were identified in the promoter of RPO26 for the above-mentioned mutant alleles, it was necessary to confirm that the phenotypes conferred by these mutants were not due to mutations in the vector sequences. Such mutations could lead to a reduction in the amount of the RPO26 gene product simply by lowering the plasmid copy-number. To test this possibility, the mutant promoter fragments from plasmids corresponding to RPO26 (from pRPO26HAT), rpo26-27 and rpo26-24 were isolated and were used to replace corresponding sequences in the unmutagenized plasmid pSN261. The mutant promoter fragments were both necessary and sufficient to confer a synthetic-lethal phenotype to JAY472 and a cold sensitive phenotype to JAY476 (not shown).

**Binding of Abf1p to the RPO26 promoter**

Mobility-shift analyses were performed with purified Abf1p (Buchman and Kornberg, 1990) in combination with the wild-type or mutant promoter fragments containing the Abf1p binding-site. Abf1p was able to bind to the wild-type promoter fragment (Fig. 3, lane 3); binding was competed with a double-stranded oligonucleotide (1-32) containing a well characterized Abf1p binding-site from the MATa locus (McBroom and Sadowski, 1994a) (Fig. 3, lanes 4 to 6). A similar-sized double-stranded oligonucleotide (33-63), which lacked an Abf1p binding site, was unable to compete in this binding reaction (Fig. 3,
Fig. 1. Alleles of *RPO26* with mutations in an upstream Abflp-binding consensus-sequence. The region of the genomic clone present on the mutagenized plasmid (*HindIII*-748 to *HindIII* +754) is indicated. *RPO26* is transcribed divergently from *TFC2*, the gene encoding the RNAPIII transcription initiation factor, TFIIIA. Large arrows indicate the direction in which each gene is transcribed. In pRPO26HAT, *TFC2* is truncated at the *HindIII* (-748) site. IVS in the *RPO26* ORF refers to the intron in this gene. Small arrows designated PE2 and D34 indicate primers used for PCR amplification of the promoter fragment from the mutagenized plasmids. The sequence of the *RPO26* promoter containing the mutations is shown, and each mutant allele of *RPO26* is aligned with the corresponding base pair changes. The boxed region is the consensus binding-sequence for Abflp within the *RPO26* promoter. The T-rich sequence often found in association with Abflp binding-sites in a number of yeast promoters is underlined. Numbering of the nucleotides is based on assigning the A of the *RPO26* ATG as +1.
Fig. 2. Phenotypes generated by mutations in the Abf1p binding sequence. A) A growth test showing the synthetic-lethal phenotype conferred by the *RPO26* promoter mutations on JAY472 (*rpo21-4, pGAL1-RPO26*), when the expression of chromosomal wild-type *RPO26* is repressed in the presence of glucose. A drop containing a suspension of ~2000 cells was applied to solid medium containing either glucose or galactose and was tested for growth at 30°C. B) Phenotype generated by the *RPO26* promoter mutations in the presence of wild-type *RPO21* in strain JAY476 (*RPO21, pGAL1-RPO26*). Growth tests were performed at four different temperatures on solid medium in the presence of glucose (chromosomal *RPO26* is repressed). Each row indicates five-fold serial dilutions of a starting cell suspension of 2000 cells per 10 μl (volume of each drop). Vector refers to pFL39 (Bonneaud, et al., 1991).
lanes 7 to 9). A single mutation \((rpo26-27)\) in the Abf1p binding-site prevented binding of Abf1p to the promoter fragment (Fig. 3, lane 12). These results suggest that Abf1p may also bind to the \(RPO26\) promoter \textit{in vivo} and that the mutations in \(rpo26-24\) and \(rpo26-27\) inhibit this interaction.

**Quantitation of the \(RPO26\) mRNA**

Abf1p can activate transcription from a variety of yeast promoters (Dhawale and Lane, 1993). Therefore, we tested whether a mutation in the Abf1p binding-site upstream of \(RPO26\) would lower the steady-state level of \(RPO26\) mRNA. The amount of \(RPO26\) mRNA was measured by primer-extension analysis of poly-A\(^+\) mRNA prepared from strains SNYH167 \((RPO26)\), SNYH168 \((rpo26-24)\) and SNYH169 \((rpo26-27)\) (Fig. 4B). In these strains a truncated functional form (Nouraini, et al., 1996) of \(RPO26\), \(RPO26-\Delta 42\), is expressed on the chromosome from a wild-type promoter, and full-length \(RPO26\) is transcribed on a yeast episomal plasmid from either wild-type or mutagenized promoters \((rpo26-24\) and \(rpo26-27\), Fig. 4A). The amount of mRNA produced from the \(ACT1\) gene was measured as an internal control in addition to mRNA produced from chromosomal \(RPO26-\Delta 42\). The steady-state level of \(RPO26\) mRNA was reduced in the presence of mutations in the Abf1p binding-site (Fig. 4B). Quantitation of the amount of \(RPO26\) mRNA (measured as the sum of downstream and upstream initiation sites) normalized to the amount of \(ACT1\) mRNA (shown by arrow in Fig. 4B), revealed approximately a 60% reduction of \(RPO26\) transcripts (Fig 4C) in strains carrying \(rpo26-24\) (Fig 4B, lane 3) and \(rpo26-27\) (Fig 4B, lane 4), compared to wild-type (Fig 4B, lane 2). This reduction in the amount of the \(RPO26\) mRNA was essentially the same using \(RPO26-\Delta 42\) as an internal control (not shown).
Fig. 3. Binding of Abf1p to the RPO26 promoter. Mobility-shift analysis was performed with purified Abf1p (lanes 3 to 9 and 12 to 18) and $^{32}$P-labeled promoter fragments from RPO26 and rpo26-27. As a control, the experiment was also performed with no protein added (lanes 1 and 10) and BSA (lanes 2 and 11). Competition for binding of Abf1p was performed with the indicated molar excess of an oligonucleotide containing a known Abf1p binding-site (specific competitor), and a similar-sized oligonucleotide lacking an Abf1p binding-site (non-specific competitor).
New sites of transcription initiation are generated by the promoter mutations

