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Mutational Analysis of Transcription Factor IIIA
from *Saccharomyces cerevisiae*

by

Owen Rowland

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

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Mutational analysis of transcription factor IIIA from *Saccharomyces cerevisiae*. Degree of Doctor of Philosophy, 1998. Owen Rowland. Graduate Department of Biochemistry, University of Toronto.

Transcription factor (TF) IIIA is an RNA polymerase III transcription factor that binds to the internal control region (ICR) of the 5S RNA gene as the first step in the assembly of a TFIIIB-TFIIIC-TFIIIA-DNA preinitiation complex. TFIIIA of *S. cerevisiae* contains nine zinc-finger motifs with an 81-amino acid domain interrupting the otherwise repeating zinc-finger motifs between fingers 8 and 9.

The first part of my studies focused on examination of the TFIIIA-DNA interaction. I purified wild-type TFIIIA and truncated versions of TFIIIA that contained the first 3, 4, 5, 6, 7, and 8 zinc fingers of TFIIIA from bacteria. I monitored the interaction of these TFIIIA proteins with the 5S RNA gene by DNase I and methylation protection footprinting. These experiments indicated that fingers 1 to 5 were solely responsible for the footprint observed with full-length TFIIIA. Fingers 6 through 9 and the 81-amino acid domain were not in tight association with DNA. The amino-terminal four zinc fingers spanned the ICR of the 5S RNA gene and finger five made contacts in the major groove of DNA upstream of the ICR. Measurements of the apparent $K_d$ values of the TFIIIA-DNA complexes indicated that the amino-terminal three zinc fingers of TFIIIA had a binding energy similar to that of the full-length protein.

As a first step in understanding the role of the 81-amino acid domain of yeast TFIIIA in establishing an active transcription complex, I identified amino acids in this domain that are essential for the transcription factor activity of TFIIIA. I analyzed versions of TFIIIA containing mutations in this domain for their ability to support *in vitro* transcription of the 5S RNA gene and to support cell viability. Analysis of the effect of internal deletions indicated
that a short leucine-rich segment ($^{352}$NGLNLLLN$^{359}$) at the carboxyl-end of the 81-amino acid domain was essential for the ability of TFIIIA to support transcription of the 5S RNA gene in vitro and in vivo. Analysis of the effect of double and quadruple mutations in the region from a.a. 336 to a.a. 364 confirmed that hydrophobic residues in this region, particularly L343, L347, L354, L356, L357, and L358, contributed to the ability of TFIIIA to support transcription of the 5S RNA gene. These hydrophobic residues may play a role in mediating an interaction between TFIIIA and another component of the transcriptional machinery. I also assessed the effect of mutations in zinc fingers 8 and 9, which flank the 81-amino acid domain. I found that TFIIIA remained active if either finger 8 or finger 9 was disrupted by mutation, but that TFIIIA containing a disruption of both these fingers was inactive.

I then analyzed the effect of mutations that disrupt the structures of the amino-terminal zinc fingers of TFIIIA. I found that disruption of the first zinc finger of TFIIIA abolished the ability of TFIIIA to promote transcription of the 5S RNA gene in vitro and to support cell viability. TFIIIA proteins containing a disruption of the second or third zinc finger bound the 5S RNA gene less efficiently than did the version of TFIIIA containing a disruption of the first zinc finger, but they nonetheless supported in vitro transcription of the 5S RNA gene and supported cell viability. These data suggest that the first zinc finger of TFIIIA may be the only finger that is essential for the recruitment of TFIIIC to the initiation complex.
Acknowledgments

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Of course, Shelley deserves special mention. As my labmate, she helped me with nearly all aspects of my studies and I would never have survived graduate studies without her. As my wife and best friend, she makes my life complete.

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LIST OF ABBREVIATIONS

bp  base pair(s)
dNTPs  deoxynucleoside triphosphates
DSE  distal sequence element
EBER  Epstein-Barr encoded RNA
EDTA  ethylenediaminetetraacetate
EMSA  electrophoretic mobility shift assay
5-FOA  5-fluoro-orotic acid
HEPES  N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hTFIIIA  human TFIIIA
ICR  internal control region
IE  intermediate element
kDa  kilodaltons
N3RdUMP  5-[N-(p-azidobenzoyl)-3-aminoallyl]-dUMP
NMR  nuclear magnetic resonance
nt  nucleotides
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pol I  RNA polymerase I
pol II  RNA polymerase II
pol III  RNA polymerase III
PSE  proximal sequence element
rRNA  ribosomal RNA
SDS  sodium dodecyl sulfate
snRNA  small nuclear RNA
TBP  TATA binding protein
TF  transcription factor (e.g. TFIIIA = transcription factor IIIA)
Tris  Tris(hydroxymethyl)aminomethane
tRNA  transfer RNA
VA  viral associated
xTFIIIA  Xenopus TFIIIA
yTFIIIA  Saccharomyces cerevisiae or yeast TFIIIA
zf  zinc finger

xii
**LIST OF *SACCHAROMYCES CEREVISIAE* GENE NAMES**

<table>
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CHAPTER 1

Introduction
Eukaryotes have three nuclear RNA polymerases, each dedicated to the transcription of a subset of genes (reviewed in Chambon, 1975). RNA polymerase I transcribes genes coding for the 5.8S, 18S, and 28S ribosomal RNA (rRNA) molecules. RNA polymerase II transcribes genes coding for proteins and for many of the small nuclear RNAs (snRNAs). RNA polymerase III transcribes genes coding for small non-translated RNAs involved in translation (tRNAs and 5S rRNA) and various aspects of RNA metabolism such as splicing. These three nuclear RNA polymerases were first separated by chromatography on a column of DEAE-Sephadex and were named according to their order of elution from this resin (Roeder and Rutter, 1969). Although all three purified RNA polymerases bind DNA and synthesize RNA in a template-dependent manner, the purified enzymes initiate transcription randomly. Accurate initiation of transcription requires accessory transcription factors that assemble into multi-protein complexes at promoter sites; these complexes are responsible for recruitment of the appropriate RNA polymerase and for directing accurate initiation of transcription.

This thesis examines transcription of the 5S RNA gene of the yeast *Saccharomyces cerevisiae* by RNA polymerase III. In my studies, I have investigated the manner in which the yeast transcription factor IIIA (TFIIIA) binds to the promoter of the 5S RNA gene and promotes assembly of a functional transcription complex. This multi-factor transcription complex recruits RNA polymerase III for accurate initiation of transcription of the 5S RNA gene. In this INTRODUCTION, I provide an overview of transcription by RNA polymerase III (pol III). Because my research and most of the detailed characterization of pol III transcription factors and their assembly into preinitiation complexes has been done in *S. cerevisiae*, I emphasize work done with this organism. *S. cerevisiae* is a useful organism for studies on pol III transcription as it is relatively easy to manipulate both biochemically and genetically. For comparison, I also describe, as is appropriate, studies on pol III transcription done in other organisms. I first describe the pol III enzyme itself, the genes transcribed by
pol III, and the promoter elements of these genes. I next describe the transcription factors utilized by pol III and their assembly into multi-factor protein-DNA complexes. This is followed by a detailed account of the properties of TFIIIA itself, with an emphasis on TFIIIA from *Xenopus laevis* since a considerable amount of work has been dedicated to TFIIIA from this organism, especially in regards to its DNA-binding properties. I conclude this Chapter with a description of the recruitment of pol III to promoters; a summary of studies relating to initiation, elongation, termination, and reinitiation of transcription by pol III; comments on the effects of chromatin on pol III transcription; and observations on the regulation of pol III transcription through the cell cycle and in response to viral infection and to changes in growth conditions.

### 1.1 RNA Polymerase III

RNA polymerase III has been purified from a number of organisms including human (Jaehning et al., 1977), mouse (Sklar and Roeder, 1976), frog (Engelke et al., 1983; Roeder, 1983), silkworm (Sklar et al., 1976), fruitfly (Gundelfinger et al., 1980), wheat (Teissere et al., 1977), and yeast (Valenzuela et al., 1976; Hager et al., 1977) (reviewed in White, 1994). It is the largest of the nuclear RNA polymerases consisting of 9 to 16 subunits with an aggregate molecular mass of 600-700 kDa depending on the organism of origin. Pol III (also referred to as RNA polymerase C) from *S. cerevisiae* is composed of 16 subunits; nine of these subunits are unique to pol III (C160, C128, C82, C53, C37, C34, C31, C25, C11), two are shared with pol I (AC40 and AC19), and five are common to all three nuclear RNA polymerases (ABC27, ABC23, ABC14.5, ABC10α, ABC10β) (reviewed in Mosrin and Thuriaux, 1990 and Thuriaux and Sentenac, 1992). The subunits are named according to the RNA polymerase enzyme(s) (A=pol I, B=pol II, C=pol III) in which they are found and numbered according to their apparent molecular masses (kDa) as derived by SDS-PAGE analysis. The genes encoding 14 of these subunits have been identified; each of these
subunits is encoded by a different gene and each gene is essential for cell viability (Ingles et al., 1984; Allison et al., 1985; Riva et al., 1986; Mann et al., 1987; Archambault et al., 1990; Mosrin et al., 1990; Woychik et al., 1990; Woychik and Young, 1990; Carles et al., 1991; Dequard-Chablat et al., 1991; James et al., 1991; Mann et al., 1992; Stettler et al., 1992; Sadhale and Woychik, 1994). The two largest subunits of yeast pol III (C160 and C128) are similar in sequence to the two largest subunits of pol I and pol II (Allison et al., 1985; Sweetser et al., 1987; Memet et al., 1988; James et al., 1991). Furthermore, the two largest subunits of the yeast nuclear RNA polymerases have multiple regions of sequence similarity with the β' and β subunits of the *E. coli* RNA polymerase (Allison et al., 1985; Sweetser et al., 1987; Memet et al., 1988; James et al., 1991). Two of the smaller subunits, AC40 and AC19, are similar to the α subunit of *E. coli* RNA polymerase (Dequard-Chablat et al., 1991). The minimal catalytic core of the pol III enzyme likely consists of those subunits that are related to subunits of the *E. coli* RNA polymerase and of those subunits that are shared with pol I and pol II. The enzyme-specific subunits are likely responsible for recognition of pol III transcription complexes and possibly for enzyme-specific regulation. Indeed, three of the pol III-specific subunits of yeast (C82, C53, and C34) form a subcomplex (Werner et al., 1992; Werner et al., 1993) and C34 of this complex interacts with the pol III initiation factor TFIIB to establish polymerase selectivity (Werner et al., 1993; Khoo et al., 1994). Human pol III appears to possess a functionally related subcomplex that also consists of three subunits (Wang and Roeder, 1997).

### 1.2 The Genes Transcribed by RNA Polymerase III

Pol III transcribes a variety of genes that encode non-translated RNA molecules (for reviews see Geiduschek and Tocchini-Valentini, 1988; Willis, 1993; and White, 1994). These RNAs participate in cellular processes such as protein synthesis and RNA metabolism. Examples of RNAs synthesized by pol III that are components of the translational machinery
include: 5S rRNA, which is associated with the large subunit of ribosomes; the tRNAs; and the 7SL RNA, which forms the scaffold of the signal recognition particle. Examples of RNAs synthesized by pol III that contribute to RNA metabolism include: the yeast and vertebrate U6 small nuclear (sn)RNA and the plant U3 snRNA, which are involved in RNA splicing; the RNA component of RNase P (H1 RNA), which is required for processing the 5' termini of pre-tRNAs; and the RNA component of MRP, which is a mitochondrial RNase. RNA polymerase III is also responsible for the synthesis of the 7SK RNA present in the 12S ribonucleoprotein particle, the RNA component of the 150S cytoplasmic ribonucleoprotein particle known as vault RNA, and the RNA component of telomerase from *Tetrahymena thermophila*. Viral RNAs that are synthesized by pol III include the VA_I and VA_II transcripts of adenovirus and the EBER1 and EBER2 transcripts of Epstein-Barr virus. Pol III also transcribes a number of repetitive short interspersed elements (SINEs) such as the Alu gene family of humans, the B1 and B2 genes of mouse, and the BmX gene family of silkworm. The function of the SINE RNAs, if any, is presently unknown. For convenience, the genes transcribed by pol III are termed class III genes; similarly, the genes transcribed by pol I and pol II are termed class I and class II genes, respectively.

### 1.3 The Promoters of Genes Transcribed by RNA Polymerase III

The structures of promoters of class III genes have been reviewed in detail by Geiduschek and Tocchini-Valentini (1988), Gabrielsen and Sentenac (1991), Kunkel (1991), Geiduschek and Kassavetis (1992), Willis (1993), and White (1994). There is much diversity in the promoter organization of class III genes. Early studies revealed the presence of promoter elements within the transcribed portion of the genes. More recently, class III genes have been characterized that do not contain any intragenic promoter elements. For simplicity, the promoters of genes transcribed by RNA polymerase III have been divided into three types based on their structure and location (Geiduschek and Kassavetis, 1992; Willis, 1993) (see
Figure 1-1. Comparison of type 1, 2, and 3 promoters of genes transcribed by RNA polymerase III. A representative gene for each type of pol III promoter is depicted; the start site of transcription is denoted by an arrow; the transcribed portion of the gene is represented by a shaded rectangle; 5' and 3' flanking regions are represented by lines; and promoter elements are denoted by hatched boxes.

(A) The type 1 promoter of the *Xenopus* 5S RNA gene. This gene contains a tripartite internal control region (ICR) extending from nucleotides (nt) +50 to +97. The three promoter elements of the ICR are the A box (nt +50 to +64), the intermediate element (IE; nt +67 to +72), and the C box (nt +80 to +97) (Bogenhagen, 1985; Pieler *et al.*, 1985a, 1985b, 1987; Majowski *et al.*, 1987; McConkey and Bogenhagen, 1987).

(B) The type 2 promoter of a tRNA gene. This gene, exemplified by the *SUP4* tRNA<sup>Tyr</sup> gene of *Saccharomyces cerevisiae*, contains two internal promoter elements: an A box (nt +8 to +19) and a B box (nt +52 to +62) (Allison *et al.*, 1983).

(C) The type 3 promoter of the vertebrate U6 snRNA gene. The promoter of this gene consists of a TATA box element centered at nt -25, a proximal sequence element (PSE) centered at nt -60, and a distal sequence element (DSE) centered at nt -220 (Bark *et al.*, 1987; Carbon *et al.*, 1987; Das *et al.*, 1988; Kunkel and Pederson, 1988; Mattaj *et al.*, 1988; Lobo and Hernandez, 1989).
A type 1 promoter: the *Xenopus* 5S rRNA gene

A type 2 promoter: a tRNA gene

A type 3 promoter: the vertebrate U6 snRNA gene
The type 1 promoter, present only in 5S RNA genes, and the type 2 promoter, represented by the promoter of tRNA genes, consist of intragenic control elements. The type 3 promoter, represented by the vertebrate U6 snRNA gene, consists of extragenic control elements. The promoters of some pol III genes are mixed, containing both intragenic type 2 promoter elements and extragenic type 3 promoter elements (e.g., the S. cerevisiae U6 snRNA gene).

1.3.1 The type 1 promoter of the 5S RNA gene

The promoter of the Xenopus somatic 5S RNA gene has been studied extensively and serves as the prototype promoter for the vertebrate 5S RNA genes (reviewed in Geiduschek and Tocchini-Valentini, 1988). Analysis of the effects of progressive deletions of 5' and 3' flanking and coding sequences of a Xenopus 5S RNA gene on its transcription revealed the presence of an internal control region (ICR) (Sakonju et al., 1980; Bogenhagen et al., 1980). These early studies also indicated that the start site of transcription of the 5S RNA gene is primarily dictated by the position of the ICR. Transcription initiates approximately 50 bp upstream of the 5' end of the ICR regardless of the sequence of the 5' flanking region of the gene. Subsequent studies analyzing the effect of point mutations within the Xenopus 5S RNA gene on in vitro transcription of that gene defined a 48-bp ICR extending from nt +50 to +97 (Pieler et al., 1985a, 1985b, 1987; Majowski et al., 1987; McConkey and Bogenhagen, 1987). These studies also revealed that the ICR contains three separate elements that contribute to efficient transcription of the gene: the A box (nt +50 to +64), an intermediate element (IE; nt +67 to +72), and the C box (nt +80 to +97) (see Fig. 1-2). Alterations in the spacing between the three elements reduce or eliminate transcription of the gene (Bogenhagen, 1985; Pieler et al., 1987). This tripartite ICR is the site of interaction of Xenopus TFIIIA with the 5S RNA gene (Engelke et al., 1980; see also section 1.5.1.3 below). Non-essential intragenic control elements that contribute to the efficiency of transcription
Figure 1-2. Comparison of the sequences and the essential promoter elements of 5S RNA genes from various organisms. The sequences of the non-transcribed strands of 5S RNA genes from *Xenopus laevis* (X.l.), *Saccharomyces cerevisiae* (S.c.), *Neurospora crassa* (N.c.), and *Drosophila melanogaster* (D.m.) are shown. The numbers above the sequences indicate the positions of the bases relative to the start site of transcription. Spaces, denoted by dashes, were introduced into the sequences of the 5S RNA genes from *X. laevis*, *N. crassa*, and *D. melanogaster* to preserve sequence alignment. Therefore, the numbering system perfectly matches only the sequence of the *S. cerevisiae* 5S RNA gene. The sequence of the *X. laevis* somatic 5S RNA gene was obtained from Peterson *et al.* (1980), the sequence of the *S. cerevisiae* 5S RNA gene from Valenzuela *et al.* (1977), the sequence of the *N. crassa* α-type 5S RNA gene from Selker *et al.* (1981), and the sequence of the *D. melanogaster* 5S RNA gene from Sharp *et al.* (1984). The essential promoter elements of the four 5S RNA genes are denoted by open rectangles. These elements were defined by analyzing the effects of deletion mutations, linker scanner mutations, and/or point mutations on transcription of the respective 5S RNA gene (see text for details). It should be noted, therefore, that the precision with which the elements are defined differ in each case. The box C promoter element, which serves as an essential promoter element in all 5S RNA genes thus far studied, is shaded.
under some *in vitro* conditions have been identified between the transcription start site and the ICR (Keller *et al.*, 1990; Wolffe and Morse, 1990).

The one conserved feature of all 5S RNA genes studied to date is the presence of a C-box element within the ICR. Other than this, the promoter elements of the 5S RNA genes from different organisms can be quite different from each other. For example, the 5S RNA genes of *Drosophila* and *Neurospora* have ICRs that contain A-box and C-box elements, but that do not contain an intermediate element (Tyler, 1987; Sharp and Garcia, 1988) (see Fig. 1-2). The 5S RNA genes of both these species also contain additional internal promoter elements that are not found in the *Xenopus* 5S RNA gene. The 5S RNA gene of *Drosophila* contains two additional promoter elements (nt +3 to +18 and nt +37 to +44) that make significant contributions to transcription efficiency (Sharp and Garcia, 1988). Mutations in an internal element (nt +19 to +30) of the *Neurospora* 5S RNA gene, which is similar in sequence to a promoter element found upstream of the pol I-transcribed 40S rRNA gene of *Neurospora*, abolish 5S RNA synthesis *in vitro* (Tyler, 1987). In addition, the *Drosophila* and *Neurospora* 5S RNA genes have essential promoter elements, which resemble a TATA-box motif, about 30 nucleotides upstream of the start sites of transcription (Tyler, 1987; Sharp and Garcia, 1988). In *Neurospora*, this upstream promoter element has a strong effect in determining the start site of transcription for the 5S RNA gene (Tyler, 1987). The 5' flanking region of the 5S RNA gene of *Bombyx mori* (silkworm) is also essential for transcription in an homologous cell-free extract (Morton and Sprague, 1984).

The ICR of the 5S RNA gene of *S. cerevisiae*, as determined by linker-scanning mutational analysis, is a relatively short 14-bp sequence extending from nt +81 to +94 (Challice and Segall, 1989). This ICR corresponds roughly in sequence and position to the C-box element of the tripartite ICR of the *Xenopus* 5S RNA gene. Although the sequence of the yeast 5S RNA is similar to that of the *Xenopus* 5S RNA gene in the region corresponding to the A box and IE, these regions of the yeast 5S RNA gene are not essential for its transcription *in vitro* (Challice and Segall, 1989). However, it is possible that an extended
ICR is required for efficient transcription of the yeast 5S RNA gene in vivo (Lee et al., 1995). The yeast 5S RNA gene has a second promoter element, termed start site element (sse), that spans the transcription start site from nt -14 to +8 (Challice and Segall, 1989). Although the sse is required for efficient transcription of the 5S RNA gene, it does not appear to dictate the start site of transcription (Taylor and Segall, 1985; Challice and Segall, 1989). Altering the spacing between the sse and the ICR by more than a few base pairs significantly reduces transcription of the 5S RNA gene (Challice and Segall, 1989).

1.3.2 The type 2 promoter of tRNA genes

Genes that contain type 2 promoters include the tRNA genes, the 7SL gene, the Alu genes, and the VA RNA genes of adenovirus (reviewed in Geiduschek and Tocchini-Valentini, 1988 and White, 1994). The type 2 promoters are characterized by two ~10-bp intragenic elements, termed the A and B boxes, which are separated by a variable number of nucleotides (see Fig. 1-1). For example, the A box of the SUP4 tRNA^{Tyr} gene of S. cerevisiae extends from nt +8 to +19 and the B box extends from nt +52 to +62 (Allison et al., 1983). The distance between the A and B boxes ranges from about 30 to 90 bp among natural tRNA genes (Sharp et al., 1985; Geiduschek and Tocchini-Valentini, 1988). The variations in spacing are generally accounted for by the presence of introns in the primary transcripts or extra loops in the mature tRNAs. Although maximal transcription activity in vitro occurs when the A and B boxes are separated by 30 to 60 bp, artificially created separations as short as 14 bp and greater than 300 bp are tolerated, albeit with a significant decrease in promoter strength (Baker et al., 1987; Fabrizio et al., 1987). In contrast to the variability in spacing between the A and B boxes, the distance between the A box and the start site of transcription is constant at about 8 to 12 bp. The A and B boxes comprise the binding site for TFIIIC on the tRNA gene with the position of the A box dictating the start site of transcription (Ciliberto et al., 1983; Baker et al., 1987; Fabrizio et al., 1987) (see section 1.4.1.1 below).
The A and B boxes were defined for several genes by examining the effects of deletion and substitution mutations on the efficiency of either in vitro or in vivo transcription (reviewed in White, 1994). Studies of mutant tRNA genes from Xenopus (Galli et al., 1981; Hofstetter et al., 1981), D. melanogaster (Sharp et al., 1981), C. elegans (Ciliberto et al., 1982), and S. cerevisiae (Allison et al., 1983) and of mutant VA genes (Wu et al., 1987; Railey and Wu, 1988) reveal that the sequences of the A- and B-box elements are well conserved among all type 2 promoters. The consensus A-box and B-box sequences are TRGCNNAGY(N)GG and GGTTCGANTCC, respectively (Galli et al., 1981; Geiduschek and Tocchini-Valentini, 1988). It should be noted that the A- and B-box sequences encode the D- and T-stem/loops, respectively, of the mature tRNA (Gauss and Sprinzl, 1981). Therefore, the A- and B-box consensus sequences likely reflect selection for both tRNA and promoter functions. Although the A-box sequences of tRNA genes are similar to the A-box sequences of 5S RNA genes (Ciliberto et al., 1983; Geiduschek and Kassavetis, 1992), this sequence similarity is unlikely to be functionally relevant since the positions of the A-boxes differ in the two types of genes and the transcription factors that interact with them differ (see sections 1.4.1.1 and 1.5.1.3 below).