Mutations in the Abf1p binding-site led to the selection of new sites of transcription initiation in the \textit{RPO26} promoter (Fig. 4B). Primer-extension experiments were performed with two different preparations of polyA$^+$ mRNA. The new initiation sites of transcription were observed using two separate preparations of polyA$^+$ mRNA. In order to map the initiation sites, primer extension analysis was performed using the PE1 primer, which hybridizes only to full-length \textit{RPO26} and not to \textit{RPO26-Δ42} transcripts (see Fig. 4A). A \textit{BclI} fragment containing the RPO26 cDNA was used as a template for sequencing with the PE1 primer. The products of this sequencing reaction served as a size marker for the primer-extension products (Fig. 5A). The estimated positions of initiation-sites in the mutant (\textit{rpo26-24} and \textit{rpo26-27}) and the wild-type (\textit{RPO26}) promoters are shown in Fig. 5B. In wild-type \textit{RPO26}, transcription was initiated at multiple sites spanning sequences +1 (A of \textit{RPO26} ATG) to -84. Multiple initiation sites of transcription is characteristic of transcription initiation in \textit{Saccharomyces cerevisiae} (Russell, 1983, Struhl, 1989). In the promoter mutants, the window of initiation was shifted upstream beginning from position -108, 19 bp downstream of the Abf1p binding site (underlined in Fig. 5B). In this new window of initiation, previously-unused or infrequently-used start sites became major transcription initiation sites, while previous major initiation sites that lie closer to the ATG were seldom used (Fig 5A).
Fig. 4. Quantitation of the amount of *RPO26* message in the presence and absence of Abf1p binding-site mutations. A) schematic representation of the *RPO26* locus on the chromosome and on the plasmid in the strain used for preparation of RNA. A truncated and functional form of *RPO26* (*RPO26*-Δ42), which is missing the first 42 amino acids and the intron, is expressed from a wild-type chromosomal promoter. Full-length *RPO26* is expressed from either a mutant or a wild-type promoter on the plasmid. The small arrows named PE3 and PE1 indicate the primers used for primer extension experiments. B) Results of the primer-extension analysis using poly-A+ mRNA. The products corresponding to the chromosomal and plasmid *RPO26* mRNA and the *ACT1* mRNA are indicated. Lanes 2 to 4 represent results of primer-extension experiments performed with a mixture of actin E2 and PE3 primers using polyA+-mRNA from strains SNYH167 (*RPO26*), SNYH168 (*rpo26*-24) and SNYH169 (*rpo26*-27), respectively. Lane 1 shows results of primer-extension experiments performed with actin E2 primer only, using mRNA from the wild-type strain. The position of migration of end-labeled 1kb Molecular Weight DNA marker (from BRL) is shown on the left. C) Quantitation. The amount of the plasmid-encoded *RPO26* message was normalized to the level of *ACT1* message. The level of mRNA generated from each gene was measured using a Molecular Dynamics PhosphorImager. The normalization was performed by dividing the amount of RPO26 mRNA by the amount of *ACT1* mRNA. The values shown in the graph were calculated according to the following formula: \([\text{wild-type or mutant } RPO26/ACT1]/[\text{wild-type } RPO26/ACT1]\). For measurement of *RPO26* mRNA in each strain, the sum of signals generated from both native and newly used initiation sites (indicated by brackets) were quantitated for each strain (lanes 2 to 4).
If

Chromosome

Wild type

Wild type or mutant

Plasmid

Chromosome

Wild type

Wild type or mutant

Plasmid

ACC1

TFC2

GTA

TFC2

RPO26

RPO26-Δ42

ATG

PE1

PE3
Quantitation of RPO26 mRNA
Normalized to actin

Amount of transcript relative to wild-type

RPO26  rpo26-27  rpo26-24
Fig. 5. Mapping of the new start sites generated due to the mutations in the Abf1p binding-site. A) Primer extension was performed with poly-A+ mRNA and primer PE1, which recognizes only the mRNA produced from full-length RPO26. The last four lanes are a sequencing ladder generated from sequencing of a Bgl fragment of RPO26 cDNA with primer PE1. The initiation sites used in the wild-type promoter are designated by short arrows. Newly generated start sites due to rpo26-24 and rpo26-27 mutations are shown by long arrows. B) Location of initiation sites used in the RPO26 promoter. Initiation sites present in the wild-type promoter are shown by open circles; solid circles represent the initiation sites that are either enhanced or only used in the presence of the mutation in the Abf1p binding-site. The Abf1p binding consensus-sequence is underlined.
DISCUSSION

Using a genetic screen based on synthetic lethality between *rpo21-4* and mutant alleles of *RPO26*, I identified mutations in the consensus binding-sequence for the transcription factor Abf1p in the promoter of *RPO26*. In addition to the synthetic-lethal phenotype, the promoter mutants also give rise to a cold-sensitive growth defect in the presence of wild-type *RPO21*. The cold-sensitive phenotype conferred by the Abf1p binding-site mutations in the *RPO26* promoter could result from a reduction in the intracellular amount of RNAPs. This reduction could, in turn, lead to suboptimal expression of certain genes that may be required for growth of yeast at low temperatures. Change in the growth properties of yeast cells as a result of underproduction of a polymerase subunit has been observed previously for RNAPII (Archambault, et al., 1996) and RNAPIII (Mosrin, et al., 1990). In the case of RNAPII, the expression of *RPO21* was placed under control of the repressible *LEU2* promoter. When expression of *RPO21* was repressed by adding leucine to the growth medium, yeast cells acquired a slow-growth and a temperature sensitive phenotype (Archambault, et al., 1996). Underproduction of Rpc3lp, the 31kD subunit of yeast RNAPIII, also led to a slow-growth phenotype and a decrease in the steady-state levels of tRNA in the cell (Mosrin, et al., 1990). The artificial underproduction of Rpc3lp was achieved when a nonsense mutant allele of *RPC31* was suppressed partially using a tRNA suppressor (Mosrin, et al., 1990).

Using electrophoretic-mobility-shift assays and purified Abf1p, we have shown that Abf1p binds to the *RPO26* promoter in vitro and that mutations in the Abf1p binding-site abolish protein binding. The *SPT2* promoter also contains an Abf1p binding-site (5'-ATCATGTTAAACG-3'), which closely resembles the binding site in the *RPO26* promoter (5'-ATCATACTATAAC-3') (Buchman and Kornberg, 1990). The affinity of Abf1p for this binding-site has been measured by mobility-shift assays (Buchman and Kornberg, 1990); a mutation analogous to that of *rpo26-27* in the *SPT2* promoter reduced the affinity of Abf1p for its binding site by more than 500-fold, abolishing binding
(Buchman and Kornberg, 1990). This is consistent with the results of our mobility-shift assays and suggests that the occupancy of the Abf1p binding-site in the promoter of 
\textit{RPO26} is likely to be dramatically reduced \textit{in vivo} in SHN168 (rpo26-24) and SHN169 (rpo26-27) strains.

Abf1p alone is not a potent transcriptional activator and it normally requires the cooperation of other weak activators (Buchman and Kornberg, 1990, Della Seta, et al., 1990b, Halfter, et al., 1989, Hamil, et al., 1988, Kraakman, et al., 1991, Sinclair, et al., 1994, Trawick, et al., 1992, Willett, et al., 1993). For example, Abf1p binding-sites are often associated with a T-rich sequence, which functions in a variety of yeast promoters as an upstream activating sequence (Buchman and Kornberg, 1990, Gonçalves, et al., 1995, Iyer and Struhl, 1995, Lue, et al., 1989, Schultes and Szostak, 1991). In the \textit{RPO26} promoter the Abf1p binding-site is 9 bps upstream of a T-rich sequence proximal to the \textit{RPO26} ORF (Fig. 1). The Abf1p binding-site mutations in the \textit{RPO26} promoter resulted in only a three-fold reduction in the amount of \textit{RPO26} mRNA (Fig. 4C). This marginal reduction is consistent with the known weak transcriptional activity of Abf1p and suggests that other promoter sequences, such as the T-rich region, contribute to the expression of \textit{RPO26}. The contribution, if any, of the T-rich region to the expression of \textit{RPO26} would not have been detected by our mutagenesis, since hydroxylamine only mutagenizes cytosine.

Primer-extension analysis of \textit{RPO26} mRNA showed that the mutations in the Abf1p-binding sequence reduce the expression of this gene (Fig. 4) and that the transcription start sites shift upstream (Fig. 5). The sites of transcription initiation from the \textit{RPO26} promoter were analyzed by Woychik \textit{et al} (1990), who reported four major initiation sites (-10, -15, -25, -26), only three of which (-15, -25, -26) were detected by our primer-extension analyses (Fig. 5). However, we did identify other initiation sites in addition to those previously reported (Woychik, et al., 1990). This discrepancy might be due to differences in the yeast strains used for the preparation of mRNA in the two studies.
For example, the number of transcription initiation sites has been shown to be different for the \textit{ADH1} gene amongst different wild-type yeast strains (Bennetzen and Hall, 1982, Pinto, et al., 1992, Pinto, et al., 1994).

The role of Abf1p binding-sites in the expression of a variety of genes has been studied (Buchman and Kornberg, 1990, Della Seta, et al., 1990b, Della Seta, et al., 1990a, Dequard-Chablat, et al., 1991, Halfter, et al., 1989, Hamil, et al., 1988, Herruer, et al., 1989, Kraakman, et al., 1991, Mager and Planta, 1990, Sinclair, et al., 1994, Trawick, et al., 1992, Willett, et al., 1993). However, to our knowledge, this is the first report of a shift in the initiation sites of transcription due to a mutation in an Abf1p binding-site. In other studies, promoters have often been used to drive expression of a \textit{lacZ} reporter gene and the effects of Abf1p binding-site mutations have been analyzed by monitoring \(\beta\)-galactosidase activity in cellular extracts. Thus, possible shifting of the initiation sites of transcription due to mutations in an Abf1p binding-site would have escaped detection.