Sequences 5' to the start site have some influence on transcription of most, if not all, tRNA genes. Examples have been reported for genes from Saccharomyces cerevisiae (Shaw and Olsen, 1984; Raymond et al., 1985), Drosophila melanogaster (Schaack et al., 1984; Sajjadi and Spiegelman, 1987), Bombyx mori (Sprague et al., 1980; Larson et al., 1983), Xenopus (Hipskind and Clarkson, 1983), and Homo sapiens (Arnold et al., 1986). The 5' elements that affect transcription of a tRNA gene are generally located within 50 bp of the start site. Usually, the 5' flanking sequences have a stimulatory effect on transcription, but repressive effects can also occur. The 3' flanking regions of tRNA genes from Drosophila melanogaster (Schaack et al., 1984), Bombyx mori (Wilson et al., 1985), and Saccharomyces cerevisiae (Allison and Hall, 1985) have also been found to influence the efficiency of transcription.
1.3.3 The type 3 promoter of the vertebrate U6 snRNA gene

In contrast to the predominately intragenic type 1 and 2 promoters, type 3 promoters consist of extragenic elements. The prototypical genes containing a type 3 promoter are the vertebrate U6 snRNA genes and the human 7SK gene. The entire transcribed region of these genes, including a sequence similar to an A-box element, can be removed with no apparent effect on the efficiency of transcription in vitro (Murphy et al., 1987; Das et al., 1987) or in vivo (Kleinert and Benecke, 1988; Kunkel and Pederson, 1989; Lobo and Hernandez, 1989).

Although the vertebrate U6 snRNA gene is transcribed by pol III (Kunkel et al., 1986; Krol et al., 1987; Reddy et al., 1987), its promoter is similar to other snRNA genes (U1-U5) that are transcribed by pol II (reviewed in Hernandez, 1992). All of the vertebrate snRNA genes contain a proximal sequence element (PSE) centered at nt -60 and a distal sequence element (DSE) centered at nt -220 (reviewed in Kunkel, 1991 and Hernandez, 1992). Mutational analysis of the PSE indicates that it is essential for transcription of the U6 gene (Carbon et al., 1987; Das et al., 1988; Kunkel and Pederson, 1988; Mattaj et al., 1988; Lobo and Hernandez, 1989). The DSE operates as an enhancer of expression of the U6 snRNA gene (Bark et al., 1987; Carbon et al., 1987; Das et al., 1988; Kunkel and Pederson, 1988). The DSE enhances pol III transcription only in the presence of the PSE (Parry and Mattaj, 1990). The PSE (Lobo and Hernandez, 1989; Parry et al., 1989) and DSE (Bark et al., 1987; Kunkel and Pederson, 1988) motifs from U6 and U2 snRNA genes are interchangeable. In addition to the PSE and the DSE, the U6 snRNA gene contains a TATA box at nt -25 that is essential for its transcription by pol III (Mattaj et al., 1988; Lobo and Hernandez, 1989). A TATA box element is not present in the pol II-transcribed U1-U5 snRNA genes. Interestingly, it is the presence of the TATA box motif that specifies an snRNA gene to be transcribed by pol III; mutation of the TATA box in the U6 snRNA gene causes it to be transcribed by pol II (Mattaj et al., 1988; Lobo and Hernandez, 1989) and insertion of a TATA box into the U2 snRNA gene causes it to be transcribed by pol III (Lobo and Hernandez, 1989). Both the PSE and the TATA box of the U6 snRNA gene are involved in
start site selection, although neither element exerts a dominant role (Parry and Mattaj, 1990; Goomer and Kunkel, 1992). For pol II-transcribed snRNA genes, the PSE alone takes on the role of determining the start site of transcription (Skuzeski et al., 1984).

1.3.4 The mixed promoter of the U6 snRNA gene of *S. cerevisiae*

The *S. cerevisiae* U6 snRNA gene (SNR6) contains both internal and external promoter elements: an intragenic A-box element positioned as in tRNA genes, a B-box element positioned at an unusually distant location 234 bp downstream of the transcription start site, and a consensus TATA box element at nt -30 (Brow and Guthrie, 1988; Brow and Guthrie, 1990; Bordonné and Guthrie, 1992; Burnol et al., 1993b; Eschenlauer et al., 1993). Sequences upstream of the TATA box of the SNR6 gene, including a sequence with similarity to the PSE of vertebrate U6 snRNA genes, have no apparent role in transcription of the gene *in vivo* or *in vitro* (Burnol et al., 1993b; Eschenlauer et al., 1993). The promoter requirements of the SNR6 gene differ depending on the transcription conditions. Transcription *in vivo* is dependent on the presence of the A- and B-box elements as is also the case for transcription *in vitro* using a crude cell extract (Brow and Guthrie, 1990; Burnol et al., 1993b; Eschenlauer et al., 1993). Although the TATA box is not essential for transcription *in vivo*, its removal reduces transcript accumulation and results in an altered start site of transcription (Burnol et al., 1993b; Eschenlauer et al., 1993; Gerlach et al., 1995). The A box has a major role in start site selection *in vivo* (Eschenlauer et al., 1993; Gerlach et al., 1995). In contrast, *in vitro* transcription of a naked SNR6 template in the presence of purified components does not require the A- and B-box elements (Margottin et al., 1991; Burnol et al., 1993b; Joazeiro et al., 1994). These *in vitro* conditions permit TFIIIB to direct its own assembly onto the SNR6 gene via interactions with the TATA box (see section 1.4.3 below).
1.4 Transcription Factors Utilized by RNA Polymerase III and Assembly of Transcription Complexes on Class III Genes

Purified RNA polymerase III initiates transcription randomly on naked DNA templates (Jaehning et al., 1977; Parker and Roeder, 1977; Sklar and Roeder, 1977). Accurate initiation of transcription in vitro by pol III requires the participation of transcription factors, which are present in a crude extract of cells, that serve to recruit pol III to the start site of transcription (Parker and Roeder, 1977; Wu, 1978; Ng et al., 1979; Weil et al., 1979; Segall et al., 1980). These factors were first identified by chromatographic fractionation of a crude extract prepared from human KB cells (Segall et al., 1980). Two factors, designated TFIIB and TFIIC because they elute from a phosphocellulose column in the PC-B (0.1-0.35 M KCl) and PC-C (0.35-0.6 M KCl) fractions, respectively, were found to be required for reconstitution of accurate transcription of tRNA and VA RNA genes in vitro. A third factor, which elutes from the phosphocellulose column in the flowthrough (PC-A) and which is designated TFIIB, was found to be required in addition to TFIIB and TFIIC for specific in vitro transcription of the 5S RNA gene. Factors of similar function have since been identified on fractionation of extracts derived from cells of Xenopus (Shastry et al., 1982), mouse (White et al., 1990), silkworm (Ottonello et al., 1987), Drosophila (Burke et al., 1983, 1985), and S. cerevisiae (Klekamp and Weil, 1982; Taylor and Segall, 1985).

In vitro studies with relatively crude mammalian, amphibian, and yeast factors have shown that these factors can assemble sequentially onto their target genes to form stable preinitiation complexes that recruit pol III to the start sites of transcription (Lasser et al., 1983; Bieker et al., 1985; Setzer and Brown, 1985; Carey et al., 1986; Segall, 1986). The order of assembly has been deduced principally from template commitment assays (Lasser et al., 1983; Bieker et al., 1985; Segall, 1986). For this assay, various subsets of transcription factors, one of which is limiting, are preincubated with a saturating amount of one template. A second functionally equivalent template, whose transcripts can be distinguished from those
of the first template, is then added with the remaining factors. If only the first template is transcribed, then the limiting factor must have been stably sequestered on this template during the preincubation. On both tRNA and VA RNA genes, the first step in the assembly of an initiation complex is the stable binding of TFIIC to the DNA template. The prebound TFIIC then allows sequestration of TFIIB into the complex. The TFIIB-TFIIC-tDNA complex then recruits RNA polymerase III. On the 5S RNA gene, TFIIB is the first factor to bind the DNA template. Subsequent incorporation of TFIIC stabilizes the TFIIB-tDNA interaction and allows recruitment of TFIIC. Finally, the TFIIB-TFIIC-TFIIB-tDNA complex serves to recruit pol III to the gene.

Recent studies have elaborated on the subunit structure of the transcription factors and the molecular architecture of the transcription complexes. Most of this detailed information has come from studies with factors from the yeast S. cerevisiae. Therefore, I first describe the transcription complexes formed on class III genes in yeast. I then give an overview of studies with mammalian and amphibian TFIIC and TFIIB. A detailed description of TFIIB, which is the subject of my thesis, is presented separately in Section 1.5.

1.4.1 Assembly of a transcription complex on an S. cerevisiae tRNA gene

1.4.1.1 TFIIC and its interaction with the tRNA genes

Properties of yeast TFIIC - Yeast TFIIC, also called τ, binds to tRNA genes. It protects the entire length of these genes against cleavage by DNase I (Stillman and Geiduschek, 1984; Camier et al., 1985; Kassavetis et al., 1989). Because mutations in the A-box and B-box elements of tRNA genes, but not elsewhere, decrease the gene's affinity for TFIIC, Baker and colleagues (Baker and Hall, 1984; Baker et al., 1986) concluded that most of the site-specific interactions between TFIIC and tRNA genes occur within the A-box and the B-box elements. This conclusion was supported by the results of a methylation protection analysis of a TFIIC-tDNA complex (Camier et al., 1985). The mutational
analyses indicated that the interaction of TFIIIC with the B box provides most of the affinity for the TFIIIC-tDNA interaction; the interaction with the A box was found to make only a minor contribution to the stability of the TFIIIC-tDNA complex (Baker et al., 1986). Also, a plasmid containing the 3' half, that is the B-box portion, of a tRNA gene was found to compete efficiently for the TFIIIC-tDNA interaction, whereas the 5' half, or A-box portion, of a tRNA gene did not (Stillman and Geiduschek, 1984). Additional experiments revealed that the role of the A box in transcription of a tRNA gene is in start-site selection rather than in recruitment of TFIIIC (Ciliberto et al., 1983; Baker et al., 1987) and that the TFIIIC-A box interaction serves to appropriately position TFIIIC at the 5' end of the gene (see below).

Yeast TFIIIC is a large, multi-protein complex. Selective proteolysis experiments indicated that TFIIIC (τ) consists of two subcomplexes called τ_A and τ_B (Marzouki et al., 1986). The τ_B subcomplex binds to the B box with high affinity while the τ_A subcomplex binds with lower affinity to the A box (Marzouki et al., 1986). UV-induced crosslinking of TFIIIC-tDNA complexes revealed that polypeptides of 95 kDa and 138 kDa interact with the A box and B box, respectively (Gabrielsen et al., 1989; Bartholomew et al., 1990). TFIIIC appears to be remarkably flexible. It can bind tDNA constructs that have widely different A-to B-box distances (Baker et al., 1987; Fabrizio et al., 1987). Moreover, the relative orientation of the A and B boxes on the DNA helix is not important (Baker et al., 1987). This flexibility may reflect the loose tethering between the τ_A and τ_B subcomplexes of TFIIIC (Camier et al., 1990). Another property of TFIIIC is its ability to bend DNA (Stillman et al., 1985; Léveillard et al., 1991). Using methods based on the anomalous electrophoretic mobility of bent DNA in a tight gel network, it was found that the binding of TFIIIC to a tRNA gene induces a bend in the DNA of 90° to 100° centered near the start site of transcription (Léveillard et al., 1991).

Yeast TFIIIC was initially estimated to have a molecular mass of ~300 kDa based on glycerol gradient centrifugation (Ruet et al., 1984) and gel filtration experiments (Stillman et al., 1985). Analysis by scanning transmission electron microscopy, however, suggested that
each of the two globular subcomplexes of TFIIIC was ~300 kDa, giving a total molecular mass of ~600 kDa (Schultz et al., 1989). This larger mass is consistent with the polypeptide composition of yeast TFIIIC that has been extensively purified by ion-exchange chromatography followed by affinity chromatography on specific tDNA or B-box columns (Camier et al., 1985; Kassavetis et al., 1989; Gabrielsen et al., 1989; Parsons and Weil, 1990; Bartholomew et al., 1990; Swanson et al., 1991). Six polypeptides with apparent molecular masses of 138(145), 131(135), 95(100), 90(91), 62(65), and 55 kDa as estimated by SDS-PAGE analysis consistently copurify with TFIIIC transcription factor activity and DNA-binding activity (Gabrielsen et al., 1989; Parsons and Weil, 1990; Bartholomew et al., 1990; Swanson et al., 1991). The four largest of these polypeptides have been shown to be authentic components of TFIIIC by biochemical, immunological, genetic, and cloning approaches.

Subunits of TFIIIC - The genes encoding the subunits of 138, 131, 95 and 90 kDa of yeast TFIIIC have been identified (Swanson et al., 1991; Parsons and Weil, 1992; Lefebvre et al., 1992; Marck et al., 1993; Arrebola et al., 1998). The four genes are single copy and are essential for cell viability. In support of a role in DNA-binding, the 138-kDa subunit or B-box-binding subunit is highly basic and contains two regions, one at the amino-terminal end of the protein and the other at the carboxyl-terminal end, that resemble the HMG box (Lefebvre et al., 1992). The HMG box is a common DNA-binding motif first found in the high-mobility group proteins (reviewed in Ner, 1992). Other studies suggest, however, that the DNA-binding domain of the 138-kDa subunit of TFIIIC resides outside the putative HMG boxes and in the middle of the protein (Lefebvre et al., 1994; Marsolier et al., 1997). The remainder of the deduced amino acid sequence of the 138-kDa subunit did not reveal any extensive sequence similarity to other known proteins (Lefebvre et al., 1992). The deduced amino acid sequence of the 131-kDa subunit reveals the presence of eleven copies of the 34-

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1 The two masses given, one of them in brackets, reflects the different size estimates that were reported for these polypeptides from different laboratories. From herein, I use the non-bracketed size estimate when referring to an individual subunit of TFIIIC.
amino acid tetratricopeptide repeat (TPR) motif (Marck et al., 1993). TPR units are thought to interact with each other and together serve as a protein-protein interaction domain (reviewed in Lamb et al., 1995). The 131-kDa subunit also contains a putative helix-loop-helix (HLH) dimerization motif preceded by a basic sequence resembling the DNA-binding domain of many HLH-containing transcription factors. The 95-kDa subunit contains a potential helix-turn-helix DNA-binding motif as well as a highly acidic region (Swanson et al., 1991; Parsons and Weil, 1992). Although the 95-kDa and 138-kDa subunits directly interact with tDNA as shown by the photocrosslinking experiments described below, versions of these proteins produced by bacteria or by in vitro-synthesis do not bind DNA on their own (Swanson et al., 1991; Parsons and Weil, 1992; Lefebvre et al., 1992). It is possible that the DNA-binding properties of these proteins are only realized in the context of an intact TFIIIC complex. Indeed, genetic studies suggest that the 90-kDa subunit stabilizes the interaction of the 138-kDa subunit with DNA (Arrebola et al., 1998). Also, post-translational modifications of these proteins may be necessary for their DNA-binding activities. In this regard, it has been observed that the 138, 131, and 95 kDa subunits of TFIIIC are phosphorylated \textit{in vivo} (Conesa et al., 1993).

**Topography of TFIIIC subunits in the TFIIIC-tDNA complex** - The topography of TFIIIC subunits on a yeast tRNA gene has been analyzed in detail by DNA-protein photocrosslinking with templates in which a photoactive nucleotide, such as 5-[N-(p-azidobenzoyl)-3-aminoallyl]-dUMP (N3RdUMP), has been enzymatically incorporated at specific positions in association with one or more radioactive nucleotides (Bartholomew et al., 1990, 1991). For these photocrosslinking experiments, protein-DNA complexes are exposed to ultraviolet light and the resultant nucleotide-derived nitrene rapidly forms a covalent bond with adjacent polypeptides. The N3RdUMP probe places the photoactive substituent approximately 0.9 to 1.0 nm away from the pyrimidine ring on a relatively stiff tether and allows the space just outside the DNA major groove to be probed for nearby proteins. The 138-kDa subunit is accessible for reaction with N3RdUMP residues located in
Figure 1-3. Transcription factor topography on the *Saccharomyces cerevisiae* tRNA and 5S RNA genes. Transcription factor complexes formed on a tRNA gene (A) and the 5S RNA gene (B) of *Saccharomyces cerevisiae* are depicted as deduced from DNase I footprinting and protein-DNA photocrosslinking data (adapted from Braun et al., 1992a). The three subunits of TFIIIB, which are TFIIIB70/Brf1 (labeled 70 kDa), the TATA binding protein (labeled TBP), and TFIIIB90/Tfc5 (labeled 90 kDa), are shaded light gray. The six subunits of TFIIIC, which have apparent molecular weights of 138, 131, 95, 90, 62, and 55, are labeled by molecular mass and are shaded medium gray. TFIIIA, which is specific for the 5S RNA gene, is shaded dark gray. The arrows denote a region of DNA that is in the vicinity of a subunit, as assessed by protein-DNA photocrosslinking (for a detailed summary of the photocrosslinking data see Braun et al., 1992a). Note that many of the protein-protein interactions that are depicted are hypothetical ones.

(A) The approximate positions of subunits of TFIIIC and TFIIIB on a tRNA gene. In the 3' half of the tRNA gene, the 138-kDa subunit of TFIIIC interacts with DNA in the region of the B-box element, and the 90-kDa subunit of TFIIIC interacts with DNA near the transcription terminator of the tRNA gene. The 95- and 55-kDa subunits of TFIIIC are in the vicinity of the A box, likely on opposite sides of the helix. The 131-kDa subunit of TFIIIC extends along DNA from the A box to upstream of the transcription start site. The position of the 62-kDa subunit of TFIIIC in relation to the other subunits is unclear as it was not photocrosslinked to any of the tDNA probes used. The three subunits of TFIIIB are all located in the 5' flanking region of the tRNA gene.

(B) The approximate locations of TFIIIA and the subunits of TFIIIC and TFIIIB on the 5S RNA gene. The positions of the subunits of TFIIIC on the 5S RNA gene are similar to those observed on the tRNA gene, with the exception of the 55-kDa subunit, which was not photocrosslinked to any of the 5S DNA probes used. On the 5S RNA gene, however, TFIIIA extends along DNA from the region of the C-box element (nt +81 to +94) to about nt +50, and serves as an adaptor to recruit TFIIIC to the appropriate position on the gene. The TFIIIB subunits are found in analogous positions to those in the tRNA gene transcription complex, upstream of the transcription start site. The interactions shown between TFIIIA and subunits of TFIIIC are hypothetical.
the vicinity of the B-box (Bartholomew et al., 1990). This is consistent with the observation that antibodies against the 138-kDa subunit recognize τB-tDNA complexes (Gabrielsen et al., 1989). Photocrosslinking analysis indicates that the 95-kDa and 55-kDa subunits of TFIIC are in the vicinity of the A-box element and are likely located on opposite sides of the DNA helix (Bartholomew et al., 1990). The presence of the 95-kDa subunit in τA has been confirmed by electron microscopy (Conesa et al., 1993). The 131-kDa subunit can be crosslinked to residues in the region of the A box (Bartholomew et al., 1990) and to residues upstream of the start site of transcription (Bartholomew et al., 1991). The 90-kDa subunit is at the 3' end of the gene (Braun et al., 1992a). A summary of the topography of the subunits of yeast TFIIC on a tRNA gene is diagrammed in Figure 1-3A.

1.4.1.2 TFIIB and its recruitment to the TFIIC-tDNA complex

Properties of yeast TFIIB - Recruitment of yeast TFIIB to a tRNA gene requires that TFIIC already be bound to the gene (Kassavetis et al., 1989). Addition of purified yeast TFIIB to a TFIIC-tDNA complex results in extension of the DNase I footprint to include ~40 bp of contiguous 5' flanking sequence (Kassavetis et al., 1989). A similar DNase I protection pattern is observed in the 5' flanking region of a tRNA gene in vivo (Huibregtse and Engelke, 1989). Whereas binding of TFIIC is complete within about a minute (Camier et al., 1990; Kassavetis et al., 1989), binding of TFIIB requires ~20 minutes to reach completion (Kassavetis et al., 1989). Once recruited, however, TFIIB binds tightly and stabilizes the interaction of TFIIC with DNA. A TFIIC-tDNA complex has a half life of 17 minutes, whereas a TFIIB-TFIIC-tDNA complex has a half life of more than 10 hours (Kassavetis et al., 1989). Once bound to DNA, TFIIB is resistant to dissociation by high salt concentrations and to displacement by the polyanion heparin; conditions which readily strip TFIIC from the TFIIB-TFIIC-tDNA complex (Kassavetis et al., 1989, 1990). In the resultant TFIIB-tDNA complex, TFIIB remains positioned upstream of the start site of transcription and suffices to direct multiple rounds of accurate transcription by pol III
Another feature of TFIIIB binding is that DNA becomes bent by about 120° (Léveillard et al., 1991; Braun et al., 1992b). This bend is centered in the middle of the TFIIIB-binding site (Léveillard et al., 1991). The significance of this bending for pol III transcription is currently unknown, but it may be important for recognition of the TFIIIB-DNA complex by pol III and/or the rate of open complex formation.

**Subunits of yeast TFIIIB** - Yeast TFIIIB consists of three proteins: the TATA-binding protein (TBP); a 70-kDa polypeptide termed TFIIIB70 (or Brf1); and a 90-kDa polypeptide termed TFIIIB90 (or Tfc5) (Kassavetis et al., 1992b, 1995; reviewed in White, 1994). Purification of TFIIIB from yeast revealed that it could be chromatographically separated into two components, termed B' and B" (Kassavetis et al., 1991). The B' fraction contains TBP and TFIIIB70, and the B" fraction contains TFIIIB90 (Kassavetis et al., 1991, 1992b). Both of these fractions are necessary, in addition to purified TFIIIC and pol III, for in vitro transcription of a tRNA gene. The 70 and 90-kDa polypeptides were first identified by DNA-protein photocrosslinking analysis of TFIIIB-tDNA complexes (Bartholomew et al., 1991). TFIIIB70 preferentially crosslinks to the tRNA gene between nt -5 and -35; TFIIIB90 preferentially crosslinks further upstream between nt -30 and -40 (Bartholomew et al., 1991; Kassavetis et al., 1991) (see Fig. 1-3A). Several independent lines of evidence led to the realization that the TATA-binding protein (TBP), which was previously known to be the DNA-binding component of the pol II transcription factor TFIIID, is a component of yeast TFIIIB (Margottin et al., 1991; Huet and Sentenac, 1992; Kassavetis et al., 1992b; Poon and Weil, 1993; reviewed in Hernandez, 1993). These studies of yeast TFIIIB were paralleled by studies that indicated that TBP is a subunit of human TFIIIB (Lobo et al., 1992; Simmen et al., 1992; Taggert et al., 1992; White and Jackson, 1992). Indeed, in vivo and in vitro studies indicate that TBP is required for transcription of all nuclear genes in *S. cerevisiae* (Cormack and Struhl, 1992; Schultz et al., 1992; Poon et al., 1993). Studies with recombinant proteins established that reconstitution of TFIIIB activity requires TBP, TFIIIB70, and TFIIIB90 (Kassavetis et al., 1995; Roberts et al., 1996; Rüth et al., 1996).
The gene encoding the 70-kDa subunit of yeast TF\textsubscript{mB} was cloned by two separate groups as an allele-specific, high-copy suppressor of temperature sensitive mutations in TBP (Buratowski and Zhou, 1992; Colbert and Hahn, 1992). The gene was also independently isolated as a suppressor of a mutation in the A box of a tRNA gene (López-De-León \textit{et al.}, 1992). The deduced amino acid sequence revealed that the amino-terminal half of TF\textsubscript{mB}70 is related to the pol II basal transcription factor TF\textsubscript{II}B. The region of similarity between the two proteins is characterized by a putative zinc finger motif, followed by two imperfect repeats. A major difference between TF\textsubscript{II}B and TF\textsubscript{mB}70 is the presence of a carboxyl-terminal extension in TF\textsubscript{mB}70 that almost doubles the size of the polypeptide when compared to TF\textsubscript{II}B. Deletion of the carboxyl-terminal portion of TF\textsubscript{mB}70 abolishes its ability to function \textit{in vivo} (Buratowski and Zhou, 1992; Colbert and Hahn, 1992).