Abf1p is a highly abundant transcriptional activator and is required for constitutive or regulated transcription of a large number of genes (see references in the preceding paragraph). For these genes Abf1p appears to facilitate the access of other regulatory factors to promoters, thereby mediating their regulatory effects (Gonçalves, et al., 1995, Trawick, et al., 1992, Willett, et al., 1993). The ability of Abf1p to create a sharp bend in DNA (McBroom and Sadowski, 1994b) and to position nucleosomes on the promoter (De Winde, et al., 1993) might underlie the mechanism by which it facilitates access to DNA for other regulatory factors. The positioning of transcription initiation-sites on the \textit{RPO26} promoter might represent another aspect of transcriptional regulation by Abf1p.

One way that establishing the initiation sites of transcription could perform a regulatory role for the short (only \(\sim 250 \text{ bp}\)) divergent \textit{RPO26/TFC2} promoter is by controlling the traffic of RNAP molecules that transcribe in opposite directions; those that transcribe \textit{RPO26} and the ones that transcribe \textit{TFC2} (Fig. 6). This form of regulation could be particularly important for transcription initiation in \textit{Saccharomyces cerevisiae}. In
this organism, the initiation sites of transcription lie 30 to 120 bp downstream of the TBP binding site, while in fission yeast and in higher eukaryotes, a single site is found 30 bp downstream of the TBP binding site (Russell, 1983; Li et al., 1994). It has been suggested that initiation sites of transcription are selected in Saccharomyces cerevisiae by downstream scanning of the promoter by the RNAPII that has been recruited to the promoter by TBP (Guardina & Lis, 1993). This form of initiation site selection could be problematic for a short divergent promoter with more than one TBP binding-site such as the RPO26/TFC2 promoter. Indeed, the appearance of upstream initiation sites for this promoter in the absence of Abf1p-binding strongly suggests that there are alternative TBP binding sites. A problem could arise since assembly of the transcription initiation complex on the alternative upstream TBP-binding sites and scanning for suitable initiation sites downstream could block the movement of the RNAPII scanning in the opposite direction (Fig. 6A). Binding of Abf1p to this promoter could potentially inhibit formation of initiation complexes on the alternative upstream TBP binding sites. In this way, Abf1p may normally confine the RNAPII enzyme transcribing RPO26 to one side of the promoter, and preventing it from interfering with the polymerase transcribing TFC2 (Fig. 6B).

There are a number of ways Abf1p may segregate the oppositely transcribing polymerases on the RPO26/TFC2 promoter. Abf1p might sterically block formation of initiation complexes on nearby TBP-binding sites. Alternatively, Abf1p may be able to bend the RPO26/TFC2 promoter in such a way that formation of initiation complexes are energetically more favorable for some TBP binding sites than others. Finally, Abf1p may position nucleosomes on the RPO26/TFC2 promoter so as to selectively block certain TBP binding sites. It should be noted that the models presented above are purely speculative since there are no data available concerning the TBP binding sites on the RPO26/TFC2 promoter, the position of nucleosomes on this promoter, bending of this promoter in the absence and presence of Abf1p binding, or the initiation sites of transcription for TFC2.
Fig. 6. Possible regulatory role for establishment of transcription initiation sites by Abf1p in the RPO26/TFC2 promoter. A) In the absence of Abf1p binding, the initiation complex transcribing RPO26 might assemble on alternative upstream TBP binding-sites, which could potentially interfere with the movement or assembly of initiation complexes primed to transcribe TFC2. B) Binding of Abf1p to the promoter could prevent formation of initiation complexes on the alternative upstream TBP binding-sites, thus preventing transcription in the direction of RPO26 that would block transcription in the direction of TFC2. For an explanation of possible mechanisms by which might be able to segregate oppositely RNAPs see Discussion.
REFERENCES


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CHAPTER V
SUMMARY AND FUTURE DIRECTIONS
THEESIS SUMMARY

Isolation of mutant alleles of RPO26

Rpo26p is a subunit common to the three yeast nuclear RNA polymerases. Although the existence of common subunits has been known for some time, their function remains largely unknown. In this thesis, I explore the functional role of Rpo26p. The impetus for these experiments came from two genetic interactions between RPO26 and the gene that encodes the largest subunit of RNAPII (RPO21). First, an increase in the gene dosage of RPO26 suppresses the ts phenotype of a RPO21 mutant allele, rpo21-4, in an allele-specific manner (Archambault, et al., 1990). Second, a combination of the ts rpo21-4 allele with non-lethal mutant alleles of RPO26 leads to cell death at all temperatures tested, a phenomenon known as synthetic lethality (Archambault, et al., 1990). Allele specific suppression of rpo21-4 by overexpression of RPO26 was interpreted as follows: rpo21-4 imposes a defect on the stability/assembly of RNAPII which can be overcome by increasing the amount of Rpo26p, perhaps by pushing the equilibrium for the assembly of subunits towards complex formation (Archambault, et al., 1990). The observation of synthetic lethality between rpo21-4 and various non-lethal RPO26 mutant alleles provided the framework for a sensitive genetic screen for isolating a collection of functionally defective mutant alleles of RPO26. As described in Chapter II, I screened a library of randomly mutagenized RPO26 for synthetic lethality in an rpo21-4 strain in which chromosomal RPO26 is expressed from the inducible GAL10 promoter. I obtained thirty-four mutant alleles of RPO26 that conferred lethality in the absence of chromosomal RPO26 expression (in the presence of glucose). Based on the phenotype that they conferred in the presence of wild-type RPO21, the rpo26 mutant alleles were divided into three classes: ts, null (lethal), and neutral (conferred no detectable growth defect). All except five of the mutant alleles contained mutations in the RPO26 open reading frame (ORF). The remaining five had mutations in the promoter of RPO26. All of the amino
acid alterations, except for an allele with a change of the initiator methionine, clustered in
the C-terminal two-thirds of Rpo26p, suggesting that the residues in the N-terminal end of
Rpo26p are not required for function. To test this hypothesis a deletion allele of \textit{RPO26}
was constructed (\textit{rpo26A42}) which was missing the codons for the first 42 amino acids of
Rpo26p. This truncated form of \textit{RPO26} was capable of supporting growth in the presence
of \textit{RPO21} and all of the mutant alleles of \textit{RPO21} that were tested, reinforcing the idea that
the N-terminal 42 amino acids of Rpo26p provide no detectable function.

With the collection of \textit{RPO26} mutant alleles at my disposal, I could begin to
address the function of Rpo26p. Ts mutants are particularly useful for this purpose since
they often are related to defects in protein-protein interactions. Among the five \textit{RPO26} ts
alleles, I chose \textit{rpo26-31} for further study. Compared to the other ts mutants, \textit{rpo26-31}
showed the most severe ts growth defect, with faster growth arrest upon a shift to the non-
permissive temperature. In addition, protein-blot analysis showed that \textit{rpo26-31} showed a
drastic decrease in the amount of Rpo26p in response to an increase to temperature,
whereas the remainder of the mutant alleles showed a marginal decrease (less than or equal
to twofold) under these conditions. In addition to a reduction of Rpo26p, the steady-state
levels of Rpo21p and the largest subunit of RNAPI (A190p) were also reduced at the non-
permissive temperature in the presence of \textit{rpo26-31}. In chapter II, I have shown that the
amount of newly assembled RNAPII and RNAPI was reduced at the non-permissive
temperature, which is a natural consequence of degradation of the above-mentioned
subunits. The assembly defect of RNAPII and the ts phenotype of \textit{rpo26-31} were
suppressed only partially by overexpression of the mutant subunit, even though the steady-
state level of \textit{rpo26-31}p was greater than the level found in a wild-type strain. As
suggested in chapter II, these results could be interpreted in two ways. First, \textit{rpo26-31}p
may have a reduced affinity for the largest subunits of RNAPI and RNAPII or for a
complex of proteins that contains these subunits. This decreased affinity could lead to
accumulation of free unassembled subunits which are prone to degradation. Alternatively,
the rpo26-3lp may be an intrinsically unstable subunit whose accumulation is dependent on assembly into the RNAP complex. At high temperature, the instability of rpo26-3l is then exaggerated, which leads to rapid degradation of the subunit. In the first model, overexpression of rpo26-3lp compensates for the reduced binding affinity by pushing the equilibrium of assembly towards complex formation. According to the second model, overexpression of rpo26-3l compensates directly for a rapidly degrading subunit. In either case, rpo26-3l needs to assemble into the polymerase complex in order to accumulate. This conjecture is supported by the observation that the stability of rpo26-3lp is jeopardized by the presence of a source of wild-type Rpo26p compared to its absence. Presumably, Rpo26p competes with rpo26-3lp for assembly into the polymerase and leads to accumulation of unassembled rpo26-3lp which is prone to degradation.

**Identification of a catalytic subunit of the yeast proteasome as a suppressor of rpo26-3l**

The observation that a defect in assembly is paralleled by a reduction in the steady-state level of polymerase subunits suggests that there is a cellular mechanism that removes unassembled subunits. To identify components of this cellular response I isolated spontaneous suppressors of rpo26-3l mutation. In chapter III, I described the isolation and characterization of one suppressor strain, scs32, which allowed partial suppression of the rpo26-3l ts phenotype and accumulation of rpo26-3lp to wild-type levels at the non-permissive temperature. scs32 contains a mutation in a gene (PUP3) that encodes a catalytic subunit of the 20S proteasome, which is the catalytic component of the 26S proteasome, the major proteolytic machinery of the cell (Coux, et al., 1996). As I reported in chapter III, scs32 suppressed other ts mutations in RPO26 which also reduce the stability of Rpo26p; however, scs32 did not suppress the ts phenotypes of various ts mutations in RPO21, nor did it allow accumulation of unstable rpo21-4p. This observation of substrate specificity by a component of the 20S catalytic core of the 26S proteasome is surprising since, although the latter recognizes specific substrates by their ubiquitin
modification, the 20S catalytic core has no known substrate specificity (Coux, et al., 1996). In order to explain this substrate specificity, in Chapter III I have suggested that unassembled or unstable Rpo21p might become rapidly recognized and ubiquitinated at a faster rate than mutant forms of Rpo26p. In fact, ubiquitin modification of Rpo21p has been observed in both yeast (Jon Huibregtse, pers. communication) and mammalian cells (Bregman, et al., 1996). Rsp5p is the ubiquitin ligase (see Hochstrasser, 1996 for a review of ubiquitin ligases) responsible for modification of Rpo21p in yeast and binds to Rpo21 through the CTD (Jon Huibregtse, pers. communication). Since the CTD is unique to Rpo21p, Rsp5p might selectively ubiquitinate and target unassembled Rpo21p for degradation. Whether A190p- or Rpo26p-specific ubiquitin ligases exist is not known.