Two groups independently isolated the gene encoding the 90-kDa subunit of yeast TF\textsubscript{mB} through use of protein microsequence data (Kassavetis \textit{et al.}, 1995; Roberts \textit{et al.}, 1996). The gene was also independently isolated as a high-copy suppressor of temperature-sensitive mutations in the two largest subunits of TF\textsubscript{II}IC (Rüth \textit{et al.}, 1996). The deduced amino acid sequence of TF\textsubscript{mB}90 did not reveal any global sequence similarity to other proteins in the databases. However, it does contain an ~50-amino acid motif termed the SANT motif, which is found in a number of other proteins that participate in transcription (e.g., Swi3, Ada2, and N-CoR) (Aasland \textit{et al.}, 1996). The SANT motif of TF\textsubscript{mB}90 is postulated to be involved in protein-protein and/or protein-DNA interactions (Aasland \textit{et al.}, 1996; Kumar \textit{et al.}, 1997).

\textbf{Incorporation of TF\textsubscript{mB} into the TF\textsubscript{II}IC-tDNA complex} - The individual subunits of TF\textsubscript{mB} can assemble in a stepwise fashion onto a TF\textsubscript{II}IC-tDNA complex (Kassavetis \textit{et al.}, 1992b; Kassavetis \textit{et al.}, 1995; Roberts \textit{et al.}, 1996). Although it does not appear to be stably incorporated, TF\textsubscript{mB}70 can be recruited to a TF\textsubscript{II}IC-tDNA complex by an interaction with the 131-kDa subunit of TF\textsubscript{II}IC (Kassavetis \textit{et al.}, 1992b; Khoo \textit{et al.}, 1994; Chaussivert \textit{et al.}, 1995). This is the only subunit of TF\textsubscript{II}IC that was detected in protein-DNA photocrosslinking studies to project upstream of the start site of transcription (Bartholomew
et al., 1991). The TFIIB-related amino-terminal portion of TFIIB70 interacts with the 131-kDa subunit of TFIIIC (Chaussivert et al., 1995; Kassavetis et al., 1997). TFIIB70-dependent entry of TBP results in a relatively stable TBP-TFIIB70-TFIIIC-DNA complex whose DNase I footprint now includes nt -32 to +5 (Kassavetis et al., 1992b). Incorporation of TBP into the complex also dramatically increases the photocrosslinking efficiency of the 131-kDa subunit of TFIIIC to DNA and expands the photocrosslinking footprint of TFIIB70 on DNA (Kassavetis et al., 1992b). TBP and TFIIB70 can interact with each other in the absence of DNA (Huet et al., 1994; Khoo et al., 1994; Chaussivert et al., 1995) and TFIIB70 can assemble onto a TBP-TATA box complex (Roberts et al., 1995; Librizzi et al., 1996). It is the carboxyl-terminal portion of TFIIB70, which is not present in TFIIB, that has the strongest interaction with TBP (Khoo et al., 1994; Chaussivert et al., 1995; Kassavetis et al., 1997); only weak interactions between the direct repeat-containing region of the amino-terminal portion of TFIIB70 and TBP have been observed through in vitro protein-protein binding experiments (Khoo et al., 1994). TFIIB90 binds to a TFIIIC-tDNA complex only in the presence of TBP and TFIIB70 (Kassavetis et al., 1992b). A number of changes occur upon entry of TFIIB90 into the TBP-TFIIB70-TFIIIC-DNA complex. The DNase I footprint is extended to nt -41 and is lost at the transcription start site; TFIIB becomes resistant to dissociation from DNA upon exposure of the complex to heparin or to high salt concentrations; and the complex gains the ability to recruit pol III for transcription (Kassavetis et al., 1991, 1992b, 1995). The recruitment of TFIIB90 appears to be mediated by both TFIIB70-TBP (Huet et al., 1994; Roberts et al., 1996) and the 131-kDa subunit of TFIIIC (Rüth et al., 1996).

Apart from being AT-rich, the sequences in the region upstream of tRNA genes are not conserved (Geiduschek and Kassavetis, 1992). This suggests that the TFIIB interaction with DNA is sequence independent. Joazeiro et al. (1996) showed that even the AT-richness is not essential. These investigators demonstrated that TFIIB can be recruited, albeit with reduced efficiency, to a TFIIIC-tDNA complex formed on an artificial tRNA gene that
contains only G and C nucleotides in its upstream sequence. TFIIIB in the resultant complex is resistant to being stripped by exposure to high ionic strength and predominately directs initiation of transcription by pol III from the normal start site (bp +1). The introduction of a minimal TATA-box element at various positions within the GC-rich region shifted the predominant start site of transcription to a position about 30 bp downstream of the 5' end of the introduced TATA-box element. Shifts in the selection of the start site correlated with shifts in the position of TFIIIB binding. Significantly, TFIIIB-binding remained TFIIIC-dependent in these experiments although the TATA-box element was found to serve as a binding site for TBP. The precise placement of TFIIIB on a tRNA gene, and consequently the start site of transcription, is codirected, therefore, by both TFIIIC and TBP. Once TFIIIB is positioned on DNA, a variety of contacts between each of the three subunits of TFIIIB and DNA are likely to account for the remarkable stability of TFIIIB-DNA complexes (Kassavetis et al., 1990, 1992b; Bartholomew et al., 1991; Joazeiro et al., 1996; Huet et al., 1997).

1.4.2 Assembly of a transcription complex on an S. cerevisiae 5S RNA gene

1.4.2.1 TFIIIA and its interaction with the 5S RNA gene

The first step in the in vitro assembly of a transcription complex on the yeast 5S RNA gene is the binding of TFIIIA to the gene via interactions with the 14-bp ICR (Challice and Segall, 1989; Wang and Weil, 1989). In the TFIIIA-DNA complex, a 34-bp region from nt +63 to +96 is protected from cleavage by DNase I (Braun et al. 1989). Photocrosslinking studies suggest that TFIIIA is positioned over a longer region than that detected by DNase I footprinting (Braun et al., 1992a). However, some of the more outlying contacts may result from motion of the DNA bringing it transiently into contact with the bound TFIIIA molecule. Guanine residues at positions +73, +74, +82, +85, +87, +88, +89, +91, +93, +94 of the yeast 5S RNA gene are protected against methylation by dimethyl sulfate in the TFIIIA-5S DNA
complex (Challice and Segall, 1989). Each of these same guanine residues, when methylated, reduce or prevent TFIIIA-5S DNA complex formation (Wang and Weil, 1989). Therefore, most of the guanine residues that are in close contact with yeast TFIIIA are within the 14-bp ICR. The binding of yeast TFIIIA to the 5S RNA gene induces a modest bend of about 45° in the 5S DNA (Braun et al., 1992b). Yeast TFIIIA contains nine Cys2/His2 zinc-finger DNA-binding motifs, with an 81 amino acid region interrupting the otherwise repeating zinc finger motifs between zinc fingers 8 and 9 (Archambault et al., 1992; Woychik and Young, 1992) (see section 1.5 below for a more detailed description of yeast TFIIIA and zinc fingers). As is the case for *Xenopus* TFIIIA, a polypeptide containing only the amino-terminal three zinc fingers of yeast TFIIIA retains the ability to specifically bind the 5S RNA gene (Milne and Segall, 1993).

1.4.2.2 Recruitment of TFIIIC and TFIIIB to the TFIIIA-5S DNA complex

**Recruitment of TFIIIC** - Formation of the TFIIIA-5S DNA complex is a prerequisite for recruitment of TFIIIC, which in turn stabilizes the TFIIIA-DNA interaction (Segall, 1986). Binding of yeast TFIIIC to the TFIIIA-5S DNA complex is readily detectable in electrophoretic mobility shift assays (Braun et al., 1989; Challice and Segall, 1989). Addition of TFIIIC also extends the DNase I footprint on the 5S RNA gene, but almost exclusively in the 3' direction (Braun et al., 1989). In addition to those guanines that had been identified as being in close contact with TFIIIA in the TFIIIA-5S DNA complex, guanine residues at nucleotides +57, +58, and +113 fail to be methylated in the TFIIIC-TFIIIA-5S DNA complex (Challice and Segall, 1989). Electrophoretic mobility shift assays and DNase I footprinting show that yeast TFIIIC on its own does not specifically bind the 5S RNA gene (Braun et al., 1989; Challice and Segall, 1989). However, DNA probably contributes to the interaction of TFIIIC with the TFIIIA-5S DNA complex. Indeed, truncation of the 5S RNA gene at nucleotide +110 compromises TFIIIC binding, although it has no major effect upon transcription levels (Braun et al., 1992b).
Recruitment of TFIIIB - Formation of the TFIIIC-TFIIIA-5S DNA complex allows recruitment of TFIIIB. The addition of TFIIIB extends the DNase I footprint of the complex 45 bp upstream of the start site of transcription (Braun et al., 1989; Kassavetis et al., 1990). Once TFIIIB enters the transcription complex, TFIIIC and TFIIIA can be selectively removed by high salt or heparin, leaving DNA-bound TFIIIB behind (Kassavetis et al., 1990). The position that TFIIIB occupies on DNA in the TFIIIB-5S DNA complex, relative to the start site of transcription, is nearly identical to its position in a TFIIIB-tDNA complex. As is the case for a TFIIIB-tDNA complex, TFIIIB also acts as the initiation factor for multiple rounds of transcription on the 5S RNA gene (Kassavetis et al., 1990).

Topography of TFIIIC and TFIIIB subunits on the 5S RNA gene - The topography of TFIIIC and TFIIIB subunits on the 5S RNA gene, as assessed by site-specific protein-DNA photocrosslinking experiments, is similar to that observed on a tRNA gene (Braun et al., 1992a; Bartholomew et al., 1990, 1991; see section 1.4.1.1 above and Fig. 1-3). The 138-kDa subunit of TFIIIC, which contacts the downstream B-box promoter element on tRNA genes, is located at the downstream end of the 5S RNA gene. The 95-kDa subunit of TFIIIC, which contacts the A-box promoter element on tRNA genes, crosslinks to 5S DNA upstream of TFIIIA, from nt +20 to +35. This placement of the 95-kDa subunit, which is similar to its placement on a tRNA gene, is interesting because an A-box sequence is not present at this position in the 5S RNA gene. An A-box-like sequence is present between nt +50 and +60 of the S. cerevisiae 5S RNA gene, but it is not required for in vitro transcription of this gene (Challice and Segall, 1989). The photocrosslinking analyses clearly indicate that no subunit of TFIIIC is in the vicinity of this A-box-like sequence; instead, TFIIIA crosslinks to this region of the 5S RNA gene. As on the tRNA gene, the 131-kDa subunit of TFIIIC protrudes upstream of the transcription start site into a region that is also occupied by TFIIIB. The positions of TFIIIB70 and TFIIIB90 upstream of the 5S RNA gene are similar to their positions on a tRNA gene, despite an absence of any sequence similarity in the upstream regions of these two genes. Finally, as is the case on a tRNA gene, recruitment of TFIIIB to
the 5S RNA gene induces a sharp bend of about 120° centered at approximately bp -30 (Léveillard et al., 1991; Braun et al., 1992b).

**Model for the role of yeast TFIII A in recruitment of TFIII C** - The above studies indicate that TFIII C and TFIII B interact with DNA at analogous positions on the tRNA and 5S RNA genes and that TFIII B functions in a similar manner on both of these templates. On a tRNA gene, the A- and B-box promoter elements interact with the τA and τB subcomplexes of TFIII C, respectively, to fix the two loosely tethered subcomplexes of TFIII C on DNA. Positioning of TFIII C at the A box is crucial for the correct placement of TFIII B upstream of the transcription start site of a tRNA gene. On a 5S RNA gene, TFIII A serves as an adapter molecule by binding to the ICR, thereby replacing the role of the A and B boxes of the tRNA gene in positioning TFIII C. TFIII A serves indirectly to position TFIII B upstream of the transcription start site, by fixing the position of TFIII C on the 5S RNA gene.

Milne and Segall (1993) mapped regions of TFIII A that are essential for DNA binding, for recruitment of TFIII C to the TFIII A-DNA complex, and for promoting transcription. These authors found that a truncated polypeptide containing only the amino-terminal three zinc fingers of TFIII A suffices for specific binding to the ICR of the 5S RNA gene and retains the ability to recruit TFIII C to the TFIII A-DNA complex. However, the resultant complex is transcriptionally inactive. The 81-amino acid domain that is present between zinc fingers 8 and 9 of yeast TFIII A was shown to be essential for its transcription factor activity (Milne and Segall, 1993). On the basis of these results and taking into consideration the known topography of TFIII C on the 5S RNA gene, these authors proposed that the amino-terminal three zinc fingers of TFIII A serve to recruit TFIII C to the TFIII A-DNA complex through protein-protein interactions with the τB subcomplex of TFIII C. They hypothesized that once TFIII C has entered the complex, its τA subcomplex interacts with the 81-amino acid domain of TFIII A. This second interaction would serve to dock TFIII C with the proper topography for subsequent assembly of TFIII B into the complex. In this model, the amino-terminal three zinc fingers of TFIII A functionally replace the B-box promoter element of a
tRNA gene and the 81-amino acid domain of TFIIIA replaces the A-box promoter element of a tRNA. Thus, TFIIIC-DNA interactions that occur on a tRNA gene are replaced by protein-protein interactions on a 5S RNA gene.

In addition to the internal control region, the yeast 5S RNA gene has an essential promoter element, the *sse*, spanning the transcription start site (Challice and Segall, 1989; see section 1.3.1 above on promoter elements). The role of the *sse* is unknown. This element does not appear to play a role in the assembly of transcription complexes, because footprinting and gel mobility shift experiments with a 5S RNA gene containing a linker-scanning mutation within the *sse* (LS2/9, Challice and Segall, 1989) indicated that transcription complex assembly was normal (data not shown in Braun *et al.*, 1992a). However, these assays may not have detected a subtle defect in the assembled complex. Alternatively, the *sse* element may reflect a preferred sequence for initiation by yeast pol III.

### 1.4.3 Assembly of a transcription complex on an *S. cerevisiae* U6 snRNA gene

**TFIIIC-independent transcription** - The *S. cerevisiae* U6 snRNA gene (*SNR6*) contains a consensus TATA box at nt -30, an intragenic A box, and a downstream B box (Brow and Guthrie, 1990; see section 1.3.4 above). In the presence of highly purified TFIIIB, the A- and B-box elements are dispensable for *in vitro* transcription of the gene. Under these conditions accurate transcription can be achieved in the absence of TFIIIC; TFIIIB directs its own assembly onto the *SNR6* gene through recognition of the TATA box by its TBP subunit (Margottin *et al.*, 1991; Burnol *et al.*, 1993b; Joazeiro *et al.*, 1994). All three subunits of TFIIIB (TBP, TFIIIB70, and TFIIIB90) are required for reconstitution of this distinctive TATA box-directed transcription of the *SNR6* gene (Joazeiro *et al.*, 1994; Kassavetis *et al.*, 1995). Despite its unique assembly pathway, this TFIIIB-*SNR6* gene complex is similar to the TFIIIB-tDNA and TFIIIB-5SDNA complexes; for example, the location and extent of the DNase I footprint attributable to TFIIIB are similar and TFIIIB is not dissociated from the *SNR6* gene by heparin (Joazeiro *et al.*, 1994).
Although TFIIIB90, TFIIIB70, and TBP are all essential subunits of TFIIIB, a detailed deletion analysis of TFIIIB90 indicated that no region of this subunit is absolutely required for in vitro transcription of the SNR6 gene (Kumar et al., 1997). Simultaneous deletion, however, of two regions of TFIIIB90, termed domains I and II, abolishes transcription of the SNR6 gene. In contrast, deletion of either one of these domains abolishes TFIIIC-dependent transcription of a tRNA gene (Kumar et al., 1997; also see section 1.4.1.2 above). The requirement for both domains I and II of TFIIIB90 for tRNA gene transcription likely reflects an essential interaction of TFIIIB90 with TFIIIC; this interaction would be required to recruit TFIIIB to the TATA-less tRNA gene but would be dispensable for TFIIIC-independent transcription of the SNR6 gene. If transcription of the SNR6 gene is carried out in the presence of a version of TFIIIB70 that lacks its putative zinc finger and first TFIIIB-related repeat segment, then both domains I and II of TFIIIB90 are required (Kassavetis et al., 1997). These results suggest that there is some functional complementarity between the TFIIIB90 and TFIIIB70 subunits of TFIIIB.

**TFIIC-dependent transcription** - Although in vitro transcription of the SNR6 gene with purified components requires neither TFIIIC nor the A- and B-box elements of the SNR6 gene, both TFIIIC and all promoter elements are required for transcription in vivo and for transcription in vitro in the presence of crude cell extracts (Brow and Guthrie, 1990; Burnol et al., 1993b; Eschenlauer et al., 1993). The observation that promoter and factor requirements depend on the conditions of transcription prompted Burnol et al. (1993a) to examine the role of TFIIIC in promoting access of TFIIIB to DNA that is part of chromatin. These authors found that nucleosomes preassembled on the SNR6 gene block TFIIIB-directed transcription; this repression by nucleosomes can be relieved on templates containing the downstream B-box element by the addition of TFIIIC.

Several observations suggest that TFIIIC has additional roles in SNR6 gene transcription in vivo. Gerlach et al. (1995) found that the A box actually plays a stronger role than the TATA box in directing start site selection for SNR6 transcription in vivo. The TATA box of
the SNR6 gene does contribute to start site selection, but only by serving to direct the precise choice of start site in the window defined by the A box. Thus, TFIIIB placement on the SNR6 gene in vivo is primarily directed by protein-protein interactions with TFIIIC. The TFIIIC-mediated recruitment of TFIIIB appears to be critical for determining polymerase specificity of SNR6 transcription in vivo. Roberts et al. (1995) found that the upstream promoter of the SNR6 gene is pol II-specific in vivo and that it is only converted to a pol III promoter when TFIIIC is bound at the downstream A- and B-box promoter elements (Roberts et al., 1995). The preferential recruitment of TFIIIB by TFIIIC, rather than by the TATA box, probably excludes the pol II general factors and promotes pol III transcription, thereby determining polymerase specificity. The TFIIIC-dependent mechanism of TFIIIB assembly also provides directionality to the placement of TFIIIB on the SNR6 gene. Whitehall et al. (1995) found that TATA box-directed assembly of TFIIIB results in bidirectional transcription from the SNR6 promoter. This is because TFIIIB can bind to the SNR6 gene in either direction since its constituent TBP cannot discern the polarity of the TATA box (Whitehall et al., 1995). When TFIIIC directs assembly, it dictates the orientation of TFIIIB on the SNR6 gene, presumably by an interaction between the 131-kDa subunit of TFIIIC and the TFIIIB70 subunit of TFIIIB. This results in TFIIIB directing transcription in only the appropriate direction for synthesis of the U6 RNA (Whitehall et al., 1995).

1.4.4 Mammalian and amphibian TFIIIC

Human TFIIIC activity can be resolved into two components, termed TFIIIC1 and TFIIIC2, by chromatography on either a Mono Q column (Yoshinaga et al., 1987), or a sequence-specific oligonucleotide affinity column (Dean and Berk, 1987). TFIIIC2 binds DNA directly, protecting the B box of the VAI RNA gene, a gene with a type 2 promoter, against cleavage by DNase I (Yoshinaga et al., 1987). The addition of TFIIIC1 extends the DNase I footprint on the VAI RNA gene to include the A box (Yoshinaga et al., 1987).
split DNA binding activity is similar to that observed for the proteolytically produced $\tau_A$ and $\tau_B$ subcomplexes of yeast TFIIIC (see section 1.4.1.1 above). TFIIIC2 can also interact with the human TFIIA-5S DNA complex as assessed by gel mobility shift assays (Oettel et al., 1997). The TFIIA-5S DNA complex generates a DNase I footprint which spans nt +45 to +99; incorporation of TFIIIC2 into this complex extends the footprint to nt +20. The addition of B box-containing DNA fragments to TFIIIC2-TFIIA-DNA complexes results in additional, specific protein-DNA complexes that have reduced mobility in native polyacrylamide gels relative to TFIIIC2-TFIIA-DNA complexes (Oettel et al., 1997). This suggests that the subunit(s) or domain(s) of TFIIIC2 required for interaction with the TFIIA-5S DNA complex is different from that required for binding to the B-box sequence.

Extensively purified fractions of TFIIIC2 contain polypeptides of 230, 110, 100, 80, and 60 kDa (Yoshinaga et al., 1989; also see, Schneider et al., 1989 and Kovelman and Roeder, 1992). The 230-kDa polypeptide can be photocrosslinked to the B-box element of the VA1 RNA gene (Yoshinaga et al., 1989; Kovelman and Roeder, 1992). The cDNA encoding this DNA-binding subunit of TFIIIC2 has been cloned from both rat and human sources. The deduced amino acid sequence of the rat and human proteins, termed rTFIIIC$\alpha$ and hTFIIIC$\alpha$, respectively, show 74% identity to each other, but had no extensive similarity to other proteins in the databases (Lagna et al., 1994; L'Etoile et al., 1994), which included the B box-binding subunit of S. cerevisiae TFIIIC (Tfc3).

Two forms of TFIIIC2 exist, termed TFIIIC2a and TFIIIC2b. They form distinct complexes with the VA1 RNA gene as assessed by their electrophoretic mobility on a nondenaturing polyacrylamide gel (Hoeffler et al., 1988). Each binds VA1 DNA with equal affinity and generates an equivalent DNase I footprint. However, only TFIIIC2a supports transcription of the VA1 RNA gene (Kovelman and Roeder, 1992). TFIIIC2a and TFIIIC2b both copurify with polypeptides of 220, 102, 90 and 63 kDa; TFIIIC2a, however, copurifies with a 110-kDa polypeptide that is not present in TFIIIC2b and TFIIIC2b copurifies with a 77-kDa polypeptide that is not present in TFIIIC2a (Kovelman and Roeder, 1992; Sinn et al.,
The cDNA corresponding to the 110-kDa subunit of TF\textsc{IIC}2\textsc{a} has been cloned (Sinn et al., 1995). Again, the deduced amino acid sequence of this protein, termed TF\textsc{IIC}\textsc{β}, had no sequence similarities with any of the subunits of \textit{S. cerevisiae} TF\textsc{IIC} whose genes had been cloned. The only notable feature of TF\textsc{IIC}\textsc{β} is the presence of five WD40 repeats, which are thought to facilitate protein-protein interactions (reviewed in Neer et al., 1994). Antibodies raised against TF\textsc{IIC}\textsc{β} do not crossreact with any of the polypeptides present in TF\textsc{IIC}2\textsc{b} (Sinn et al., 1995).

The exact composition of TF\textsc{IIC}1 is still not clear, but it appears to be a multisubunit complex that has an aggregate molecular mass of ~200 kDa (Yoshinaga et al., 1987; Wang and Roeder, 1996). Wang and Roeder (1996) reported the presence of a second chromatographically distinct form of TF\textsc{IIC}1, designated TF\textsc{IIC}1', that possesses all of the known properties of TF\textsc{IIC}1. These authors also found that TF\textsc{IIC}1 and TF\textsc{IIC}1' can each bind to the termination region of VA\textsubscript{1} RNA gene and tRNA genes in the absence of TF\textsc{IIC}2. Other investigators, however, do not detect an interaction of TF\textsc{IIC}1 with the termination region of class III genes (Dean and Berk, 1987; Yoshinaga et al., 1987; Oettel et al., 1997). Additionally, Oettel et al. (1997) have described a factor that is distinct from TF\textsc{IIC}1, termed TF\textsc{IIC}0, that binds specifically to the termination region of class III genes; TF\textsc{IIC}0 can substitute for TF\textsc{IIC}1 in reconstituting transcription of class III genes. Clearly, further characterization of human TF\textsc{IIC} is needed to resolve these discrepancies and to determine the mechanism of action of TF\textsc{IIC}1.