**Characterization of RPO26 promoter mutations: Contribution of Abf1p to transcription of RPO26**

As mentioned earlier, among the collection of RPO26 mutant alleles that I isolated through the synthetic-lethal screen (Chapter II), five were mutated in the RPO26 promoter (Chapter IV). Two of these, rpo26-24 and rpo26-27, contained mutations in a consensus sequence for binding of Abf1p, suggesting that binding of this transcription factor to the RPO26 promoter is required for optimal expression of RPO26. Using purified Abf1p and mobility shift assays, I found that Abf1p specifically bound the RPO26 promoter in vitro, and that the Abf1p binding-site mutations in rpo26-27 abolished this binding. Furthermore, using primer extension analysis of RPO26 mRNA from wild-type and mutant promoters, I showed that the mutations in the Abf1p-binding site reduced the levels of RPO26 mRNA by approximately 60%. In addition to a reduction of RPO26 transcripts, the initiation sites of transcription were shifted upstream in the promoter. Binding of Abf1p to the RPO26 promoter may help set the sites of transcription initiation, a phenomenon that has not been reported previously for Abf1p. Whether this is an isolated case specific to the RPO26 promoter, or if it represents a new regulatory role for Abf1p, is not known.
FUTURE DIRECTIONS

Through the experiments described in Chapter II, I have shown that Rpo26p is required for the assembly of RNAPI and RNAPII (and perhaps RNAPIII) and for the stability of the largest subunits of these enzymes. Is this the only role played by Rpo26p or does it contribute to other aspects of the RNAP function? In the following pages, I describe experiments aimed at exploring the possibility of other functions for Rpo26p. Furthermore, as described in Chapter IV, binding of the Abf1p to the RPO26/TFIIDA promoter is required for normal expression of RPO26. Abf1p also seems to participate in setting the sites of transcription initiation in this promoter. I have suggested a model describing a possible physiological function for initiation site selection by Abf1p. I will propose an experiment to further investigate the validity of the model proposed in Chapter IV.

Investigation of a role for Rpo26p in transcription elongation

There is indirect evidence that Rpo26p might be involved in the regulation of transcription elongation. Rpo26-specific antibodies can inhibit non-specific elongation activity of yeast RNAPs (Breant, et al., 1983, Buhler, et al., 1980, Huet, et al., 1985, Sawadogo, et al., 1980). This inhibition can be blocked by preincubation of RNAPs either with DNA (Breant, et al., 1983, Huet, et al., 1985) or with the transcription elongation factor SII (Sawadogo, et al., 1980). Rpo26p may be involved in transcription elongation in two ways. First, the zinc-binding domain of the E. coli β' subunit contributes to the stability of the ternary elongation complex by binding to DNA (Nudler, et al., 1996). Therefore, DNA binding by Rpo26p might perform a similar function. Second, the surface on the RNAPII molecule to which SII binds might include some region of Rpo26p, in addition to the binding-sites identified in Rpo21p (Rappaport, et al., 1988, Wu, et al., 1996). Mutant alleles of RPO26 that abolish either DNA binding by Rpo26p or interaction with SII might be identified by a genetic screen based on sensitivity to the drug 6-AzaUracil
6AU is converted to 6-AZA UMP inside the cell and reduces the concentration of intracellular UTP and GTP by inhibiting enzymes that are involved in the pathways of synthesis of these nucleotides (Exinger and Lacroute, 1992, Handschumacher, 1960). Three observations show a direct correlation between 6-AU sensitivity of mutant strains defective in RNAPII transcription and a defect in elongation of transcription. First, the gene encoding SII in yeast (PPR2) is not required for cell viability but its absence leads to a 6-AU$^S$ phenotype (Clark, et al., 1991, Exinger and Lacroute, 1992, Hubert, et al., 1983, Nakanishi, et al., 1992). Second, it is known that a reduction of nucleotide substrate concentrations in vitro, in effect mimicking the effect of 6AU in vivo, reduces the rate of elongation by yeast RNAPII (Nagamine, et al., 1981). Third, 6-AU$^S$ mutations have been identified in RPO21 and RPO22, each of which stems from a defect in a different aspect of RNAPII elongation (Archambault, et al., 1992b, Powell and Reines, 1996). RPO21 6-AU$^S$ mutations can be suppressed by overexpression of SII, which correlates with the fact that RNAPII enzymes purified from these strains have a reduced binding affinity for the elongation factor SII (Wu, et al., 1996), and RPO22 6-AU$^S$ mutants show a reduced rate of elongation and increased frequency of pausing (Powell and Reines, 1996) in vitro. The 6AU$^S$ phenotype is thought to stem from the additive effects of a reduced intracellular concentration mainly of GTP, and an elongation deficient polymerase (Archambault, et al., 1992b). In fact, the 6-AU sensitivity due to the absence of SII function or mutations in RPO21 can be suppressed by addition of guanine to the growth medium (Archambault, et al., 1992b, Exinger and Lacroute, 1992).