\textit{Xenopus} TF\textsc{IIC} appears to be a large multisubunit complex with a native molecular mass of ~400 kDa as determined by gel filtration (Keller et al., 1992). Its incorporation extends the footprint of a TF\textsc{IIIA}-5S DNA complex an additional 25 bp towards the 5' end of the gene (Sturges et al., 1995). Although \textit{Xenopus} TF\textsc{IIC} has been partially purified by B-box DNA affinity chromatography, it remains poorly characterized. The partially purified factor is enriched in five proteins with apparent molecular masses of 200, 160, 85, 75 and 31 kDa.
(Keller et al., 1992). The 85-kDa polypeptide specifically binds a B box-containing oligonucleotide as assessed by UV crosslinking (Keller et al., 1992).

1.4.5 Mammalian and amphibian TFIIIB

Apart from TBP, the composition of TFIIIB from vertebrates is unclear. Human TFIIIB preparations have been reported that contain, in addition to TBP, polypeptides of (i) 150, 82, and 54 kDa (Lobo et al., 1992), (ii) 190, 96, 87, and 60 kDa (Chiang et al., 1993), and (iii) 172 kDa and a TBP-associated factor of undefined size (Taggart et al., 1992). TFIIIB from *Xenopus* is reported to contain TBP and at least two additional polypeptides of 92 and 75 kDa (McBryant et al., 1996). Some of these TFIIIB-associated polypeptides could originate from either pol III or from other transcription factors (see Wang and Roeder, 1995). As a further complication to the characterization of mammalian TFIIIB, human cells appear to possess distinct TFIIIB complexes that are specific for transcription of class III genes with external promoters or for transcription of class III genes with internal promoters (Lobo et al., 1992; Teichmann and Seifart, 1995; Wang and Roeder, 1995). A human cDNA encoding a non-TBP component of the TFIIIB complex (hTFIIIB90) has been cloned. The amino-terminal half of hTFIIIB90 is similar in sequence to yeast TFIIIB70 (Brf1) and to the pol II transcription factor TFIIIB. Each of these proteins contain a putative zinc finger at the amino terminus followed by two imperfect direct sequence repeats. A recombinant protein containing the nonconserved carboxyl-terminal region of hTFIIIB90 was found to interact strongly with hTBP, whereas an amino-terminal fragment of hTFIIIB90 was found to interact relatively weakly with hTBP (Wang and Roeder, 1995). These authors also made the observation that recombinant hTFIIIB90 plus recombinant hTBP are sufficient to replace human TFIIIB in transcription of the VA₁ gene (a gene with a type II promoter). This suggests that human TFIIIB lacks a functional equivalent of the third component of yeast TFIIIB, yTFIIIB90 (Tfc5). In contrast, Teichmann et al. (1997) found that a partially purified yeast TFIIIB" fraction, which contains yTFIIIB90 and yeast TFIIIE (see section
1.4.6.3 below on yeast TFIIIE), can substitute for human TFIIIB in transcription of the human U6 snRNA gene (a gene with a type III promoter). These discrepancies may be explained either by the differential dissociation of human TFIIIB under different purification schemes or by the possibility that different forms of TFIIIB exist in human cells, each dedicated to the transcription of a particular type of class III gene. Determination of the exact composition(s) of human TFIIIB and the precise relationship between the components of human TFIIIB and yeast TFIIIB requires further studies.

1.4.6 Other RNA polymerase III transcription factors

1.4.6.1 TFIIID, a factor unique to Bombyx mori

Fractionation of extracts from the silkworm Bombyx mori identified an essential protein factor, termed TFIIID, that is required in addition to TFIIIB, TFIIIC, and TFIIIA for accurate initiation of transcription by pol III on tRNA and 5S RNA genes (Ottonello et al., 1987). TFIIID was found to play a critical role in the assembly of the other factors into stable transcription complexes. Neither TFIIIC, TFIIIB, nor TFIIID alone is able to form a stable complex with the silkworm tRNA\textsuperscript{Ala}C gene; however, a combination of TFIIIB and TFIIID or a combination of TFIIIC and TFIIID leads to the formation of a stable protein-DNA complex (Ottonello et al., 1987; Young et al., 1991b). This indicates that more than one pathway exists for the assembly of transcription complexes on tRNA genes, at least in the reconstituted silkworm system. TFIIIC and TFIIID together protect most of the tRNA\textsuperscript{Ala}C gene against cleavage by DNase I (Young et al., 1991b; Young et al., 1996). The DNase I footprint extends over the region from nt -11 to nt +136 and includes 3' flanking sequence (Young et al., 1996). In the silkworm system, the 3' flanking region plays an essential role, in addition to the internal promoter elements, in the recruitment of TFIIIC and TFIIID (Wilson et al., 1985; Young et al., 1991b). The TFIIIB/TFIIID interaction with DNA is not as well characterized as the TFIIIC/TFIIID interaction, but a specific TFIIIB/TFIIID-tDNA
complex is detectable by gel shift analysis and it is clearly different from that of the TFIIIC/TFIID-tDNA complex (Young et al., 1996). The relationship between silkworm TFIID and factors from other organisms has not yet been determined, but it has been suggested that silkworm TFIIIC and TFIID may represent subcomplexes of components that are associated solely within TFIIIC in other organisms (Young et al., 1991b).

1.4.6.2 TFIIIR, an inhibitor of DNase activity

A novel factor, termed TFIIIR, was discovered upon fractionation of extracts of silk glands from Bombyx mori (Young et al., 1991a). It is required for in vitro transcription of tRNA\textsubscript{Ala}, 5S RNA, and BmX genes in combination with the other general transcription factors from silkworm (Young et al., 1991a). The resistance of TFIIIR activity to heat, detergent, phenol, proteases, and DNases, along with its sensitivity to alkali and RNase led to the conclusion that TFIIIR is made of RNA (Young et al., 1991a). The RNA that provides TFIIIR activity was found to be a specific tRNA, tRNA\textsuperscript{IleIAU} (Dunston et al., 1994a), which acts indirectly to enhance transcription by inhibiting a low-frequency DNA-cleavage activity that is present in the silkworm TFIIIB fraction (Dunston et al., 1994b). For this reason, TFIIIR is no longer considered to be a transcription factor (Smith et al., 1995).

1.4.6.3 TFIIIE

Another transcription factor that may be an additional core component of the S. cerevisiae pol III transcription system has been described (Dieci et al., 1993). This factor, termed TFIIIE, is separable from TFIIIB, TFIIIC, TFIIIA, and pol III at an early stage of fractionation of yeast nuclear extracts and is essential for transcription of both 5S RNA and tRNA genes in vitro. Although TFIIIE copurifies with TFIIIB under some conditions and is present in both the TFIIIB' and TFIIIB" fractions used by Kassavetis et al. (1991), TFIIIE is distinct from TFIIIB' and TFIIIB". TFIIIE is functionally distinct from silkworm TFIIIR (see above); it is inactivated by treatment with proteinase K and it does not act by inhibiting an
irreversible negative activity (Dieci et al., 1993). TFIIIE does not appear to bind to the tRNA gene either alone or in combination with any of the other known factors nor does it appear to be involved in the assembly of a stable TFIIIB-TFIIIC-tDNA complex. It is required for both single and multiple rounds of transcription, and is therefore not a reinitiation factor. Dieci et al. (1993) proposed that TFIIIE may be involved in the utilization of the preinitiation complex by pol III. The gene encoding TFIIIE has not yet been identified.

The essential role of TFIIIE in pol III transcription has been questioned by Kassavetis et al. (1995). These authors found that TFIIIB reconstituted from proteins (TBP, TFIIIB70, and TFIIIB90) produced in E. coli was functional for directing tRNA (TFIIIC-dependent) and U6 snRNA (TFIIIC-independent) synthesis by pol III. Therefore, these experiments eliminated the possibility of yeast-derived TFIIIB fractions providing an essential TFIIIE activity. Rüth et al. (1996) found that TFIIIE was required for full activity of TFIIIB assembled from recombinant subunits, but specific, albeit weak, transcription activity was nonetheless observed in the absence of TFIIIE. This implies that TFIIIE may serve to increase the activity of TFIIIB, possibly by preventing inactivation or facilitating the correct folding of a TFIIIB component(s). However, it is still possible that TFIIIE plays an essential role in class III gene transcription because a small amount of TFIIIE activity may have been associated with the purified yeast-derived pol III fractions used by Kassavetis et al. (1995) and Rüth et al. (1996).

1.5 TFIIIA

TFIIIA is specifically required for initiation of transcription on the 5S RNA gene (Engelke et al., 1980; Segall et al., 1980; Shastry et al., 1982; Taylor and Segall, 1985). This protein binds to the internal control region of this gene as the first step in the assembly of a multifactor transcription complex (see section 1.4 above on assembly of pol III transcription complexes).
1.5.1 Xenopus TFIIIA

1.5.1.1 Isolation and initial characterization of Xenopus TFIIIA

TFIIIA from Xenopus laevis has been extensively characterized, especially with respect to its DNA- and RNA-binding properties (for reviews see Pieler and Theunissen, 1993; White, 1994; Shastry, 1996). Xenopus TFIIIA (xTFIIIA) was the first gene-specific eukaryotic transcription factor to be purified; its isolation was facilitated by its presence in the abundant 7S ribonucleoprotein storage particle of immature Xenopus oocytes (Picard and Wegnez, 1979; Pelham and Brown, 1980; Engelke et al., 1980). It was also the first eukaryotic transcription factor for which the cDNA was cloned and sequenced (Ginsberg et al., 1984).

Xenopus TFIIIA is a single polypeptide of about 38.5 kDa that protects the entire 50-bp ICR of the amphibian 5S RNA gene against cleavage by DNase I (Engelke et al., 1980). The essential contact points are mostly on the non-template strand at the 3' end of the internal control region (Sakonju et al., 1981; Sakonju and Brown, 1982). Careful measurements indicate that the TFIIIA:gene stoichiometry in the xTFIIIA-5S RNA gene complex is 1:1 (Bieker and Roeder, 1984; Smith et al., 1984). Physical characterization of native xTFIIIA indicates that it is an extremely elongated molecule (Bieker and Roeder, 1984).

Limited proteolysis of xTFIIIA releases a 30-kDa amino-terminal portion that suffices for DNA binding and a 10-kDa carboxyl-terminal portion that is required for the transcription activity of TFIIIA but is dispensable for its DNA-binding activity (Smith et al., 1984; Miller et al., 1985; Vrana et al., 1988). Extensive proteolysis of xTFIIIA generates ~3-kDa peptides that are resistant to further digestion, suggesting that TFIIIA is made up of repeats of a tightly folded 3-kDa unit (Miller et al., 1985). Close inspection of the amino acid sequence of xTFIIIA, as deduced from its cDNA (Ginsberg et al., 1984), revealed the presence of nine variant copies of an ~30 amino acid sequence that are repeated in tandem and are characterized by two invariant pairs of cysteines and histidines (Brown et al., 1985; Miller et
al., 1985). Comparison of the nine repeating units revealed the consensus motif Tyr/Phe-X-Cys-X2.4-Cys-X3-Phe-X5-Leu-X2-His-X3.4-His-X5 (Miller et al., 1985) (see Fig. 1-4A). Miller et al. (1985) also confirmed an earlier study (Hanas et al., 1983b) that had indicated that xTFIIIA contains bound zinc ions and that these ions are needed for specific DNA-binding; the stoichiometry of zinc in TFIIIA was measured to be 7 to 11 zinc ions per molecule (Miller et al., 1985). Since zinc is often tetrahedrally liganded in proteins by cysteine and histidine residues, it was proposed that the two cysteines and two histidines of each repeating unit coordinate a zinc ion to form a tightly folded domain (Brown et al., 1985; Miller et al., 1985). The 3-kDa peptides that are generated on extensive proteolysis of xTFIIIA likely each represent one of these "zinc fingers" (Miller et al., 1985). The coordination of zinc ions by cysteines and histidines in xTFIIIA was later confirmed by extended X-ray absorption fine structure (EXAFS) analysis (Diakun et al., 1986).

1.5.1.2 Zinc finger structure

The Cys2/His2 zinc-finger motif first found in Xenopus TFIIIA has since been identified in hundreds of other proteins and is now considered to be a major structural motif in for DNA-binding (Berg, 1990; Jacobs, 1992; Böhm et al., 1997) (see Fig. 1-4). Based on comparisons to metalloproteins with known structures, Berg (1988) and Gibson et al. (1988) proposed that a zinc finger consists of a short antiparallel β-sheet followed by an α-helix. This model was later supported by NMR analysis of a single zinc finger from the Xenopus protein Xfin (Lee et al., 1989). The two cysteines are in the turn of the β-sheet region and the two histidines are positioned on the inner face of the carboxyl-terminal portion of the α-helix. The structure is stabilized by the tetrahedrally coordinated zinc ion and a hydrophobic core containing three conserved hydrophobic residues. Circular dichroism and NMR studies show that zinc and its coordinating residues are essential for folding the domain into a defined structure (Frankel et al., 1987; Párraga et al., 1988). Subsequent NMR studies of polypeptides containing one or two zinc fingers revealed that this fold is conserved in zinc
Figure 1-4. The structure of the zinc-finger motif and interaction of *Xenopus* TFIllA with DNA.

(A) Diagram of the primary sequence of a zinc finger. Conserved amino acids of the zinc-finger motif are circled. Non-conserved amino acids are indicated by Xs. The zinc ion, which is tetrahedrally coordinated by two cysteine and two histidine residues, is circled in the center of the diagram.

(B) Diagram of the three-dimensional structure of a single zinc finger (taken from Figure 1 of Berg and Godwin, 1997). The amino terminal portion of the zinc finger forms a short anti-parallel β-sheet with a hairpin turn and the carboxyl-terminal portion of the zinc finger forms an α-helix that usually fits into the major groove of the target DNA. The tetrahedrally coordinated zinc ion is depicted as a dark sphere. The four metal-binding amino acids (Cys and His residues) and the three conserved hydrophobic residues are shown.

(C) Current model for the interaction of the nine zinc fingers of *Xenopus* TFIllA with the ICR of the 5S RNA gene (adapted from Figure 5 of Clemens et al., 1992). The sequence of the non-transcribed strand of the *Xenopus* 5S RNA gene from nt +40 to +100 is shown. The essential promoter elements of this gene, the A box, the IE, and the C box, are indicated below the nucleotide sequence by broken lines. Zinc fingers 1 to 3 (at right) wrap around the major groove of DNA for approximately one turn of the helix in the region of the C box. Zinc fingers 4, 5, and 6 interact predominately with one face of the DNA helix. Finger 5 binds in the major groove in the region of the IE. Fingers 4 and 6 each cross the minor groove interacting with extended regions of DNA. Fingers 7 to 9 wrap around the major groove for approximately one helical turn of the DNA helix in the region of the A box. This diagram does not represent structural changes in the DNA that may occur on TFIllA-binding, nor the precise orientation of zinc fingers spanning the minor grooves.

(D) Amino acids in the first three zinc fingers of *Xenopus* TFIllA that contact DNA. These data are from the NMR structure of a polypeptide containing residues 11 to 101 of *X. laevis* TFIllA, plus the amino-terminal methionine and a Cys35-Ser mutation, bound to the C box sequence of the *Xenopus* 5S RNA gene (taken from Figure 1(a) of Wuttke et al., 1997). The conserved zinc ligands (Cys and His) are underlined and secondary structural elements are indicated schematically above the corresponding positions in the sequences of the zinc fingers (the β-strands are indicated by arrows, and the α-helix by a cylinder). Boxed residues make specific contacts to DNA bases, and circled residues contact the DNA phosphate backbone.
fingers from different proteins (Klevit et al., 1990; Omichinski et al., 1990, 1992; Kochoyan et al., 1991; Neuhaus et al., 1992).

Molecular modeling studies and mutational analyses suggested that the amino-terminal residues of the α-helix are involved in DNA recognition and typically make contact with 3 bp in the major groove of DNA (Berg, 1988; Gibson et al., 1988; Nardelli et al., 1991). In agreement with this prediction, the first co-crystal structure solved for a Cys2/His2 zinc finger polypeptide, that of the three zinc fingers of Zif268 (also known as NGFI-A) bound to its cognate DNA-binding site, revealed that the three zinc fingers wrap partway around the DNA double helix with each zinc finger binding in the major groove of B-DNA (Pavletich and Pabo, 1991). In this structure, each of the three zinc fingers appears to bind DNA independently, with each finger having a similar orientation and with amino acids in the amino-terminal portion of its α-helix contacting 3 bp. The amino acids that contact the bases mainly occur at three positions: -1, +3, and +6, where +1 is the first residue of the α helical region of the zinc finger. In the Zif268-DNA complex, most of the contacts are along one strand of the DNA (the guanine-rich strand). The β-sheet of each zinc finger is on the back of the α-helix and is not involved in specific recognition of DNA.

The relatively simple mode of interaction in the Zif268-DNA complex suggested that a simple recognition code may exist for zinc finger-DNA interactions. To a certain extent, the existence of a recognition code is supported by database analyses and studies examining mutations of the three recognition residues that lead to a change of specificity (Desjarlais and Berg, 1992a, 1992b, 1993; Jacobs, 1992; Nardelli et al., 1992). However, it is clear that a base contact by one amino acid can be affected by neighboring amino acids; for example, the amino acid at position +2 of the α-helix can often modulate the specificity of the interaction of a zinc finger with DNA. Selection of zinc fingers that bind to given a DNA sequence from a phage display library encoding zinc fingers with randomized residues has provided further insight into possible recognition codes; this approach, however, has been limited by the size of the libraries (Choo and Klug, 1994a, 1994b; Rebar and Pabo, 1994; Wu et al., 1995;
Greisman and Pabo, 1997). Thus, the importance of context-dependent interactions in zinc finger-DNA recognition has yet to be fully appreciated.

The complexities of DNA recognition by zinc fingers are also highlighted by other mutational studies which show that the linkers between the zinc fingers make an important contribution to the affinity of the interaction (Wilson et al., 1992; Choo and Klug, 1993; Clemens et al., 1994). Furthermore, crystallographic studies of protein-DNA complexes formed with the zinc finger-containing polypeptides GLI (Pavletich and Pabo, 1993), Tramtrack (Fairall et al., 1993), YY1 (Houbaviy et al., 1996), and a designed protein (Kim and Berg, 1996) indicate that zinc fingers can interact with DNA in slightly different ways. These structural studies also indicate that context-dependent interactions are important for zinc finger-DNA recognition. Contacts can occur between neighboring zinc fingers and overlapping subsites can be recognized by adjacent zinc fingers.

1.5.1.3 Interaction of Xenopus TFIIIA with the 5S RNA gene

A model of the Xenopus TFIIIA-5S DNA complex - Xenopus TFIIIA binds to the 5S RNA gene such that its amino terminus is oriented towards the 3' end of the ICR and its carboxyl terminus is towards the 5' end of the gene (Miller et al., 1985; Vrana et al., 1988). Early models of the interaction between the nine zinc fingers of xTFIIIA and the 50-bp ICR of the 5S RNA gene assumed that all nine zinc fingers make similar contacts with DNA (Fairall et al., 1986; Churchill et al., 1990). However, subsequent studies clearly showed that the dispositions of the nine zinc fingers of xTFIIIA are not uniform (Clemens et al., 1992; Fairall and Rhodes, 1992; Hayes and Clemens, 1992; Hayes and Tullius, 1992; Hansen et al., 1993). The binding sites of the individual zinc fingers of xTFIIIA have been defined by analyzing the interaction of recombinant polypeptides containing differing numbers of xTFIIIA zinc fingers with the 5S RNA gene by DNase I footprinting, methylation interference, hydroxyl-radical footprinting, and by binding experiments with mixtures of 5S DNA fragments differing in length by a single bp (Vrana et al., 1988; Clemens et al., 1992;
Hayes and Clemens, 1992; Hansen et al., 1993). In the model based on these data (see Fig. 1-4C), zinc fingers 1, 2 and 3 bind in the major groove of the C-box promoter element, and zinc fingers 7, 8 and 9 bind in the major groove of the A-box promoter element. These three amino-terminal and three carboxyl-terminal fingers of xTFIIIA are thought to wrap around the major groove of the DNA helix at each end of the ICR in a manner similar to that observed in the crystal structure of the three zinc fingers of Zif268 bound to DNA (Pavletich and Pabo, 1991). The 20 bp of DNA between the A box and the C box of the ICR is contacted by zinc fingers 4, 5 and 6 of xTFIIIA, which appear to lie on one side of the DNA helix. Zinc finger 5 is proposed to bind in the major groove of the intermediate element and zinc fingers 4 and 6 are each proposed to bind in, or across, the minor groove to bridge the major groove-binding fingers. Similar models were proposed on the basis of hydroxyl-radical footprinting and missing nucleoside experiments with full-length xTFIIIA bound to the 5S RNA gene (Hayes and Tullius, 1992) and on re-examination of the DNase I footprinting data for the xTFIIIA-5S DNA complex taking into account the crystal structure of the DNase I-DNA complex (Fairall and Rhodes, 1992).

The recently reported NMR and crystal structures of xTFIIIA zinc fingers bound to 5S DNA have confirmed the main features of these models (see below).

**NMR structure of a zf1-3-DNA complex** - The NMR structure of the amino-terminal three zinc fingers of *Xenopus* TFIIIA (zf1-3) bound to a 15-bp oligonucleotide duplex corresponding to its binding site in the 5S RNA gene has been reported (Foster et al., 1997; Wuttke et al., 1997). As expected, each zinc finger adopts the characteristic structure of the Cys2/His2 zinc finger, which consists of a small anti-parallel β-sheet packed against an α-helix in a globular structure that is stabilized by a central zinc ion. Although the three zinc fingers of zf1-3 bind DNA in the major groove in a manner reminiscent of Zif268 (Pavletich and Pabo, 1991), the zf1-3-DNA complex does exhibit a number of novel features. As predicted by earlier biochemical analyses (Liao et al., 1992; Clemens et al., 1994), the three zinc fingers of zf1-3 bind to a larger site than those of Zif268. They make contacts with 13
bp of DNA (nucleotides T80 to C92 of the *Xenopus 5S* RNA gene). The subsites occupied by the individual zinc fingers of zf1-3 partially overlap, particularly with regards to DNA backbone contacts. There are substantial protein-protein contacts between adjacent fingers. One consequence of these protein-protein interactions is that the orientation of finger 1 in the major groove is substantially different from that of fingers 2 and 3; the \(\alpha\)-helix of finger 1 extends across the major groove rather than lying along it. This orientation extends the finger 1 binding site towards the 3' end of the C box.

Specific recognition of the C box by the zf1-3 polypeptide is achieved through a complex network of hydrophobic and hydrogen-bonding interactions between amino acid side-chains and DNA bases (see Fig. 1-4D for a summary of amino acids that contact bases in the NMR structure). Finger 1 makes base-specific contacts to 5 bp of DNA (bp 88 to 92 of the *Xenopus 5S* RNA gene); finger 2 makes base-specific contacts to the central GGG triplet (bp 85 to 87); and finger 3 makes base-specific contacts with 4 bp of DNA (bp 81 to 84). Contacts are primarily to the purine-rich, non-transcribed strand, but some contacts are also made to bases on the opposite strand. In finger 1, the recognition residues are Lys26, Trp28, and Lys29 at positions -1, +2, and +3 of the \(\alpha\)-helix, respectively. The hydrophobic and hydrogen bond contacts to DNA bases made by Trp28 are unlike any found in the previously determined structures of zinc finger-DNA complexes. In finger 2, the recognition residues are His59 (position +3), Arg62 (position +6), and potentially His58 (position +2). In finger 3, the recognition residues are Thr86 (position -1), Asn89 (position +3), Lys92 (position +6), and Arg96 (position +10). Interaction between a side-chain at position +10 of the \(\alpha\)-helix of a zinc finger and a base is also unprecedented. The additional contacts made by Arg96 at position +10 are largely responsible for the extended DNA sub-site contacted by finger 3. The lysine and histidine side-chains of zf1-3 that are involved in base recognition are dynamically disordered in the NMR solution structure; these side-chains may make fluctuating hydrogen bonds to different DNA bases. Foster *et al.* (1997) proposed that the
retention of some degree of side-chain mobility in the protein-DNA interface would confer an entropic advantage for DNA-binding.