I have analyzed my collection of RPO26 mutant alleles, including those generated previously by site-directed mutagenesis (Archambault, et al., 1990), for sensitivity to 6AU. Three mutant alleles showed sensitivity to 6AU: rpo26-31 (E144K), rpo26-35 (LK118) and rpo26-36 (LK125) (the results for rpo26-35 are shown in Fig. 1). Although the sensitivities of the two latter mutants was reversed by adding guanine to the medium, rpo26-31 remained sensitive to the drug even in the presence of guanine, suggesting that
Fig. 1- 6-AU$^+$ phenotype of a mutant allele of $RPO26$- An equivalent of 200 cells was spread on solid medium in the presence and absence of 50 μg/ml 6AU and incubated at 30°C for 5 days. The ability of guanine to rescue the 6-AU sensitivity of $rpo26-35$ was tested by adding guanine to the growth medium.
the drug sensitivity of \textit{rpo26-31} might be a secondary consequence of reduced amounts of RNAPII in this strain (Chapter II). The 6AU sensitivity of none of these strains was reversed by overexpression of SII, suggesting that, unlike the \textit{RPO21} 6AU\textsuperscript{s} mutants, the drug sensitivity is not related to defects in binding to SII. Therefore, the possibility remains that the \textit{rpo26} 6AU\textsuperscript{s} mutants have a reduced elongation rate. Purification of RNAPI, II and III from cells containing these \textit{RPO26} mutants and measurements of the rate of elongation, frequency of pausing and ability to respond to SII-mediated read-through should determine whether the 6-AU\textsuperscript{s} phenotype is a result of reduced elongation rate or if it stems from a defective response to SII.

\textbf{An alternative strategy for isolation of new \textit{rpo26} mutant alleles.} In my screen, it is unlikely that I identified all functionally disruptive mutations in \textit{RPO26}. Hydroxylamine targets only cytosine (for de-amination to uracil); therefore, there remain a large number of codons in \textit{RPO26} that were not mutated and screened for synthetic lethality (Fig. 2). A less biased mutagenesis scheme, for example one based on PCR, might allow identification of new and potentially interesting mutant alleles of \textit{RPO26}. Furthermore, it is possible that I limited the screen to those \textit{rpo26} mutants that have a defect in RNAP assembly/stability by screening for synthetic lethality in combination with \textit{rpo21-4}, which has a similar defect. To address this possibility, I analyzed a number of \textit{rpo26} neutral mutant alleles for lethality in strains carrying several different alleles of \textit{RPO21}. These included some linker-insertion mutant alleles (\textit{rpo21-5, -7, -8}) (Archambault, et al., 1992a), and \textit{rpo21R11} which contains only 11 out of 23 CTD repeats (Xiao, et al., 1994). The positions of the mutational alterations within these alleles and their associated phenotypes are shown in Figure 3. It is obvious from these results that the synthetic lethality effect seems to be specific to \textit{rpo21-4}. For example, although \textit{rpo21-4} and \textit{rpo21R11} have similar growth rates, synthetic lethality is observed only in combination with \textit{rpo21-4} (Fig. 3). The lethality observed in combination with \textit{rpo21-7} might not be
Fig. 2- A large number of potential \textit{RPO26} mutants are not recovered from the genetic screen described in Chapter II. The distributions of mutations in Rpo26p that are expected from hydroxylamine mutagenesis and those actually obtained from the synthetic lethal screen are shown. In some cases, the same codon in \textit{RPO26} can be mutated in more than one position, giving rise to two expected amino acid changes. Nonsense mutations that change an amino acid into a translational stop codon are shown by *.
Potential Obtained

Rpo6p Potential Obtained

a. a.# 10 20 30 40 50
| | | | |
Rpo6p MSEYEFAPNDGNENPEFDVFHEFSDDETYEERKPQFQDGGETDANGKVT
Potential LNI.KKT..NS.K..KN.NK.Y.FMKK..KK.S*..NSKI.INT..S.I.II
V D | L D V D
Obtained I NV I

a. a.# 60 70 80 90 100
| | | | |
Rpo6p GGNGPEDIQQHEQIRRKLDEKAIQPDQKATTPYMTKERYRARIILGTRALQ
Potential SS.SSKN.**YK*.KK.IF.K.T.S.N*KIIS.II..KKT..SIKT.*
L D
Obtained K ** * *K I S I I K D * L

a. a.# 110 120 130 140 150
| | | | |
Rpo6p ISMNAPVFVDLEFETDPRLIAMKELAEKKIPLVIRRLPDGSFEDWSVEE
Potential .FI.TSI.IN.KSKIN.S.C.TI.K.TK..S.I.KK..SNSF.KN*NMKK
VL D L H V V L L D
Obtained F V L T KK L K * V

a. a.# 155
| |
Rpo6p LIVDL
Potential F.MN.

Obtained F
Fig. 3- A test of synthetic lethality between *RPO26* mutant alleles and various mutant alleles of *RPO21*. An equivalent of 200 cells was spotted on solid YPD medium and the cells were incubated at 30°C for two days. The strain used for this experiment contained a replacement of *RPO26* with *LEU2*. A copy of *RPO26*, either mutant or wild-type, was supplied on a low copy plasmid. Also, in this strain, chromosomal *RPO21* was expressed from the inducible *GAL1* promoter. This strain was unable to grow in the presence of glucose (expression of *RPO21* repressed) unless supplied with a plasmid encoding either a wild-type or mutant copy of *RPO21*. Strains containing various combinations of *RPO26* and *RPO21* alleles were tested for synthetic lethality in the presence of glucose and galactose (not shown). Schematic representations of Rpo21p and Rpo26p along with the approximate position of the amino acid alteration for each mutant allele are shown at the top.
significant since strains containing rpo21-7 grow even more poorly than those containing rpo21-4. Therefore, it is possible that screening a library of mutagenized RPO26 for synthetic lethality with rpo21IR11 might reveal a functional interaction between Rpo26p and the CTD. However, it is also possible that the reason mutations in RPO26 are not synthetic lethal with rpo21IR11 is because Rpo26p plays no role in CTD-related functions.

Does Rpo26p interact with the other common subunits? Lalo et al. (1993) have provided evidence for a network of interactions among the common subunit ABC10βp (RPB10p) and the subunits common to RNAPI and RNAPIII (AC40p and AC19p). Overexpression of ABC10β suppresses ts mutations in RPC40 and RPC19, genes that encode AC40p and AC19p, respectively. Furthermore, overexpressing each of the latter subunits can suppress the phenotypes of ts mutant alleles of the other (Lalo, et al., 1993). This network of interactions suggests the existence of a core common-subunit complex. If Rpo26p participates in this core common-subunit complex, the ts phenotype of rpo26 mutants might be suppressed by overexpression of the other common subunits. Conversely, overexpression of RPO26 might be able to rescue the ts phenotypes of mutations in other common subunits. These are very simple experiments which can shed light on important protein-protein interactions in the RNAP complex.

Does Rpo26p function uniformly in all RNAPs?

As mentioned in Chapter II, rpo26-31 affects the assembly of RNAPI and RNAPII and the stability of their largest subunits. An interesting question that remains to be answered is whether mutations in RPO26 affect all three RNAPs in the same way. A simple way to test this for rpo26-31 would be to raise an antibody to the largest subunit of RNAPIII (Rpo31p) and to monitor the steady-state level of this subunit. Since the remainder of ts rpo26 mutant alleles reduce the amount of Rpo26p, they might also reduce the steady-state levels of the largest subunits of RNAPs. Therefore, the uniformity of the
RNAP defects generated by these mutants could also be tested by western-blot analyses of mutant cell extracts at the non-permissive temperature.

An alternative strategy for identifying possible polymerase-specific mutations would be to screen a library of randomly-mutagenized *RPO26* for mutant alleles that generate synthetic-lethality in combination with mutations in a subunit of only one RNAP, i. e., a form of polymerase-specific synthetic lethality.

**Coordination of the expression of RNAP subunits**

Proper cellular transcription is dependent on synthesis and assembly of a specific stoichiometric ratio of RNAP subunits. Therefore there needs to be a proper amount of each subunit synthesized at any one time. It is possible that synthesis of the RNAP subunits are coordinated at the level of transcription and/or translation of the genes encoding these subunits. The *RPO26* promoter mutations provide an opportunity for testing the possibility of coordination between the amount of transcription of *RPO26* and genes encoding the other subunits of yeast RNAPs. This transcriptional coordination can be tested by primer extension analysis of the amount of transcription of other RNAP subunit genes in the presence and absence of *RPO26* promoter mutations.

**Investigation of the generality of initiation site selection by Abf1p**

In Chapter IV, I suggested that the role of Abf1p in setting the sites of transcription initiation might be required for controlling the traffic of oppositely transcribing polymerases in short divergent promoters. If this truly represents a physiological role for Abf1p, then other short divergent promoters that are activated by Abf1p should show a shift in the initiation sites of transcription upon mutagenesis of their Abf1p-binding sites. An example of such a promoter is the short (349 bp) *YPT1/TUB2* promoter which contains an Abf1p binding-site half way between the start of the ORFs of the two genes (Halfter et al., 1989). Mutation of the Abf1p binding site reduces the amount of transcription in both directions.
(Halfter et al., 1989). However, as the amount of transcription was measured from promoter-fusions to \textit{LACZ}, it was not determined if the sites of transcription initiation were shifted in the presence of mutations in the Abf1p binding-site (Halfter et al., 1989). Primer extension analysis of \textit{LACZ} mRNA from strains containing the above \textit{LACZ}-fusion constructs should determine if setting the initiation sites of transcription by Abf1p can be extended to the \textit{YPT1/TUB2} promoter, or whether it is an isolated case occurring only for the \textit{RPO26/TFC2} promoter.

Occurrence of divergently transcribed genes is common in yeast. In order to further test the generality of the model presented in Chapter IV, one can search for the Abf1p binding-site consensus sequence in other previously identified divergent promoters. Then, contribution of Abf1p to the levels and selection of initiation sites of transcription for genes driven by these divergent promoters should be determined. Finally, contribution of Abf1p to the activity and initiation site selection of the \textit{TFC2} promoter should be determined to complete the analysis of the \textit{RPO26/TFC2} promoter. An experimental strategy similar to that described above for the \textit{YPT1/TUB2} promoter should also be suitable for the promoters discussed in this paragraph.
REFERENCES