In the absence of DNA, zf1-3 is intrinsically flexible; the individual zinc fingers domains display rigid-body motions with significant flexibility in the linkers between the fingers (Brüschweiler et al., 1995). The NMR structure of the zf1-3-DNA complex clearly shows that the linkers lose their flexibility upon binding to DNA and adopt highly ordered structures with close packing interactions between side chains within the linkers and also with side chains of the neighboring zinc fingers. Protein-protein interactions that are associated with the structural organization of the linkers appear to indirectly contribute to high affinity DNA-binding by helping to lock the zinc fingers in the correct position for DNA-binding. Previous studies had shown that DNA-binding by zf1-3 is markedly impaired by cassette, or single residue, mutations of linker residues (Choo and Klug, 1993; Clemens et al., 1994), showing that the linkers have an important role in the zf1-3-DNA interaction despite having only limited direct contacts to DNA. In summary, the NMR structure of the zf1-3-DNA complex supports and expands upon the large body of footprinting and mutagenesis data available on the interaction of xTFIIIA with DNA.

Crystal structure of a zf1-6-DNA complex - The crystal structure of the amino-terminal six zinc fingers of Xenopus TFIIIA (zf1-6) bound with 31 bp of the 5S RNA gene promoter has been reported (Nolte et al., 1998). The crystal structure confirms that zinc fingers 4-6 adopt a completely different configuration on DNA than do zinc fingers 1-3. Zinc fingers 4-6 lie along one face of the DNA double helix to form an open, extended structure. Only zinc finger 5 makes contacts with bases in the major groove. Zinc fingers 4 and 6 instead sit above the neighboring minor grooves and appear to serve primarily as spacer elements in DNA recognition. In spanning the minor grooves, a few contacts are made by residues of zinc fingers 4 and 6 with the DNA backbone.

Further structural studies will be required to elucidate the detailed mechanism of interaction of full-length xTFIIIA with DNA.
**Energetics of the xTFIIIA-DNA interaction** - Full-length xTFIIIA binds to the 5S RNA gene with an apparent dissociation constant ($K_d$) of about 0.5 nM as measured in a nitrocellulose filter binding assay (Romaniuk, 1990). Under slightly different binding conditions, a similar $K_d$ value for the xTFIIIA-5S DNA complex was measured with an electrophoretic mobility shift assay (Del Rio and Setzer, 1991). The C-box has been defined as the primary determinant of TFIIIA binding by chemical interference and protection experiments (Sakonju and Brown, 1982; Fairall et al., 1986) and by measurement of the effect of mutations within the ICR on TFIIIA-binding affinity (Pieler et al., 1987; You et al., 1991; Veldhoen et al., 1994; Rawlings et al., 1996). Veldhoen et al. (1994) performed a selected amplification and binding assay (SAAB) and found that the wild-type sequence from nt 79 to 92, which contains the C box, is preferentially selected.

Various studies have indicated that the interaction of the amino-terminal zinc fingers of xTFIIIA with the C-box element provides most of the binding energy for the TFIIIA-DNA interaction (Darby and Joho, 1992; Liao et al., 1992; Theunissen et al., 1992; Bogenhagen, 1993; Clemens et al., 1994). A polypeptide containing only the amino-terminal three zinc fingers of xTFIIIA binds specifically and with high affinity to a minimal DNA-binding site consisting of nt +80 to +92 of the 5S RNA gene, which corresponds to the C-box promoter element (Liao et al., 1992). The third zinc finger is a major contributor to the affinity of xTFIIIA for DNA (Liao et al., 1992; Theunissen et al., 1992; Del Rio et al., 1993; Zang et al., 1995). Detailed studies on the energetics of TFIIIA-5S DNA binding using truncated forms of TFIIIA (Clemens et al., 1994) or using mutant forms of TFIIIA in which the structure of individual zinc fingers has been disrupted (Del Rio et al., 1993) confirm that individual zinc fingers make differing contributions to the binding energy of complex formation. The study of Del Rio et al. (1993) also suggested that the energetic contribution made by each zinc finger is influenced by the simultaneous binding of the other zinc fingers to DNA. Indeed, Kehres et al. (1997) provide evidence for energetically unfavorable interactions between zinc fingers on binding of full-length TFIIIA to the 5S RNA gene. The
degree of interference appears to be greatest on binding of the zinc fingers at each end of the protein to DNA. The exact source of this interference is unknown, but it likely results from energetically unfavorable distortions in the DNA and/or the protein that are necessary to allow simultaneous binding of all nine zinc fingers to DNA.

**Bending of DNA by Xenopus TFIIIA** - The binding of full-length xTFIIIA to the 5S RNA gene induces a bend in the DNA of about $60^\circ$ near the center of the TFIIIA-binding site (Schroth et al., 1989, 1991; Brown et al., 1996; Kehres et al., 1997). It is possible that DNA bending is required for simultaneous occupancy of the ICR of the 5S RNA gene by all nine zinc fingers of xTFIIIA.

### 1.5.1.4 Transcriptional activation by Xenopus TFIIIA

**Role of the carboxyl-terminal region** - The portion of xTFIIIA following the ninth zinc finger, which is referred to as the carboxyl-terminal region, is essential for generation of a functional initiation complex on the 5S RNA gene. Deletion of the carboxyl-terminal region does not alter the affinity of TFIIIA for 5S DNA, nor does it alter the size of the DNase I footprint on the gene (Smith et al., 1984; Vrana et al., 1988; Hayes et al., 1989; Xing and Worcel, 1989). This truncated TFIIIA, however, cannot promote transcription of the 5S RNA gene (Smith et al., 1984; Vrana et al., 1988; Mao and Darby, 1993). Mutational analysis of the carboxyl-terminal region of xTFIIIA defined a 14-amino acid segment, from residues 291 to 304, as essential for transcriptional activation (Mao and Darby, 1993). The ability of this segment to contribute to the transcription factor activity of xTFIIIA is dependent on its position relative to the ninth zinc finger. Deletion of the eight amino acids that separate the transcription-activating region from the ninth zinc finger abolishes its activity; insertion of residues into this eight amino acid segment reduces the activity of TFIIIA, but non-conservative substitutions in this segment are well tolerated (Mao and Darby, 1993). The role of this position-dependent transcription-activating region of xTFIIIA is unknown. It may function by recruiting TFIIIC to the 5S RNA gene. Indeed, a direct
interaction of this region with TFIIIC is suggested by the reduced stability of transcription complexes formed with a version of xTFIIIA lacking the entire carboxyl-terminal region (Hayes et al., 1989). Another possibility is that the transcription-activating domain functions by inducing a conformational change in a target polypeptide that is required for the formation of an active initiation complex.

**Role of the carboxyl-terminal zinc fingers** - A role for the carboxyl-terminal zinc fingers of xTFIIIA in transcription of the 5S RNA gene that is distinct from their role in DNA-binding has been suggested from studies that analyzed the effects of structural disruptions of individual zinc-finger domains (see section 1.5.1.3 above; Del Rio et al., 1993; Rollins et al., 1993). Disruption of any one of zinc fingers 1 through 6 has no discernible effect upon the ability of xTFIIIA to promote *in vitro* transcription of the 5S RNA gene (Del Rio and Setzer, 1993), despite reductions in the binding affinity of the mutant TFIIIA's for 5S DNA (Del Rio et al., 1993). For example, xTFIIIA containing a disruption of zinc finger three has a 27-fold reduction in its affinity for 5S DNA, but supports a wild-type level of 5S RNA synthesis *in vitro*. This implies that DNA-binding is not a limiting step in transcription of the 5S RNA gene. In contrast, disruption of any one of zinc fingers 7 to 9 results in a moderate reduction to a complete loss of transcriptional activity. For example, disruption of zinc finger nine causes a severe reduction in *in vitro* transcription of the 5S RNA gene even though the DNA-binding affinity of this version of xTFIIIA is reduced only 2.7-fold. Analysis of the effect of these zinc finger disruptions on the ability of xTFIIIA to direct transcription of the 5S RNA gene *in vivo* gave similar results with the exception of disruption of zinc finger seven (Rollins et al., 1993). Mutation of this finger led to reduced transcription activity *in vitro* but had no apparent effect *in vivo*. These data suggest that zinc fingers 9 and 8, and perhaps finger 7, contribute both to the binding of TFIIIA to the A box of the 5S RNA gene and to higher-order interactions that are either directly or indirectly required for the formation of a functional transcription complex. For example, these zinc fingers may be involved in protein-protein interactions that are essential for the assembly or the activity of
the transcription complex; alternatively, these fingers may serve to appropriately position the essential carboxyl-terminal region of xTFIIIA on DNA.

1.5.1.5 Interaction of Xenopus TFIIIA with 5S RNA

One of the remarkable features of Xenopus TFIIIA is its ability to specifically bind both the 5S RNA gene and its transcript, the 5S RNA (Honda and Roeder, 1980; Pelham and Brown, 1980; reviewed in Pieler and Theunissen, 1993). Point mutations in loop A, helix II, and helix V of 5S RNA, which form the central core of its structure, abolish the 5S RNA-xTFIIIA interaction (Sands and Bogenhagen, 1987; You et al., 1991; Theunissen et al., 1992; Rawlings et al., 1996). In contrast to the base specificity in the TFIIIA-5S DNA interaction, the main determinants for high affinity binding of xTFIIIA to 5S RNA are the secondary and tertiary structure of the 5S RNA and not its primary sequence (Romaniuk et al., 1987; Baudin and Romaniuk, 1989; Romaniuk, 1989; You and Romaniuk, 1990; Baudin et al., 1991; Theunissen et al., 1992). The zinc fingers of xTFIIIA are responsible for RNA-binding (Smith et al., 1984; Sands and Bogenhagen, 1991), with fingers 4 and 6 making the greatest contributions to the energy of the TFIIIA-RNA interaction (Theunissen et al., 1992; Clemens et al., 1993; Setzer et al., 1996; Friesen and Darby, 1997). A polypeptide consisting of zinc fingers 4 through 7 of xTFIIIA binds with high affinity to the central core of the 5S RNA molecule (McBryant et al., 1995). These studies suggest that different subsets of zinc fingers within xTFIIIA confer specificity for binding to DNA (zinc fingers 1 to 3) and to RNA (zinc fingers 4 to 6 or 7). This model, however, is likely to be an oversimplification because in each case the remaining zinc fingers interact with either RNA or DNA and they may influence the overall interactions (Del Rio et al., 1993; Setzer et al., 1996).

The ability of xTFIIIA to bind 5S RNA serves a number of functions. In Xenopus, 5S RNA is synthesized in excess over other ribosomal components in immature oocytes (Pelham and Brown, 1980). The excess 5S RNA is exported from the nucleus to the cytoplasm in a TFIIIA-5S RNA complex (Guddat et al., 1990). In the cytoplasm, the 5S RNA is stabilized
in ribonucleoprotein particles until it is needed for the assembly of ribosomes in the later stages of oogenesis (Picard and Wegnez, 1979; Pelham and Brown, 1980). The ability of xTFIIIA to bind the 5S RNA transcript could also serve to regulate the level of 5S RNA gene expression by a mechanism of feedback inhibition (Pelham and Brown, 1980). Indeed, in vitro transcription of the 5S RNA gene is inhibited by the addition of excess, endogenous 5S RNA (Pelham and Brown, 1980; Rollins et al., 1993). A study of 5S RNA gene expression in Xenopus embryos that have been microinjected with mRNAs encoding wild-type and mutant forms of xTFIIIA suggests that feedback regulation also occurs in vivo (Rollins et al., 1993). Microinjection of mRNA coding for xTFIIIA containing a disruption of its sixth zinc finger causes an increase in in vivo expression of the 5S RNA genes that is greater than that observed following injection of mRNA coding for wild-type TFIIIA. Because the sixth zinc finger of xTFIIIA plays an important role in high affinity RNA-binding, but only a minor role in DNA-binding, the enhanced in vivo transcription of the endogenous 5S RNA genes might reflect a higher abundance of free TFIIIA due to reduced sequestration of the mutant TFIIIA into a TFIIIA-5S RNA complex (Theunissen et al., 1992; Del Rio et al., 1993; Setzer et al., 1996).

1.5.2 Other amphibian TFIIIA proteins

The cDNAs encoding TFIIIA from four other frog species (Xenopus borealis, Rana catesbeiana, Rana pipiens, and Bufo americanus) have been cloned and the deduced amino acid sequences of these TFIIIA proteins have been compared with that of TFIIIA from Xenopus laevis (Gaskins and Hanas, 1990; Gaskins et al., 1992). Although these TFIIIA s all contain nine zinc finger motifs, there is a surprising amount of sequence divergence (see Fig. 1-5 for a comparison of the amino acid sequences of TFIIIA from X. laevis and R. catesbeiana). TFIIIA from X. borealis has ~84% identity with TFIIIA from X. laevis. Whereas TFIIIA from R. catesbeiana and R. pipiens are ~94% identical to each other, they are only ~60% identical to TFIIIA from X. laevis. The amino-terminal two zinc fingers are
Figure 1-5. Comparison of the amino acid sequences of TFIIIA from various organisms. Shown are the deduced amino acid sequences of TFIIIA from *Xenopus laevis* (X.l.) (Ginsberg *et al.*, 1984), *Rana catesbeiana* (R.c.) (Gaskins and Hanas, 1990), *Homo sapiens* (H.s.) (Arakawa *et al.*, 1995; Drew *et al.*, 1995), and *Saccharomyces cerevisiae* (S.c.) (Archambault *et al.*, 1992; Woychik and Young, 1992). The four sequences have been aligned to compare the zinc finger motifs and the flanking amino- and carboxyl-terminal sequences. The consensus amino acids of the zinc finger motif, (Y/F)-X-C-X2.4-C-X3-F-X5-L-X2-H-X3.4-H-X5 (Miller *et al.*, 1985), are in boldface on the line labeled consensus a.a.. Residues within the zinc fingers listed below that conform to this consensus motif are also in bold. Other identical amino acids are shaded. For any position that contains only two different residues among the four proteins, only the pair between the more closely related TFIIIA*s*, in terms of overall identity, is shaded. Dashes indicate gaps introduced to maintain alignment of the four proteins. The sequence of the 81-amino acid region between zinc fingers 8 and 9 of *S. cerevisiae* TFIIIA is shown at the bottom of the figure.
the most highly conserved of the nine zinc fingers. Although the sequences of corresponding zinc fingers vary, the spacings of the cysteine and histidine residues for the corresponding zinc fingers are highly conserved. The differences in the sequences among various amphibian TFIIIAAs are reflected in functional differences (Gaskins et al., 1989). TFIIIA from *X. laevis* and from *X. borealis* protect the entire ICR (nt +43 to +96) of the 5S RNA gene of *X. borealis* from cleavage by DNase I, whereas TFIIIA from *R. catesbeiana* protects only a portion of the ICR (nt +78 to +96) of the 5S RNA gene of *X. borealis*. Furthermore, TFIIIA from *R. catesbeiana* is considerably less efficient than is TFIIIA from *X. laevis* or from *X. borealis* in promoting transcription of an *X. borealis* 5S RNA gene.

### 1.5.3 Human TFIIIA

Early studies indicated that human and *Xenopus* TFIIIA proteins are functionally and structurally related. *Xenopus* 5S RNA genes are transcribed accurately in extracts from human cells (Segall et al., 1980); both human and mouse 5S RNA genes are transcribed accurately in *Xenopus* extracts (Emerson and Roeder, 1984; Moorefield and Roeder, 1994). Moreover, in a reconstituted *in vitro* transcription system *Xenopus* TFIIIA can substitute for human TFIIIA in promoting transcription of a 5S RNA gene in the presence of human TFIIIB and TFIIIC (Carey et al., 1986).

Moorefield and Roeder (1994) purified TFIIIA from HeLa cells and found that hTFIIIA activity cofractionated with a single polypeptide of 42 kDa. This protein, following gel-purification and renaturation, directed specific *in vitro* transcription of a human 5S RNA gene, supporting its identity as hTFIIIA. The DNase I footprint obtained with purified hTFIIIA extends from nt +43 to +97 of the human 5S RNA gene (Moorefield and Roeder, 1994). This is similar to the pattern of DNase I protection observed with xTFIIIA on its homologous gene, suggesting that the two proteins are structurally related. This structural relationship was confirmed by the observation that antibodies raised against TFIIIA from *Xenopus laevis* cross-react with hTFIIIA (Moorefield and Roeder, 1994).
Human cDNAs encoding a protein highly related to xTFIIIA have been identified (Arakawa et al., 1995; Drew et al., 1995). Although the protein product of these cDNAs has yet to be formally shown to possess TFIIIA activity, the presence of nine contiguous zinc fingers and the extensive amino acid sequence identity with *Xenopus laevis* TFIIIA suggest that these cDNAs do indeed encode hTFIIIA (see Fig. 1-5). The molecular mass predicted for hTFIIIA from the cDNA is 47 kDa, which is similar to the apparent molecular mass of purified hTFIIIA (Moorefield and Roeder, 1994). The amino acid sequences of the corresponding zinc fingers of xTFIIIA and the putative hTFIIIA are highly similar and the spacings between zinc fingers are generally conserved. A notable difference between the two proteins is the size of the amino-terminal region before the first zinc finger; xTFIIIA has 14 amino acids in this region whereas hTFIIIA has 99 amino acids. The 14-amino acid sequence in the carboxyl-terminal portion of xTFIIIA that is essential for its transcription activity (Mao and Darby, 1993; see section 1.5.1.4 above) is conserved in the putative hTFIIIA clone.

1.5.4 *Saccharomyces cerevisiae* TFIIIA

Two forms of TFIIIA can be purified from *S. cerevisiae*, one of 51 kDa and the other 48 kDa, each of which has transcription factor activity (Wang and Weil, 1989). Hydrodynamic studies suggest that yeast TFIIIA, like the *Xenopus* protein, is an asymmetric polypeptide (Wang and Weil, 1989). Several observations suggest, however, that TFIIIA from *S. cerevisiae* (yTFIIIA) is not closely related to *Xenopus* TFIIIA. Antibodies raised against xTFIIIA do not cross-react with TFIIIA purified from yeast and cDNA probes encoding *Xenopus* TFIIIA do not hybridize with genomic yeast DNA (Wang and Weil, 1989). The affinity of xTFIIIA for a yeast 5S RNA gene is 100- to 1000-fold lower than is the affinity of yTFIIIA (Wang and Weil, 1989). Similarly, yTFIIIA has very low affinity for a *Xenopus* 5S RNA gene and this gene is not transcribed in a yeast cell-free extract that actively transcribes the yeast 5S RNA gene (Struksnes et al., 1991).
The gene encoding *S. cerevisiae* TFIIIA, termed *TFC2*, has been identified and shown to be essential for cell viability (Archambault *et al.*, 1992; Woychik and Young 1992). It is located in the yeast genome adjacent to *RPO26/RPB6*, the gene encoding the 23-kDa subunit that is shared by all three RNA polymerases (Archambault *et al.*, 1990; Woychik *et al.*, 1990). The proximity and head to head positioning of *TFC2* and *RPO26*, both of which encode proteins involved in transcription, raise the possibility that their expression may be coordinately controlled. The deduced amino acid sequence of *yTFIIIA* predicts a protein of ~50 kDa containing nine zinc-finger motifs (see Fig. 1-5). As suggested by the biochemical studies described above, the amino acid sequences throughout the corresponding zinc fingers of *yTFIIIA* and *xTFIIIA* differ extensively (see Fig. 1-5). The spacings of the Cys-Cys, His-His, and His-Cys residues in corresponding zinc fingers of the two proteins also differ. Additionally, the two proteins differ in the size and sequence of the amino- and carboxyl-terminal regions that flank the zinc fingers. The most striking feature of *yTFIIIA* is the presence of 81 amino acids between zinc fingers 8 and 9; this "insert" is not present in amphibian TFIIIAs nor in the putative hTFIIIA. This 81-amino acid region of *yTFIIIA* is required for its transcription factor activity (Milne and Segall, 1993). No sequence similarity is apparent between the 81-amino acid region of *yTFIIIA* and the carboxyl-terminal region of *xTFIIIA*, which is required for *xTFIIIA* transcription activity. See section 1.4.2 above for a description of the DNA-binding properties of *S. cerevisiae* TFIIIA and its role in assembly of a transcription complex on the yeast 5S RNA gene.

Camier *et al.* (1995) generated a yeast strain that allows synthesis of 5S RNA *in vivo* in the absence of TFIIIA. This was accomplished by fusing the 5S RNA gene with the tRNA-like promoter sequence of the yeast *RPR1* gene. *In vivo* processing of the transcript of this fusion gene liberates a 5S RNA that is only slightly longer than 5S RNA from the wild-type gene. Cells of a yeast *tfc2Δ* strain, which are devoid of TFIIIA and therefore do not synthesize any 5S RNA from the endogenous chromosomal 5S RNA genes, survive if the hybrid *RPR1-5S* RNA gene is present on a multicopy plasmid. This means that the only
essential function of TFIIIA in yeast is in promoting transcription of the 5S RNA genes. Yeast tfc2Δ cells containing the RPR1-5S RNA gene, however, do not grow as well as wild-type cells, are thermostensitive at 37°C, and exhibit a cell morphology reminiscent of pseudohyphal growth. The 5S RNA that is produced from the RPR1-5S RNA gene is modified at its 5' and 3' ends and could be, therefore, less active than wild-type 5S RNA; this could account for the growth defects. It is also possible that yeast TFIIIA is involved in other non-essential cellular processes. For example, 5S RNA can compete with 5S DNA for yeast TFIIIA-binding, suggesting that yeast TFIIIA, like Xenopus TFIIIA, could have a role in the nucleocytoplasmic transport of 5S RNA (Klekamp and Weil, 1982; Brow and Geiduschek, 1987).

1.6 Transcription by RNA Polymerase III

1.6.1 Recruitment of RNA polymerase III

Kassavetis et al. (1990) demonstrated that a salt- or heparin-stripped yeast transcription complex, which contains TFIIIB bound to the upstream region of a tRNA gene or a 5S RNA gene, is fully competent to recruit pol III for multiple rounds of accurately initiated transcription. Therefore, TFIIIB is the only essential initiation factor, at least in the yeast system, and serves to recruit pol III to the start site of transcription. When yeast pol III enters the transcription complex, it generates a DNase I footprint from nt -3 to nt +21 on the SUP4 tRNA Tyr gene and from nt -10 to nt +13 on the 5S RNA gene (Kassavetis et al., 1990). Once pol III has been recruited to a promoter, several of its subunits can be crosslinked specifically to SUP4 tRNA Tyr probes containing the photoreactive nucleotide N3RdUMP (see section 1.4.1.1 above) (Bartholomew et al., 1993). The two largest subunits of pol III, C160 and C128, extend along DNA and are accessible to photoreactive nucleotides from bp -17 to +20. The C34 subunit also makes extensive contacts with DNA and can be crosslinked to photoreactive nucleotides from bp -21 to +6. This places the C34 subunit at the back (that is,
upstream) end of pol III, which makes it a strong candidate for interaction with TFIIIB (see below). The ABC27 subunit, in contrast, is accessible to photoreactive nucleotides at the leading (that is, downstream) edge of the pol III-binding site. The C82 and C53 subunits interact with nucleotides at central positions within the pol III-binding site. A similar crosslinking pattern was obtained with a photoreactive deoxycytidine analog, 4-[N-(p-azidobenzoyl)-2-aminoethyl]-dCMP, which, like N3RdUMP, has a tether distance of ~0.9 nm (Lannutti et al. 1996). The use of a "zero distance" photocrosslinker, 4-thiothymine, indicated that at least ten subunits of pol III, including C160, C128, C82, C53, AC40 or C37 (these polypeptides were not clearly resolved), C34, C31, and C27, make direct contacts with tDNA and 5S DNA templates (Bartholomew et al., 1994). The addition of pol III to a TFIIIIB-TFIIIC-tDNA complex reduces the photo-affinity labeling efficiency of the 90-kDa subunit of TFIIIIB at bp -26/-21. Because no subunit of pol III becomes cross-linked to a photoactive probe at bp -26/-21, it is unlikely that binding of pol III to the initiation complex directly displaces TFIIIIB90; it is more likely that recruitment of pol III causes a conformational change in TFIIIIB90 that removes it from the vicinity of bp -26/-21 (Persinger and Bartholomew, 1996).

The C82, C53, C34 and C31 subunits of yeast pol III have no counterparts in yeast pol I or pol II, and are thus likely candidates for recognizing class III transcription complexes (Sentenac et al., 1992). A temperature sensitive form of yeast pol III that contains a mutation in the amino-terminal zinc-binding region of the C160 subunit simultaneously loses three of these pol III-specific subunits (C82, C34, and C31) upon heat inactivation (Werner et al., 1992). The notion that the C82, C34, and C31 subunits might form a subcomplex is supported by a yeast two-hybrid analysis, which indicates that each of these subunits interacts with the other two subunits (Werner et al., 1993). In addition, the C34 subunit, which interacts with DNA at an upstream position in the promoter near TFIIIIB (Bartholomew et al., 1993), interacts with TFIIIIB70 (Brf1) as assessed by two-hybrid analysis (Werner et al., 1993) and by an in vitro protein-protein interaction assay (Khoo et
These results suggest that the putative C82-C34-C31 subcomplex of yeast pol III is responsible for recognizing TFIIIB in the preinitiation complex. This hypothesis is supported by the observation that conditional mutations in the C31 subunit and in the C34 subunit impair specific transcription of class III genes, but do not affect the catalytic properties of pol III on a poly[d(A-T)] template (Thuillier et al., 1995; Brun et al., 1997).

Like yeast pol III, human pol III contains a dissociable, three-subunit subcomplex that interacts with components of human TFIIIB (Wang and Roeder, 1997). Although the human pol III enzyme that lacks this subcomplex retains all catalytic functions for RNA synthesis and is fully competent in elongation and termination of transcription, the core enzyme is unable to initiate transcription in a promoter-dependent manner in the presence of the appropriate accessory factors.

It has recently been reported that all components required for transcription of VA and tRNA genes (TFIIB, TFIIIC, and pol III) can be coimmunopurified from a HeLa cell line that expresses an epitope-tagged subunit of human pol III (Wang et al., 1997). This raises the possibility that pol III is preassembled with its cognate transcription factors (collectively referred to as an RNA polymerase III holoenzyme) prior to promoter recognition in vivo.

### 1.6.2 Initiation and Elongation

Once bound to the promoter, pol III melts the DNA at the start site of transcription in a process that can be monitored by footprinting with KMnO$_4$ (Kassavetis et al., 1990, 1992a). KMnO$_4$ oxidizes T residues in single-stranded DNA, but has little effect on T residues in duplex DNA (Hayatsu and Ukita, 1967). Although yeast pol III can stably associate with the preinitiation complex at 0°C, the DNA remains in duplex form and transcription does not occur (Kassavetis et al., 1992a). Melting at the promoter and formation of a transcription bubble is a reversible, temperature-dependent process that occurs progressively over a temperature range of 10°C to 40°C (Kassavetis et al., 1992a). The transcription bubble of the
binary complex (pol III + DNA template) on the SUP4 tRNA^{Tyr} gene includes 20 to 22 bp of DNA, maximally extending from nt -11 to +11 (Kassavetis et al., 1990, 1992a).

When pol III initiates transcription of the SUP4 tRNA^{Tyr} gene under conditions that limit the nascent RNA chain to less than six nucleotides, the transcription bubble, which is not displaced, becomes irreversibly open even at low temperatures (Kassavetis et al., 1992a). When the RNA chain has been extended to nt +17, the transcription bubble diminishes in size to between 13 and 17 bp of DNA, and is shifted downstream to maximally encompass nt +3 to +19 (Kassavetis et al., 1990, 1992a). The ternary complex (pol III + DNA template + RNA) arrested at position 17 is resistant to dissociation by the polyanion heparin (Kassavetis et al., 1989, 1990, 1992a), in contrast to ternary complexes arrested at either position 2 or 5, which are sensitive to heparin (Kassavetis et al., 1992a). This suggests the existence of an intermediate step in the pathway from the binary complex to the productively elongating ternary transcription complex. There is also evidence for distinct steps in the transcription initiation process by mammalian pol III (Kovelman and Roeder, 1990).

A comparison of the DNase I footprint and the protein-DNA photocrosslinking patterns of the ternary complex arrested 17 bp into the SUP4 tRNA^{Tyr} gene with those of the non-elongating binary complex (see section 1.6.1 above) clearly depicts the discrete progression of pol III along the template. In the ternary complex arrested at +17, the DNase I footprint attributable to pol III extends to nt +40 and the start site of transcription is once again accessible to cleavage by DNase I (Kassavetis et al., 1989, 1990). Also, all of the pol III subunits are out of range of photoreactive nucleotides placed at nt -8 or further upstream (Bartholomew et al., 1993, 1994; Lannutti et al., 1996; Persinger and Bartholomew, 1996). The most efficient crosslinking of the C128 subunit of pol III is near the site of RNA catalysis at nt +16 and +17 in the paused elongation complex (Bartholomew et al., 1993; Lannutti et al., 1996). The C160 subunit of pol III extends along much of the length of the shifted transcription bubble (Bartholomew et al., 1993; Lannutti et al., 1996). The C34 subunit of pol III is again at the trailing edge of the transcription bubble, but, like the rest of
the pol III subunits, is shifted ~20 bp further down the gene relative to its position in the binary complex (Bartholomew et al., 1993; Lannutti et al., 1996).

The presence of large transcription complexes within the transcribed region of most class III genes presents a potential obstacle to the elongating pol III. Detailed examination of the kinetics of RNA chain elongation on the SUP4 tRNA\(^{\text{Tyr}}\) gene in the presence of either purified components or crude extracts from *S. cerevisiae* reveals that the presence of TFIIIC does not cause any significant pausing of pol III (Bardeleben et al., 1994; Matsuzaki et al., 1994). Interestingly, the ability of TFIIIC to rapidly cede way to pol III is orientation-specific; that is, pol III approaching TFIIIC in the direction of normal transcription is not hindered by TFIIIC, but pol III elongating in the anti-sense direction toward the backside of TFIIIC has a significant pause on reaching the DNA-binding site of TFIIIC (Bardeleben et al., 1994). These authors also showed that TFIIIC, is displaced from the template by the passage of pol III, at least on templates that do not have bound TFIIIB (Bardeleben et al., 1994). Since yeast TFIIIB that is present in transcription complexes that have been stripped of TFIIIC can on its own recruit pol III for multiple rounds of transcription *in vitro* (Kassavetis et al., 1990), displacement of TFIIIC by pol III passage would not necessarily mean that transcription factors would need to be reassembled from scratch for the next round of transcription on that gene. Although it is unclear whether TFIIIC dissociates from DNA *in vivo*, genomic DNase I footprinting of a tRNA gene in yeast chromatin does show that the upstream footprint, presumably due to TFIIIB, is stronger than the internal footprint at the TFIIIC-binding site (Huibregtse and Engelke, 1989). In contrast to the above studies, *in vitro* experiments that tested for release of transcription factors with a template competition assay indicated that fully assembled transcription complexes are not removed from the internal control region by multiple passages of pol III (Bogenhagen et al., 1982; Setzer and Brown, 1985; Jahn et al., 1987). In this scenario, it is possible that only a portion of the bound transcription complex is dislodged by pol III at any given moment, with the remaining protein-DNA interactions sufficing to keep the transcription factors in the vicinity of the gene
and able to reestablish contact with DNA as pol III advances. The association of TFIIB with DNA upstream of the transcription start site may be important in preventing TFIIC and TFIID from being displaced from the template as pol III transcribes through their DNA-binding sites. At least in the case of the Xenopus 5S RNA gene, studies suggest that the continued presence of transcription factors may in fact be important in vivo to prevent nucleosomes from assembling on the gene and silencing it (Tremethick et al., 1990).

1.6.3 Termination

A series of T residues in the non-template strand of DNA usually serves as a signal sequence for termination of transcription by pol III (reviewed in Geiduschek and Tocchini-Valentini, 1988). A cluster of four or more T residues within a GC-rich sequence is sufficient to act as a termination signal during in vitro transcription of Xenopus 5S RNA genes (Bogenhagen and Brown, 1981). Oligo(dT) clusters also serve as termination signals for tRNA genes (Koski et al., 1980; Adeniyi-Jones et al., 1984; Traboni et al., 1984; Allison and Hall, 1985; Mazabraud et al., 1987). The efficiency of termination correlates with the length of the T stretch (Allison and Hall, 1985) and also with the identity of the flanking nucleotides (Bogenhagen and Brown, 1981; Mazabraud et al., 1987). There appears to be some species differences in the requirements for transcription termination. While S. cerevisiae pol III requires six or seven Ts for efficient termination (Koski et al., 1982; Allison and Hall, 1985), Xenopus pol III terminates efficiently at a sequence of four Ts (Bogenhagen and Brown, 1981). It should also be noted that not all pol III termination sites conform with the oligo(dT) consensus (Mazabraud et al., 1987). For example, human pol III can terminate at clusters of A or alternating A/T residues (Emerson and Roeder, 1984; Hess et al., 1985; Matsumoto et al., 1989).

Whereas pol III requires a variety of accessory factors to initiate transcription specifically, it can recognize termination signals accurately and efficiently in the absence of other factors. This was first demonstrated by analyzing transcripts produced by highly
purified *Xenopus* pol III that had initiated transcription randomly in the absence of accessory transcription factors (Cozzarelli *et al*., 1983). This polymerase terminated transcription efficiently at a downstream cluster of T residues, indicating that the pol III enzyme can on its own terminate transcription accurately. Analysis of transcription termination by bovine pol III led to a similar conclusion (Watson *et al*., 1984). Genetic evidence that pol III itself is important for recognition or utilization of termination signals comes from studies in *S. cerevisiae*; mutations in the second-largest subunit of pol III (C128) can lead to either reduced or increased termination by this enzyme (James *et al*., 1990, 1991; Shaaban *et al*., 1995).

Transcription termination likely occurs by a multi-step process involving first the pausing of the RNA polymerase at the termination site followed by release of nascent RNA and subsequent polymerase dissociation. In support of this, Campbell and Setzer (1992) showed that recognition of the termination signal by purified *Xenopus* pol III can be uncoupled experimentally from the process of polymerase and transcript release. Following factor-independent transcription initiation from a poly(dC)-tailed template, pol III was frequently unable to displace the nascent RNA from the template strand during transcription elongation. When no RNA displacement occurred, pol III recognized the termination signal and paused, but then continued transcription. This implies that pol III release from the template is dependent on RNA strand displacement during elongation, whereas termination signal recognition is not.

Although the above studies suggest a factor-independent mechanism for pol III termination, there is also evidence that the RNA-binding protein La is involved in termination. La protein is a human autoimmune antigen that is transiently associated with all nascent pol III transcripts (Lerner *et al*., 1981; Rinke and Steitz, 1982). La binds to these RNAs via their common U-rich 3' ends, which corresponds to the oligo(dT) termination signal for pol III (Stefano, 1984). Extracts from HeLa cells that have been immunodepleted of La are compromised in their ability to support transcription of class III genes (Gottlieb and
Steitz, 1989a). The small number of pol III transcripts synthesized in the absence of La are one to five U residues short of the authentic 3' end (Gottlieb and Steitz, 1989a, 1989b). Addition of purified La to the immunodepleted extracts restores transcript length and partially restores transcription (Gottlieb and Steitz, 1989b). These results suggest that La is required for completion of transcription and transcript release prior to recycling of pol III.

1.6.4 Reinitiation

Dieci and Sentenac (1996) found that yeast pol III, once engaged in transcription, is predominately committed to reinitiating transcription on the same template. Furthermore, a kinetic analysis indicated that yeast pol III completes additional cycles of transcription more rapidly than the initial one. This process was dependent on normal termination since runoff transcription did not allow efficient recycling. These authors proposed a mechanism involving facilitated reinitiation of transcription whereby the terminating pol III contacts a component of the preinitiation complex, likely TFIIIB, and reinitiates transcription without being released from the template. This would maximize transcriptional efficiency of class III genes, which may be essential to satisfy the high cellular needs for class III gene products.

In addition to its role in termination, the human La antigen has been implicated in facilitating recycling of human pol III onto stable preinitiation complexes (Maraia et al., 1994; Maraia, 1996). Although this may be due to La simply facilitating dissociation of pol III and template, the high efficiency with which a limiting amount of pol III is recycled in the presence of La suggests that La may actively direct pol III to reinitiate transcription (Maraia et al., 1994). The requirement of La in directing pol III to preinitiation complexes for reinitiation of transcription supports the more active role (Maraia, 1996). However, further studies are needed to determine if La is involved in facilitated recycling as described for yeast pol III.
1.7 Chromatin and RNA Polymerase III Transcription

The expression of eukaryotic genes in vivo occurs in a chromatin environment, and both biochemical and genetic evidence indicate that chromatin structure plays an active role in regulating transcription (reviewed in Felsenfeld, 1992). The fundamental repeating unit of chromatin is the nucleosome. Each nucleosome consists of ~146 bp of DNA wrapped approximately twice around an octameric protein core containing two copies each of histones H2A, H2B, H3, and H4, ~50 to 70 bp of linker DNA, and one molecule of linker histone, usually histone H1 (reviewed in Ramakrishnan, 1997). The arrays of nucleosomes further assemble into higher order structures that are stabilized by the linker histone. Chromatin is generally repressive in nature, mainly because the accessibility of a gene to transcription factors is likely be severely limited. However, the presence of positioned nucleosomes can positively influence transcription in certain circumstances (for example, see Schild et al., 1993). As described below, transcription by pol III can be influenced by chromatin structure in both a positive and negative manner.

The effect of chromatin structure on the expression of 5S RNA genes has been extensively studied. Preassembly of nucleosomes onto 5S DNA templates of any origin inhibits both transcriptional initiation and elongation by pol III (Gottesfeld and Bloomer, 1982; Shimamura et al., 1988; Morse, 1989; Felts et al., 1990; Hansen and Wolffe, 1992; Stüinkel et al., 1995). Gottesfeld and Bloomer (1982) found that preincubation of Xenopus TFIIIA with a X. laevis oocyte-type 5S RNA gene prior to nucleosome assembly counteracted the nucleosomal inhibition of transcription. In contrast, Tremethick et al. (1990) found that prebinding of Xenopus TFIIIA alone does not prevent nucleosomal inhibition of transcription of the X. laevis somatic-type 5S RNA gene and that the entire transcription complex is required to prevent nucleosomal repression of transcription. In agreement with the latter findings, Felts et al. (1990) found that prebinding of S. cerevisiae TFIIIA to a yeast 5S RNA gene is not sufficient to maintain an active transcriptional state.
following chromatin assembly. Finally, it has been found recently that human TFIIIA alone is sufficient to prevent nucleosomal repression of a homologous 5S RNA gene (Stünkel et al., 1995). These discrepancies may be due to species-specific or gene-specific differences or due to differences in the assay conditions. In all cases, however, a complete TFIIIB-TFIIIC-TFIIIA-5S DNA transcription complex is resistant to displacement by nucleosomes and hence maintains an active transcriptional state. Chromatin structure and transcription factor complexes are both disrupted by DNA replication (Wolffe and Brown, 1986; Jackson, 1990).

After DNA replication, chromatin assembly has been proposed to occur first by the formation of the (H3/H4)2 tetramer, followed by inclusion of (H2A/H2B)2 to form a histone octamer, and then formation of a higher-order chromatin structure (Smith and Stillman, 1991). TFIIIA can interact with DNA assembled with a (H3/H4)2 tetramer of histones (depending on the position of histone-DNA contacts), but not a histone octamer (Rhodes, 1985; Hayes and Wolffe, 1992; Lee et al., 1993a). It is possible, therefore, that 5S RNA genes can assemble a stable transcription complex before a histone octamer forms. The binding of TFIIIA to 5S RNA genes in a chromatin environment may also be achieved by modification of nucleosomes. The lysine rich amino-terminal tails of the core histones interact extensively with the phosphodiester backbone of DNA (reviewed in Wade et al., 1997). Acetylation of these histone tails allows access of TFIIIA to nucleosomal DNA (Lee et al., 1993a). Acetylation of histones is associated with histone deposition on newly replicated DNA (reviewed in Wolffe, 1991). The histones are progressively deacetylated as chromatin matures. It was proposed that incorporation of acetylated histones into nascent chromatin may facilitate TFIIIA access to DNA immediately following replication (Lee et al., 1993a).

Another example of a pol III gene analyzed in the context of chromatin is the S. cerevisiae U6 snRNA gene (SNR6). The SNR6 gene has three promoter elements: a TATA box at -30, an intragenic A box, and a B box downstream of the termination signal (see section 1.3.4). The A and B boxes, the binding site for TFIIIC, are essential for transcription of the SNR6 gene in vivo and for its transcription in vitro in the presence of a crude cell
extract (Brow and Guthrie, 1990; Burnol et al., 1993b; Eschenlauer et al., 1993). A mutant
SNR6 gene lacking a B box can be transcribed in vitro, however, in the presence of purified
TFIIIB and pol III; in this case, TFIIIC is not required (Margottin et al., 1991; Burnol et al.,
1993b). The different promoter requirements for transcription of the SNR6 gene in vivo or in
crude extracts and for its transcription in the presence of purified components suggest an
additional role for TFIIIC in transcription, possibly in promoting access of TFIIIB to DNA
that is part of chromatin. Consistent with this, Burnol et al. (1993a) showed that
nucleosomes preassembled on the SNR6 gene can block transcription directed by TFIIIB, but
that TFIIIC can alleviate this inhibition when the downstream B-box element of the gene is
intact. It has also been proposed that a positioned nucleosome may actually have a positive
role in transcription of the SNR6 gene in vivo. Gerlach et al. (1995) demonstrated that
TFIIIB placement in vivo is directed mainly by TFIIIC-binding elements rather than by
upstream elements. However, the A- and B-box elements of the SNR6 gene are spaced
relatively far apart as compared to A box to B box separations of most tRNA genes. Thus,
the interaction of TFIIIC with the SNR6 gene may be suboptimal. It was proposed, partly
based on chromatin footprinting data, that a positioned nucleosome brings the A- and B-box
elements closer together to facilitate the binding of TFIIIC, and consequently promote
TFIIIB recruitment. An analogous situation may also occur on the human U6 snRNA gene.
Transcription of this gene is enhanced after chromatin reconstitution, and appears to be due
to a strongly positioned nucleosome in the upstream region between the distal sequence
element (DSE) and the proximal sequence element (PSE) (Stünk et al., 1997).

1.8 Regulation of RNA Polymerase III Transcription

The regulation of class III gene expression operates principally at the level of the
transcription initiation machinery. That is, the activities of TFIIIB, TFIIIC, and TFIIIA are
directly regulated. Rather than presenting an exhaustive overview of pol III transcriptional
regulation, I present a number of recent examples in the literature that illustrate the regulatory mechanisms that affect pol III transcription.

Transcription by pol III is under cell cycle control, increasing gradually through G1, reaching a maximum during S and G2, decreasing as cells enter M, and reaching a minimum in late M (Hartl et al., 1993; White et al., 1995). The mitotic repression of pol III transcription can be reproduced in vitro with use of Xenopus egg extracts and, at least in this system, is due to the inactivation of the pol III transcriptional machinery by phosphorylation (Hartl et al., 1993). It was subsequently found that the mitotic repression involves the direct phosphorylation of a component of TFIIB (Gottesfeld et al., 1994). The phosphorylation status of TFIIB also appears to be important for its activity in S. cerevisiae. Hockman and Schultz (1996) found that casein kinase II, a highly conserved serine/threonine kinase important for cell cycle and growth control, is required for efficient pol III transcription of the yeast 5S RNA and tRNA genes, both in vitro and in vivo. It was subsequently shown that the TBP component of yeast TFIIB is the target of casein kinase II-mediated phosphorylation and that phosphorylation of TBP by casein kinase II correlates with an increase in TFIIB transcription factor activity (Ghavidel and Schultz, 1997).

The retinoblastoma tumor-suppressor protein (Rb) functions as a general repressor of pol III transcription (White et al., 1996). Since the activity of Rb is subject to cell cycle control (Weinberg, 1995), this provides another mechanism for coordinating pol III gene expression with the phases of the cell cycle (for review, see White, 1997). Transcription of every pol III template tested, including the genes encoding tRNAs, 5S rRNA, U6 snRNA, adenovirus VA1 RNA, and Epstein-Barr virus EBER2 RNA, is repressed by Rb in vitro (White et al., 1996; Larminie et al., 1997). Accordingly, there is an elevated expression of class III genes in Rb-deficient cells (White et al., 1996). The main target of Rb appears to be TFIIB. Rb specifically decreases TFIIB activity in reconstituted transcription reactions and Rb physically interacts with TFIIB (Larminie et al., 1997; Chu et al., 1997). Since Rb contains regions of sequence similarity, albeit weak, with regions in the human TFIIB subunits TBP
and hTFIIIB90, a model was proposed suggesting that Rb inactivates TFIIIB by mimicking TBP and hTFIIIB90 in order to disrupt TFIIIB (Larminie et al., 1997).

Human TFIIIC can be a target of both positive and negative regulation in response to viral infection. Pol III transcription is strongly stimulated by the expression of immediate early proteins of adenovirus (E1A and/or E1B) and pseudorabies virus (PrIE) (Berger and Folk, 1985; Gaynor et al., 1985; Hoeffler and Roeder, 1985; Yoshinaga et al., 1986). In contrast, poliovirus infection substantially decreases pol III transcription of cellular genes (Fradkin et al., 1987). Fractionation studies indicated that TFIIIC is responsible for these changes in the activity of pol III transcription (Hoeffler and Roeder, 1985; Yoshinaga et al., 1986; Fradkin et al., 1987). The relative levels of two different forms of TFIIIC2, termed TFIIIC2a (active form) and TFIIIC2b (inactive form), are affected by viral infection of cells (Hoeffler et al., 1988; Clark and Dasgupta, 1990; Sinn et al., 1995). Both forms of TFIIIC2 specifically bind the B box of a VA1 RNA gene with high affinity, but only TFIIIC2a can support pol III transcription (Kovelman and Roeder, 1992). The main physical difference between these two forms is the presence of a 110-kDa polypeptide in TFIIIC2a that is absent in TFIIIC2b (Kovelman and Roeder, 1992; Sinn et al., 1995).

The specific regulation of 5S RNA gene expression has been examined in a number of systems. During the early stages of Acanthamoeba castellanii differentiation into dormant cysts, expression of 5S RNA genes, but not tRNA genes, is down-regulated (Matthews et al., 1995). The specific down-regulation of transcription of the 5S RNA gene in the encysting Acanthamoeba cells appears to be due to a drastic decrease in the activity or level of TFIIIA (Matthews et al., 1995). Clarke et al. (1996) examined the synthesis of 5S RNA and tRNAs in extracts prepared from S. cerevisiae cells subjected to a variety of changes in growth conditions. These authors found that 5S RNA and tRNA synthesis can be either coordinately regulated or differentially regulated depending on the growth conditions. An extract prepared from yeast cells that have been starved for the essential amino acid histidine is defective for 5S RNA gene transcription but competent for tRNA gene transcription. It was
found that this differential regulation was not due to inactivation of intrinsic TFIIIA activity, but rather due to a proteinaceous inhibitor that specifically interferes with 5S RNA gene transcription. Finally, as discussed in section 1.5.1.5, TFIIIA has the distinctive ability to bind specifically to both the 5S RNA gene and the 5S RNA gene product, thereby providing a means of feedback regulation of 5S RNA gene transcription (Pelham and Brown, 1980).
Thesis Rationale

Transcription of the 5S RNA gene by RNA polymerase III requires the gene-specific factor called TFIIIA. TFIIIA binds to the internal control region of the 5S RNA gene and serves to recruit and position TFIIIC in the transcription complex. The TFIIIC-TFIIIA-DNA complex then recruits TFIIIB, which acts as an initiation factor in directing accurate transcription by pol III. TFIIIA from *Xenopus laevis* has been extensively characterized with regards to its DNA-binding properties. Difficulties in the purification of amphibian TFIIIC and TFIIIB, however, have hampered studies of the role of xTFIIIA in assembly of a functional transcription complex on the 5S RNA gene. In contrast, the pol III transcriptional machinery of the yeast *S. cerevisiae* has been extensively characterized. This is partly due to the relative ease of carrying out both biochemical and genetic studies with *S. cerevisiae*. Most, if not all, of the subunits of yeast TFIIIB, TFIIIC, and pol III have been identified and most of the corresponding genes have been cloned. As such, *S. cerevisiae* is an ideal organism in which to study the role of TFIIIA in transcription of the 5S RNA gene.

TFIIIA of *S. cerevisiae* contains nine zinc-finger motifs with an 81-amino acid segment interrupting the otherwise repeating zinc-finger motifs between fingers 8 and 9 (Archambault *et al.*, 1992). A polypeptide containing only the amino-terminal three zinc fingers of TFIIIA specifically binds to the 5S RNA gene and recruits TFIIIC; the TFIIIC-TFIIIA-DNA complex formed with this truncated form of TFIIIA, however, cannot promote transcription of the 5S RNA gene (Milne and Segall, 1993). The 81-amino acid region between zinc fingers 8 and 9 is essential for the transcription activity of TFIIIA (Milne and Segall, 1993).

The first portion of my thesis research focused on the interaction of the zinc fingers of yeast TFIIIA with the yeast 5S RNA gene (Chapter 2). I found that zinc fingers 6 to 9 of TFIIIA were not in tight association with DNA and that zinc fingers 1 to 5 made contacts in the major groove of DNA from nucleotides +73 to +94. A polypeptide containing zinc fingers 1 to 3 of TFIIIA bound with high affinity to the ICR of the yeast 5S RNA gene.
As a step towards understanding the role of the 81-amino acid domain of yeast TFIIIA in establishing an active transcription complex on the 5S RNA gene, I carried out a mutational analysis to identify amino acids within this domain that are essential for its transcription factor activity (Chapter 3). I found that the 81-amino acid domain of yeast TFIIIA was surprisingly tolerant to mutation. Only a deletion of an asparagine- and leucine-rich segment \(^{352}\text{NGLNLLLN}^{359}\) within the carboxyl-terminal portion of the 81-amino acid domain completely destroyed the transcription factor activity of TFIIIA. Alanine-scanning mutagenesis indicated that the hydrophobic residues of this segment were critical for transcription activity. In an Appendix to Chapter 3, I present preliminary data from an experiment investigating the role of the 81-amino acid domain in the formation of an active transcription complex. My results were consistent with the possibility that at least one role of the 81-amino acid region is to prevent an inappropriate interaction of TFIIIC with the 5S RNA gene.

In Chapter 3 and Chapter 4, I present experiments assessing the effects of structural disruptions of various zinc fingers on the activity of TFIIIA. I found that yeast TFIIIA remained active if either zinc finger 8 or zinc finger 9 was disrupted by mutation, but that TFIIIA containing a disruption of both these zinc fingers was unable to promote transcription of the 5S RNA gene (Chapter 3). I found that disruptions of zinc fingers 1, 2, 3, or 5 each affected the ability of TFIIIA to bind DNA to various degrees, but that only a disruption of zinc finger 1 abolished transcription factor activity (Chapter 4). This suggested that zinc finger 1 not only contributed to the DNA-binding affinity of TFIIIA but was also essential for formation of an active transcription complex on the 5S RNA gene.
CHAPTER 2

Interaction of Wild-type and Truncated Forms of Transcription Factor IIIA from Saccharomyces cerevisiae with the 5S RNA Gene

The experiments in this Chapter are presented essentially as published in:
Rowland, O. and J. Segall (1996)
Journal of Biological Chemistry 271:12103-12110
ABSTRACT

Transcription factor IIIA (TFIIIA), which contains nine zinc finger motifs, binds to the internal control region of the 5S RNA gene as the first step in the assembly of a multifactor complex that promotes accurate initiation of transcription by RNA polymerase III. I have monitored the interaction of wild-type and truncated forms of yeast TFIIIA with the 5S RNA gene. The DNase I footprints obtained with full length TFIIIA and a polypeptide containing the amino-terminal five zinc fingers (TF5) were indistinguishable, extending from nucleotides +64 to +99 of the 5S RNA gene. This suggests that fingers 6 to 9 of yeast TFIIIA are not in tight association with DNA. The DNase I footprint obtained with a polypeptide containing the amino-terminal four zinc fingers (TF4) was 14 base pairs shorter than that of TF5, extending from nucleotides +78 to +99 on the nontranscribed strand and from nucleotides +79 to +98 on the transcribed strand of the 5S RNA gene. Protection provided by a polypeptide containing the first three zinc fingers (TF3) was similar to that provided by TF4 with the exception that protection on the nontranscribed strand ended at nucleotide +80, rather than nucleotide +78. Methylation protection analysis indicated that finger 5 makes major groove contacts with guanines +73 and +74. The amino-terminal four zinc fingers make contacts that span the internal control region, which extends from nucleotides +81 to +94 of the 5S RNA gene, with finger 4 appearing to contact guanine +82. Measurements of the apparent $K_d$ values of the TFIIIA-DNA complexes indicated that the amino-terminal three zinc fingers of TFIIIA have a binding energy that is similar to that of the full length protein.
INTRODUCTION

Transcription factor IIIA (TFIIIA), a sequence-specific DNA-binding protein, binds to the 5S RNA gene as the first step in the assembly of a multifactor transcription complex. Interaction of TFIIIA with the internal control region (ICR) of the 5S RNA gene is necessary for incorporation of the multisubunit TFIIIC into the TFIIIA-DNA complex. Formation of the TFIIIC-TFIIIA-DNA complex is a prerequisite for recruitment of TFIIIB; the TFIIIB-TFIIIC-TFIIIA-DNA complex then recruits RNA polymerase III (see Chapter 1, section 1.4.2).

Since the initial purification of TFIIIA from Xenopus (Engelke et al., 1980), the mode of interaction of this TFIIIA with the Xenopus 5S RNA gene has been extensively studied (see Chapter 1, section 1.5.1). The 50-bp ICR of the Xenopus 5S RNA gene contains three elements that contribute to efficient transcription of the gene: the A-box spanning nucleotides +50 to +64, the intermediate element spanning nucleotides +67 to +72, and the C-box spanning nucleotides +80 to +97 (Pieler et al., 1985a, 1985b, 1987; Bogenhagen, 1985). The recently reported crystal structure of the amino-terminal six zinc fingers of Xenopus TFIIIA bound with 31 bp of the 5S RNA gene promoter (Nolte et al., 1998) shows that zinc fingers 1 to 3 wrap around the DNA major groove of the C-box promoter element and that zinc fingers 4 to 6 lie on one side of the DNA helix with finger 5 binding in the DNA major groove of the intermediate promoter element and fingers 4 and 6 traversing the neighboring DNA minor grooves; the carboxyl-terminal three zinc fingers of Xenopus TFIIIA are thought to wrap around the DNA major groove of the A-box promoter element (Clemens et al., 1992; Hayes and Clemens, 1992; Hayes and Tullius, 1992; Fairall and Rhodes, 1992; Hansen et al., 1993). The amino-terminal fingers of Xenopus TFIIIA interact with the C-box element with a binding energy that is similar to that of the TFIIIA-DNA interaction (Sakonju et al., 1981; Sakonju and Brown, 1982; Fairall et al., 1986; Vrana et al., 1988; You et al., 1991; Darby and Joho, 1992;
Indeed, a polypeptide containing the amino-terminal three zinc fingers of Xenopus TFIIIA binds with high affinity to the 5S RNA gene, protecting the region from approximately nucleotides +74 to +95 from cleavage by DNase I (Christensen et al., 1991; Liao et al., 1992). It should be noted that various fingers make differing contributions to the binding energy of complex formation and that the energetic contribution made by a finger can be influenced by neighboring fingers or sets of fingers (Del Rio et al., 1993; Clemens et al., 1994; Zang et al., 1995; Kehres et al., 1997).

Molecular characterization of TFIIIB and TFIIIC, and insights into the architecture of the transcription complex have been largely derived from studies with Saccharomyces cerevisiae (see Chapter 1, section 1.4.1). We have therefore pursued structure-function studies with TFIIIA from this organism. Comparison of the deduced sequences of TFIIIA from S. cerevisiae and from Xenopus laevis indicated that the two proteins are structurally similar in that they both contain nine zinc finger motifs (Ginsberg et al., 1984; Archambault et al., 1992; Woychik and Young, 1992). However, the amino acid sequences of the corresponding fingers of yeast and amphibian TFIIIA as well as the linker sequences between the fingers differ extensively. Moreover, in contrast to the 50-bp DNase I footprints of the Xenopus TFIIIA-DNA complex (Engelke et al., 1980) and the human TFIIIA-DNA complex (Seifart et al., 1989; Moorefield and Roeder, 1994), the DNase I footprint of the yeast TFIIIA-DNA complex is 35 bp, extending from nucleotides +63 to +97 (Braun et al., 1989). The DNase I footprint includes the C-box element of the yeast 5S RNA gene. This 15-bp sequence, which is positioned at nucleotides +81 to +94, is the only intragenic element that is essential for efficient in vitro transcription of the yeast 5S RNA gene (Challice and Segall, 1989).

Milne and Segall (1993) showed that a truncated polypeptide containing the amino-terminal three zinc fingers of yeast TFIIIA retains the ability to bind to the yeast 5S RNA gene. In the present study, I have analyzed in more detail the interaction of full length and truncated forms
of yeast TFIIIA with the 5S RNA gene. My data reveal that fingers 6 to 9 of yeast TFIIIA are not in tight association with DNA in the TFIIIA-DNA complex and suggest that the amino-terminal five zinc fingers make major groove contacts from nucleotides +73 to +94. A polypeptide containing the amino-terminal three zinc fingers of TFIIIA has a binding energy that is similar to that of the full length protein.

MATERIALS AND METHODS

Plasmids for Bacterial Expression of Wild-type and Truncated Forms of Yeast TFIIIA. A plasmid for bacterial expression of wild-type TFIIIA was obtained by inserting the 2.3-kilobase pair NcoI-BamHI fragment of pJA454 (Archambault et al., 1992) between the corresponding sites of pET-11d (Studier et al., 1990), which places the coding region of TFIIIA under the control of a bacteriophage T7 RNA polymerase promoter. Construction of plasmids for bacterial expression of TFIIIA truncated after the eighth (TF8), seventh (TF7*), fourth (TF4*), and third (TF3*) zinc fingers took advantage of the following restriction endonuclease recognition sites: an EcoRV site present at codon 282 at the end of finger 8, five residues after the second His residue of the zinc finger motif; an XbaI site at codon 265 within finger 8, seven residues after the second Cys residue of the zinc finger motif; a HindIII site at codon 171 within finger 5, three residues after the second Cys residue of the zinc finger motif; and a Bsp1286I site at codon 152 within finger 4, eleven residues after the second Cys residue of the zinc finger motif (see Fig. 2-1A; also see Fig. 1 of Milne and Segall (1993)). The zinc finger motif is (Tyr/Phe)-Xaa-Cys-(Xaa)2,4-Cys-(Xaa)3-Phe-(Xaa)5-Leu-
(Xaa)_2-His-(Xaa)_3,4-His-(Xaa)_5. The code name TFX indicates that the protein contains the first $X$ amino-terminal fingers; an asterisk in the name indicates that the truncation occurred within finger $X + 1$.

The first step in construction of a plasmid for expression of TFI11A truncated after the fourth zinc finger motif (TF4*) involved cloning the annealed, self-complementary oligonucleotide 5'-CTAGCTAGCTAG-3' (kindly provided by J. Ingles), which provides stop codons in all reading frames, into the Smal site of pJA453 (Archambault et al., 1992) to generate pJA453-STOP. pJA453 is a pBluescript SK+ derivative containing the TFI11A-coding region from the initiator ATG codon to the HindIII site at codon 171. The NcoI-BamHI fragment of pJA453-STOP was then inserted between the corresponding sites of pET-11d for expression of TF4. The first step in construction of plasmids for expression of TF8, TF7*, and TF3* involved subcloning the 0.85 kilobase pair KpnI-EcoRV fragment, the 0.80 kilobase pair KpnI-XbaI fragment, and the 0.46 kilobase pair KpnI-Bsp1286I fragment, respectively, of pJA454 between the KpnI and EcoRV sites of pJA453-STOP. For these constructions, the XbaI end of the KpnI-XbaI fragment and the Bsp1286I end of the KpnI-Bsp1286I fragment were blunted prior to ligation. The NcoI-BamHI fragment of each of the resultant plasmids, containing TFI11A-coding sequence, was then subcloned between the corresponding sites of the pET-11d expression vector. This cloning procedure introduced the non-TFI11A, poly linker-encoded amino acids EFLQPLAS, SNCSSP, LISNSCSP, and SNCSSP at the carboxyl-terminus of TF8, TF7*, TF4*, and TF3*, respectively.

For the DNase I and methylation protection footprint experiments, genes encoding proteins with carboxyl-terminal truncations that ended after the linker sequence of finger 6 (TF6), finger 5 (TF5), finger 4 (TF4), and finger 3 (TF3) were generated by polymerase chain reaction. Downstream primers introduced a stop codon at the end of the linker. TF6, TF5, TF4, and TF3 had a stop codon introduced after codons 223, 193, 163, and 133, respectively. To facilitate subcloning, these primers also contained a BamHI site at their 5'-ends. These primers
were used in a PCR reaction, with pJA454 as template and the universal primer as the upstream primer, to generate the truncated TFIIIA-coding sequences. The PCR products were cut with NcoI and BamHI and cloned between the corresponding sites of pET-11d.

For the DNase I experiments, pET-11d-based plasmids were also constructed for bacterial expression of amino-terminal truncated forms of TFIIIA. Plasmids JA454-2 and JA454-3 (Milne and Segall, 1993), which encode TFIIIA proteins lacking the sequence for codons 2-43 and codons 2-69, respectively, were digested with KpnI. After the resultant ends had been blunted by treatment with the Klenow form of DNA polymerase I, the DNA was digested with BamHI. The pET-11d expression vector was digested with NcoI and the resultant ends were treated sequentially with S1 nuclease and the Klenow form of DNA polymerase I prior to digestion of the DNA with BamHI. The KpnI-BamHI fragments that had been excised from pJA454-2 and pJA454-3 were then subcloned between the NcoI and BamHI sites of pET-11d; this generated plasmids for expression of variant forms of TFIIIA that lack the sequence prior to finger 1 (1FS) and that lack most of the sequence prior to finger 2 (2FS), respectively. The sequence of all PCR-amplified DNA and all subcloning junctions was verified by the chain termination method (Sanger et al., 1977).

**Purification of Yeast TFIIIA from Bacteria.** The pET-11d-derived plasmids were transformed into the *Escherichia coli* strain BL21(DE3), which contains the T7 RNA polymerase gene under the control of the lacUV5 promoter. One ml of an overnight culture was used to inoculate 100 ml of LB medium (1% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1% NaCl (pH 7.0)) containing 100 µg of ampicillin/ml. When the culture reached an A600 of 0.5-0.7, ZnSO4 and isopropyl-β-D-thiogalactopyranoside were added to 50 µM and 1 mM, respectively. Four hours after induction, cells were harvested, washed with 5 ml of buffer A (20 mM HEPES (pH 7.4), 5 mM MgCl2, 50 µM ZnSO4, 250 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol), and resuspended in 4 ml of buffer A containing 1 mM phenylmethylsulfonfonyl fluoride (PMSF). The cells were broken by sonication and TFIIIA was
then purified from inclusion bodies essentially as described by Del Rio and Setzer (1991). All steps were carried out at 4°C. The pellet obtained by centrifugation of the sonicated extract at 17,000 x g for 10 min was solubilized in 1 ml of buffer A containing 1 mM PMSF and 5 M urea with mixing by inversion for 16 h. The solubilized extract was centrifuged for 20 min at 23,500 x g, and the supernatant was taken. For further purification, the supernatant was brought to 25% saturation with (NH₄)₂SO₄ and mixed by inversion at 4°C for 1 h. Precipitated protein was removed by centrifugation. The supernatant was brought to 80% saturation with (NH₄)₂SO₄ and mixed by inversion for 2 h, and precipitated protein was recovered by centrifugation. The pellet was dissolved in 10 ml of buffer A containing 5 M urea and applied to a 2 ml column of Bio-Rex 70 that had been equilibrated with the same buffer. After the column had been washed with buffer A containing 5 M urea, bound protein was eluted with a 0.25-1 M gradient of NaCl in buffer A containing 5 M urea. TFIIIA-containing fractions were identified by electrophoresis of an aliquot of each fraction on an SDS-polyacrylamide gel followed by staining with Coomassie Blue. Protein concentration was determined using the Bradford assay (Bradford, 1976), with bovine serum albumin (BSA) as a standard. Fractions were stored in aliquots at -70°C and used without dialysis. Active TFIIIA could be obtained by dilution of the urea-containing samples.

**Electrophoretic Mobility Shift and *In Vitro* Transcription Assays.**

Electrophoretic mobility shift assays were performed as described previously (Challice and Segall, 1989). The standard 20 µl reaction contained 10 mM HEPES (pH 7.9), 110 mM KCl, 11 mM MgCl₂, 50 µM ZnSO₄, 10% glycerol, 0.65 mM dithiothreitol, 0.25 mM EDTA, 2 µg BSA, 2 µg poly(dI·dC)-poly(dI·dC), and a 270 bp end-labeled DNA fragment containing the yeast 5S RNA gene (see below). After a short incubation, an aliquot of TFIIIA was added and after an additional 5-10 min incubation the samples were applied to a 4% polyacrylamide gel running at 60V. The gel and running buffer was 45 mM Tris-borate (pH 8.3), 1 mM EDTA. Gels were prerun for 1 h at 60 V and run at 120 V at room temperature.
In vitro transcription assays were performed as described (Taylor and Segall, 1985) using the yeast 5S RNA gene (p19-5S) (Challice and Segall, 1989) or a tRNA gene as template (pPm16) (Olson et al., 1981). A 50 μl reaction contained 18 μl of a yeast-derived heparin-agarose fraction (fraction h) (Taylor and Segall, 1985), which contains TFIIIB, TFIIIC, and RNA polymerase III, and, as indicated, partially purified yeast TFIIIA or bacterially produced TFIIIA.

Measurement of Apparent Dissociation Constants ($K_d$). A series of 20-μl gel mobility shift reactions were prepared using a constant concentration of purified wild-type or truncated TFIIIA and variable concentrations of end-labeled 5S DNA prepared from p19-5S (see below). Reaction mixtures were incubated for 10 min and then subjected to electrophoresis on a non-denaturing gel to separate protein-DNA complexes from free DNA. Control experiments indicated that equilibrium was reached very quickly: the amount of protein-DNA complex present after a 1-min incubation was the same as the amount present after a 10-min incubation. The ratio of bound DNA to free DNA in each lane was determined using ImageQuant software (Molecular Dynamics, Inc.) to analyze phosphorimages of dried gels obtained with a Molecular Dynamics PhosphorImager. These ratios were then used in conjunction with the known concentration of input DNA to determine the concentrations of TFIIIA-DNA complex and free DNA. Apparent dissociation constants were determined by nonlinear regression analysis using Regression (Blackwell Scientific Publications, Inc.) and KaleidaGraph 2.1 (Abelbeck Software) to fit the data to the following equation derived from the law of mass action: $[P-D] = \frac{[P_T][D]}{K_d + [D]}$, where $P-D$, $P_T$, and $D$ refer to TFIIIA-DNA complex, total active TFIIIA and free DNA, respectively. $[P-D]$ and $[D]$ were the input variables to the non-linear regression analysis and $K_d$ and $[P_T]$ were the unconstrained output parameters. The calculation assumes that TFIIIA binds to DNA with 1:1 stoichiometry and does not require that the fraction of protein that is active be known. The standard errors for the individual $K_d$ determinations varied from 14 to 35%; the standard errors for $[P_T]$ varied from 5
to 25%. I emphasize that my measurements reflect apparent $K_d$ values since I cannot exclude the possibility that the equilibrium is perturbed during electrophoresis.

**DNase I Footprint Analysis.** DNA fragments of 270 bp containing the 5S RNA gene, referred to as 5S DNA, uniquely labeled at the 5'-end of the transcribed strand or the nontranscribed strand were prepared by digestion of p18-5S and p19-5S, respectively, with *EcoR*1 and *Xba*I (Challice and Segall, 1989). After end-filling of the digested DNA with the Klenow form of DNA polymerase I in the presence of [$\alpha$-$^{32}$P]dCTP, the fragment containing the 5S RNA gene was purified by gel electrophoresis and recovered by electroelution. An 80 µl gel mobility shift reaction contained 1.5 ng of DNA and an amount of protein that was sufficient to bind all the DNA. For these experiments, the protein solubilized from inclusion bodies (see above) was used without further purification. The reaction mixture was incubated for 5 min at room temperature prior to the addition of 8 µl of DNase I (4 µg/ml). After an additional 3 min incubation, 4 µl of 0.45 M EDTA was added to stop the reaction. The reaction was extracted with phenol/chloroform (50:50 v/v). The DNA was precipitated in the presence of 70% ethanol, 0.3 M NaOAc (pH 7.0) using oyster glycogen as carrier. The pellet was rinsed with 70% ethanol, dried *in vacuo* and resuspended in loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The samples were analyzed on a 7M urea, 8% polyacrylamide sequencing gel.

**Methylation Protection Analysis.** Standard 20 µl gel mobility shift reactions containing 2.5 ng of uniquely end-labeled 5S DNA (see above) were set up in triplicate and incubated with protein for 5 min at room temperature. Protein solubilized from inclusion bodies (see above) was used without further purification. Two µl of a 3% dimethyl sulfate solution in 0.5 M sodium cacodylate (pH 8.0) was then added and the samples were incubated for 5 min at room temperature prior to being loaded onto a 4% nondenaturing polyacrylamide gel. The positions of free DNA and protein-DNA complexes were determined by autoradiography of the wet gel. DNA was recovered from gel slices by electroelution and
precipitated with ethanol in the presence of 0.3 M NaOAc (pH 7.0) using oyster glycogen as carrier. The DNA was resuspended in H₂O, extracted with phenol/chloroform (50:50 v/v), and precipitated once more with ethanol in the presence of 0.3 M NaOAc (pH 7.0). The purified DNA was cleaved by treatment with piperidine (Maxam and Gilbert, 1980) and run on an 7M urea, 8% polyacrylamide sequencing gel. Quantification of relative intensities of bands was carried out using IPLab Gel software (Signal Analytics Corp.) to analyze phosphorimages of dried gels obtained with a Molecular Dynamics PhosphorImager.

RESULTS

**Determination of Apparent Dissociation Constants (K_d).** As a first step in further characterization of yeast TFIIIA, I determined the affinity of the protein for the 5S RNA gene. Wild-type TFIIIA, expressed in *E. coli* under the control of a phage T7 RNA polymerase promoter, was purified by subjecting protein solubilized from inclusion bodies to chromatography on Bio-Rex 70 (Fig. 2-1B; see Materials and Methods). This purified TFIIIA was active in binding to the 5S RNA gene as monitored by a gel mobility shift assay (Fig. 2-1C). As a more stringent assay of the activity of the bacterially produced transcription factor, I tested it for the ability to support *in vitro* transcription of the yeast 5S RNA gene in the presence of a yeast-derived fraction containing TFIIIB, TFIIIC, and RNA polymerase III. This fraction directs accurate transcription of a tRNA gene, but requires the addition of TFIIIA for transcription of the 5S RNA gene (Fig. 2-1D, lanes 1 - 3). Bacterially produced wild-type
Figure 2-1. Characterization of wild-type and truncated forms of TFIIIA purified from bacteria.

(A) Schematic representation of wild-type TFIIIA annotated to show the approximate positions of the carboxyl-terminal end-points of the truncated forms of TFIIIA containing seven zinc fingers (TF7*), four zinc fingers (TF4*) and three zinc fingers (TF3*). The numbered boxes denote the nine zinc fingers. The asterisks indicate that these truncated forms of TFIIIA contained additional protein sequence after the last intact zinc finger (see Materials and Methods). The hatched regions refer to the 48- and 35-amino acid sequences present at the amino and carboxyl termini of the protein, respectively. The stippled region denotes the 81-amino acid domain present between zinc fingers 8 and 9.

(B) SDS-polyacrylamide gel analysis. Aliquots of wild-type (WT) and truncated forms of yeast TFIIIA, purified from bacteria as described in the Materials and Methods, were subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie Brilliant Blue. The sizes (in kilodaltons) of molecular mass markers run in lane M are indicated on the right. These markers were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The estimated $M_r$ values of the proteins purified from E. coli, obtained by comparison with the mobilities of these molecular mass markers, are given below preceded by their predicted molecular weight: wild-type TFIIIA 49,982, 49,500; TF7* 31,126, 33,500; TF4* 20,257, 23,500; TF3* 17,912, 22,000.

(C) Binding of wild-type and truncated forms of bacterially produced TFIIIA to the 5S RNA gene. A 270-bp radioactively labeled DNA fragment containing the yeast 5S RNA gene was incubated prior to electrophoresis on a nondenaturing polyacrylamide gel with the bacterially produced proteins indicated above the lanes (see Materials and Methods). No protein was added to the reaction in lane 1. The mobilities of free DNA and TFIIIA-DNA complexes are indicated to the right of the autoradiogram.

(D) Assessment of transcription factor activity. In vitro transcription reactions (see Materials and Methods) contained a yeast tRNA gene (lane 1) or the yeast 5S RNA gene (lanes 2 - 7) as template (denoted as t and 5, respectively, above the lanes); partially purified yeast TFIIIC, TFIIIB, and RNA polymerase III; and the form of TFIIIA indicated above the lanes. ppA, TFIIIA partially purified from yeast; the other forms of TFIIIA (WT, TF7*, TF4*, and TF3*) were purified from bacteria. The RNAs synthesized in vitro were analysed on a 7M urea, 10% polyacrylamide gel. The autoradiogram shows the portion of the gel containing tRNA and 5S RNA. The various-sized transcripts represent primary and processed products.
TFIIIA was able to substitute for partially purified yeast TFIIIA in promoting transcription of the yeast 5S RNA gene (Fig. 2-1D, lanes 3 and 4).

I next measured the affinity of TFIIIA for the 5S RNA gene by incubating a constant amount of protein with increasing amounts of a radiolabeled 5S DNA fragment. Protein-DNA complexes were separated from free DNA by electrophoresis on a nondenaturing gel (Fig. 2-2A). The relative amounts of free DNA and bound DNA were determined at each input DNA concentration and the apparent dissociation constant (K_d) was derived by nonlinear regression analysis of the data (Fig. 2-2A; see Materials and Methods). From five separate experiments, I found that the average K_d of the protein-DNA complex was 0.11 ± 0.03 nM (Table 2-1). For comparison, the K_d values measured for the Xenopus TFIIIA-DNA interaction range from 0.42 to 2.2 nM (Hanas et al., 1983a; Romaniuk, 1990; Del Rio and Setzer, 1991; Liao et al., 1992; Del Rio et al., 1993).

I also determined the binding affinities of carboxyl-terminal truncated forms of yeast TFIIIA. Versions of TFIIIA that contained the first three zinc fingers (TF3*), the first four zinc fingers (TF4*), and the first seven zinc fingers (TF7*) were purified from bacteria. These truncated forms of TFIIIA contained up to 25 amino acids after the last intact zinc finger; this additional sequence (denoted by the asterisk) included a portion of the next zinc finger and six to eight amino acids introduced from vector sequence (see Materials and Methods). I considered that this sequence would be relatively unstructured and would be unlikely to have a significant effect on the affinity measurements. Each form of truncated TFIIIA eluted from Bio-Rex 70 at a unique salt concentration, ran as a single band of the expected molecular mass on an SDS-polyacrylamide gel (Fig. 2-1B), and was active in binding to the 5S RNA gene (Fig. 2-1C). Consistent with the reduction in size of the carboxyl-terminal truncated proteins, the protein-DNA complexes formed with the truncated proteins (Fig. 2-1C, lanes 3 - 5) had increased mobilities relative to the complex formed with wild-type protein (lane 2). The protein-DNA interactions visualized in the gel mobility shift assay were shown to be specific
**Figure 2-2. Determination of apparent dissociation constants.** Apparent dissociation constants ($K_d$) were determined by electrophoretic mobility shift analysis of reactions containing a constant concentration of TFIIIA and variable concentrations of end-labeled 5S DNA as described in the Materials and Methods.

(A) A nonlinear regression analysis of representative data obtained with wild-type TFIIIA (WT). The concentration of TFIIIA was ~3 nM and the concentration of 5S DNA varied from 0.04 to 0.70 nM.

(B) A nonlinear regression analysis of representative data obtained with TFIIIA containing seven zinc fingers (TF7*). The concentration of TF7* was ~4 nM and the concentration of 5S DNA varied from 0.04 to 0.70 nM.

(C) A nonlinear regression analysis of representative data obtained with TFIIIA containing four zinc fingers (TF4*). The concentration of TF4* was ~20 nM and the concentration of 5S DNA varied from 0.04 to 0.70 nM.

(D) A nonlinear regression analysis of representative data obtained with TFIIIA containing three zinc fingers (TF3*). The concentration of TF3* was ~15 nM and the concentration of 5S DNA varied from 0.07 to 1.85 nM.

The insets of panels A - D show the mobility shift experiments from which the data were derived for each plot. The reaction in the first lane in each gel inset contained no TFIIIA. The $K_d$ value obtained in each experiment is denoted. As determined from the $[P_T]$ values obtained in these examples, the preparations of wild-type TFIIIA, TF7*, TF4*, and TF3* were 11, 12, 2.5, and 13% active, respectively.
Table 2-1. Apparent dissociation constants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>K_d (nM)</th>
<th>n</th>
<th>ΔG° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type TFIIIA</td>
<td>0.11 ± 0.03</td>
<td>5</td>
<td>-13.6</td>
</tr>
<tr>
<td>TF7*</td>
<td>0.087 ± 0.004</td>
<td>3</td>
<td>-13.7</td>
</tr>
<tr>
<td>TF4*</td>
<td>0.24 ± 0.09</td>
<td>5</td>
<td>-13.1</td>
</tr>
<tr>
<td>TF3*</td>
<td>0.87 ± 0.3</td>
<td>7</td>
<td>-12.4</td>
</tr>
</tbody>
</table>

The average K_d ± S.D. was determined from n independent experiments, of which a representative example is shown in Figure 2-2. The standard Gibbs free energy change (ΔG° = -RTln(1/K_d)) was calculated using the average equilibrium dissociation constant and T = 298 K.
by monitoring the effects of addition of competitor DNAs (data not shown). As expected from
the observation that the 81-amino acid domain located between fingers 8 and 9 of yeast TFIIIA
is essential for the transcriptional activity of the protein (Milne and Segall, 1993), bacterially
purified TF7*, TF4*, and TF3* were unable to support in vitro transcription of the 5S RNA
gene (Fig. 2-1D, lanes 5 - 7).

Removal of zinc fingers 8 and 9 from yeast TFIIIA did not have a significant effect on the
apparent affinity of the protein-DNA interaction (Fig. 2-2B and Table 2-1). Removal of zinc
fingers 5 to 9 led to an ~2-fold increase in the apparent K_d (Fig. 2-2C and Table 2-1) and
removal of zinc fingers 4 to 9 led to an ~8-fold increase in the apparent K_d (Fig. 2-2D and
Table 2-1). Using the measured K_d values, I calculated the ΔG° values for the protein-DNA
interactions (Table 2-1). This representation of the data emphasizes that a polypeptide
containing the amino-terminal three zinc fingers of yeast TFIIIA has a high binding energy; the
binding energy of TF3* was 90% that of the full-length protein. Using protein synthesized in
vitro, a TFIIIA-DNA interaction was not detected with a truncated molecule containing only the
amino-terminal two zinc fingers (Milne and Segall, 1993) or with a truncated molecule
containing only zinc fingers 3 to 9 (O. Rowland, data not shown). This suggests that if these
proteins can bind to the 5S RNA gene, they do so with a relatively high K_d. It should be noted
that my analysis using truncated proteins would not necessarily detect the effects that finger-
DNA interactions in one portion of the molecule might exert on the energetics of interactions
occurring elsewhere in the complex (for example, see Del Rio et al. (1993), Clemens et al.
(1994), and Zang et al. (1995), Kehres et al., 1997).

**DNase I Footprinting.** To approximate the position of various fingers of yeast TFIIIA
on the 5S RNA gene, I compared the DNase I cleavage patterns of the gene complexed with
wild-type and truncated forms of TFIIIA (Fig. 2-3). For my first series of experiments, I used
TF7*, TF4*, and TF3* (data not shown); at the request of a reviewer, I repeated these
experiments with versions of TF8, TF6, TF5, TF4, and TF3 with carboxyl-terminal
Figure 2-3. DNase I protection analysis of TFIIIA-DNA complexes. DNA fragments containing the yeast 5S RNA gene uniquely end-labeled on the transcribed strand (panel A) or the nontranscribed strand (panel B) were incubated with bacterially produced wild-type TFIIIA (WT); carboxyl-terminal truncated forms of TFIIIA containing eight zinc fingers (TF8), seven zinc fingers (TF7*), six zinc fingers (TF6), five zinc fingers (TF5), four zinc fingers (TF4), or three zinc fingers (TF3); and amino-terminal truncated forms of TFIIIA beginning immediately before the first zinc finger (1FS) or immediately before the second zinc finger (2FS). The DNA fragments produced by brief treatment of the reactions with DNase I were analysed on a sequencing gel (see Materials and Methods). The reactions in the third lanes had no added protein. The first and second lanes contain the cleavage products of A + G and C + T chemical sequencing reactions, respectively. Nucleotide positions of the 5S RNA gene are indicated on the sides of the panels. Also shown is a schematic representation of the extent of DNase I protection in the various protein-DNA complexes (panel C). The sequence of the 5S RNA gene is given from nucleotides +58 to +112; the ICR is boxed. The extent of DNase I protection on the nontranscribed strand (NT; hatched rectangles) and transcribed strand (T; solid rectangles) in complexes formed with wild-type TFIIIA, TF8, TF7*, TF6, TF5, TF4 and TF3 proteins is shown. The shaded portions of the solid rectangles indicate uncertainty in positioning the boundary of the protected region. The solid arrowheads denote DNase I sensitive sites within the region protected by wild-type TFIIIA, TF8, TF7*, TF6, and TF5.
truncations that terminated at the end of the linker sequence (see Materials and Methods). Similar results were obtained with both sets of truncations. I also analyzed TF7* and amino-terminal truncated forms of TFIIIA that began before the first finger and before the second finger, referred to as 1FS (1st finger start) and 2FS (2nd finger start), respectively (see Materials and Methods). As seen previously with TFIIIA purified from yeast (Braun et al., 1989), I found that wild-type TFIIIA expressed in bacteria protected a 35-bp region of the 5S RNA gene from DNase I cleavage; this region extended from nucleotides +64 to +98 on the transcribed strand (Fig. 2-3A, fourth lane) and from nucleotides +66 to +99 on the nontranscribed strand (Fig. 2-3B, fourth lane). Within this region, nucleotides +70 and +71 on the transcribed strand and nucleotides +74 and +75 on the nontranscribed strand remained accessible to DNase I cleavage. The DNase I cleavage patterns of the TF8-DNA, TF7*-DNA, TF6-DNA, and TF5-DNA complexes (Fig. 2-3, A and B, fifth to eighth lanes) were identical to that of the wild-type TFIIIA-DNA complex with the exception that the TF7*-DNA complex gave rise to DNase I hypersensitive sites at nucleotides +51 and +52 on the transcribed strand, 12 bp upstream of the protected region. These data indicate that fingers 6 to 9 and the 81-amino acid domain of yeast TFIIIA either are not in close proximity to DNA in the TFIIIA-DNA complex or have such weak affinity for DNA that they are readily displaced by DNase I. It is possible that the hypersensitivity present at nucleotides +51 and +52 in the DNase I footprint of TF7* is generated by the 17 amino acid carboxyl-terminal extension present in this protein; if this is the case, it is possible that the carboxyl-terminal fingers of yeast TFIIIA are in closer proximity to DNA than is implied by the DNase I protection patterns. Indeed, the observation of Braun et al. (1992a) that yeast TFIIIA can be crosslinked to templates containing photoactive nucleotides at residues +48 to +59 of the 5S RNA gene is consistent with the possibility that a portion of TFIIIA carboxyl-terminal to finger 5 is weakly bound to DNA in the TFIIIA-DNA complex.

TFIIIA containing four intact zinc fingers generated a smaller DNase I footprint on the 5S RNA gene than did TF5. On the transcribed strand, TF4 appeared to protect a 20 bp region,
extending from nucleotides +79 to +98 (Fig. 2-3A, ninth lane). Comparison of the relative intensities of the bands at nucleotide +76 suggested that the upstream boundary of this footprint might extend to nucleotide +76. On the nontranscribed strand, the protected region extended from nucleotides +78 to +99 (Fig. 2-3B, ninth lane). Comparison of the footprints obtained with TF4 and TF3 showed that deletion of the fourth zinc finger led to a change in the DNase I footprint only on the non-transcribed strand (Fig. 2-3, A and B, tenth lanes). Whereas TF4 provided protection from nucleotides +78 to +99, TF3 did not protect residue +78 from DNase I digestion. In summary, my data confirmed, as previously suggested (Milne and Segall, 1993), that yeast TFIIIA, like its amphibian counterpart, binds to the 5S RNA gene with its carboxyl-terminus towards the 5'-end of the gene. Comparison of the DNase I protection patterns obtained with the carboxyl-terminal truncated proteins TF5, TF4, and TF3 indicated that the amino-terminal three zinc fingers span the ICR, which maps approximately from nucleotides +81 to +94 (Challice and Segall, 1989); the presence of finger 4 lead to only a modest change in the protection pattern at the 5'-end of the ICR; and the presence of finger 5 lead to protection of an additional 12 bp upstream of the ICR.

Inspection of the DNase I footprints generated by the two amino-terminal truncated proteins indicated that TFIIIA lacking the non-zinc finger amino-terminal extension (1FS) and TFIIIA lacking the first zinc finger (2FS) did not protect nucleotide +98 on the transcribed strand (Fig 2-3A, eleventh and twelfth lanes). Additionally, protection of nucleotides +96 and +94 on the transcribed strand (Fig 2-3A, twelfth lane) and +97 on the nontranscribed strand (Fig. 2-3B, twelfth lane) was reduced in the 2FS-DNA complex. These data position finger 1 at the 3'-end of the ICR.

**Methylation Protection Analysis.** I also used dimethyl sulfate to probe the protein-DNA complexes formed with wild-type and carboxyl-terminal truncated forms of TFIIIA (Fig. 2-4). In the TFIIIA-DNA complexes formed with wild-type TFIIIA, guanines +73, +74, +93 and +94 on the transcribed strand and guanines +82, +85, +87, +88, +89 and +91 on the
Figure 2-4. Determination of guanine residues protected from methylation in TFIIIA-DNA complexes. DNA fragments containing the yeast 5S RNA gene uniquely end-labeled on the transcribed strand (panel A) or on the nontranscribed strand (panel B) were incubated with wild-type TFIIIA (WT) or a truncated form of TFIIIA containing six zinc fingers (TF6), five zinc fingers (TF5), four zinc fingers (TF4), or three zinc fingers (TF3) purified from bacteria. The reactions in the second lanes had no added protein. Shortly after the addition of dimethyl sulfate, the reaction mixtures were applied to a nondenaturing gel to resolve protein-DNA complexes from free DNA. DNA recovered from free DNA (second lanes) or from protein-DNA complexes (third to seventh lanes) was cleaved at the modified G residues and analysed on a sequencing gel (see Materials and Methods). The first lanes contain the products of an A + G chemical sequencing reaction of the DNA probe. Nucleotide positions of the 5S RNA gene are indicated on the left-hand sides of the panels; the rectangles on the right-hand sides of the panels denote the G residues protected from methylation on the transcribed (open rectangles) and non-transcribed (closed rectangles) strands of the wild-type TFIIIA-DNA complex. In panel C, the sequence of the 5S RNA gene is given from nucleotides +58 to +112. The ICR is boxed, and G residues protected from methylation in the wild-type TFIIIA-5S DNA complex are indicated (o, transcribed strand; •, nontranscribed strand).
nontranscribed strand were protected against modification by dimethyl sulfate (Fig. 2-4, A and B, third lanes) (Challice and Segall, 1989). Similarly, modification of these residues interferes with binding of TFIIIA (Wang and Weil, 1989). As noted in Fig. 2-4C, 8 of the 10 G residues that are in close contact with TFIIIA are within the ICR. The methylation protection patterns obtained with TF6 and TF5 were identical to that obtained with wild-type TFIIIA (Fig. 2-4, A and B, third to fifth lanes). In the TF4-DNA complex, the G residues at positions +73 and +74 on the transcribed strand, which map upstream of the ICR, were no longer protected (Fig. 2-4A, sixth lane). These data place finger 5 in close contact with G73 and G74 on the transcribed strand. G82 on the nontranscribed strand was partially protected in the TF4-DNA complex and not protected in the TF3-DNA complex (Fig. 2-4B, sixth and seventh lanes; data not shown). Quantification of the analysis shown in Fig. 2-4 (see Materials and Methods) indicated that modification of G82 relative to modification of the unprotected residue G99 in wild-type TFIIIA-DNA, TF6-DNA, TF5-DNA, TF4-DNA and TF3-DNA complexes was 0.09, 0.12, 0.13, 0.42, and 0.75, respectively, and 0.83 in free DNA. The simplest interpretation of these results is that finger 4, or the finger 4-finger 3 linker, contacts G82 and that this interaction occurs more efficiently when finger five is docked onto DNA (see Discussion).

**DISCUSSION**

I expressed yeast TFIIIA in *E. coli* and found, as observed by others (Ottonello et al., 1994), that bacterially produced yeast TFIIIA supports accurate *in vitro* transcription of the 5S
RNA gene. Using wild-type and truncated forms of TFIIIA that had been purified from bacteria, I studied the interaction of the protein with the 5S RNA gene using DNase I and dimethyl sulfate as probes. I note that DNase I allows only approximate mapping of the boundaries of a protein-DNA complex. First, not all phosphodiester bonds are cleaved efficiently by this enzyme in naked DNA. Second, the boundary of a footprint could be influenced by a weak/transient interaction of a finger with DNA, displacement of a weakly bound finger by DNase I, or steric hindrance between the enzyme and DNA-bound protein. Despite these limitations, inspection of the protection patterns allowed me to position various portions of yeast TFIIIA along the 5S RNA gene. Although yeast TFIIIA and Xenopus TFIIIA both contain nine zinc fingers, the 40-kDa amphibian protein generates a 50-bp DNase I footprint (Engelke et al., 1980), whereas the 50-kDa yeast protein generates a 35-bp footprint (Braun et al., 1989; this study). I found that the smaller footprint of yeast TFIIIA can be accounted for by the absence of an intimate interaction of yeast fingers 6-9 with DNA. Studies of the Xenopus TFIIIA-DNA interaction (Clemens et al., 1992; Fairall and Rhodes, 1992; Hayes and Clemens, 1992; Hayes and Tullius, 1992; Del Rio et al., 1993) suggest that the carboxyl-terminal three fingers (fingers 7-9) of Xenopus TFIIIA wrap around the major groove of the DNA helix at the 5'-end of the amphibian ICR and immediately upstream of this element. The three zinc fingers in the middle of the protein lie on one side of the helix, with finger 5 contacting the major groove and fingers 4 and 6 each crossing the minor groove (Nolte et al., 1998). The amino-terminal three zinc fingers wrap around the major groove of the DNA helix contacting a 13 bp region of the C-box (Christensen et al., 1991; Hayes and Clemens, 1992; Clemens et al., 1992; Liao et al., 1992; Bogenhagen, 1993; Hansen et al., 1993; Veldhoen et al., 1994; Foster et al., 1997; Wuttke et al., 1997; Nolte et al., 1998) with an affinity that is similar to that of the entire protein (Liao et al., 1992; Choo and Klug, 1993; Zang et al., 1995). My methylation protection analysis indicated that finger 5 of yeast TFIIIA is in close contact with G73 and G74, placing it in the major groove, as is finger 5 in the Xenopus TFIIIA-DNA complex. Because the DNase protection analysis indicated that finger 5 provides protection to
nucleotide +64, the finger 5-DNA interaction presumably extends upstream of nucleotides +74 and +75. The methylation protection analysis also indicated that G^{82} was not protected from modification in the yeast TF3-DNA complex, was partially protected in the TF4-DNA complex, and was completely protected in the TF5-DNA complex. This suggests that yeast finger 4 makes a major groove contact with G^{82} and that this interaction occurs more efficiently when finger 5 is docked at G^{73}/G^{74}. If this is the case, it is possible that the 12 bp difference between the DNase I footprints of TF5 and TF4 represents loss of protection not only by finger 5 but also by finger 4, that is DNase I might partially displace finger 4 from the TF4-DNA complex. This could explain why the only difference between the DNase I protection patterns of the yeast TF4-DNA and TF3-DNA complexes was protection of nucleotide +78 in the TF4-DNA complex. The interactions suggested above, which position the major groove contacts made by fingers 4 and 5 10 bp apart on the same side of the helix, require that the finger 4-finger 5 linker, which is only five amino acids, cross the minor groove (e.g. see Kochoyan et al. (1991)). TFIIIA-induced bending of DNA (Braun et al., 1992b) might facilitate this proposed spacing. Alternatively, it is possible that the finger 4-finger 3 linker is responsible for protection of G^{82} and that most of finger 4 lies over the minor groove, extending from approximately nucleotide +82 to nucleotide +75, leaving this region accessible to DNase I. Finally, it is possible that finger 4 approaches DNA in a novel manner. In the above model, irrespective of how finger 4 interacts with DNA, fingers 3, 2, and 1 would then be responsible for the major groove contacts represented by protection of G residues from positions +85 to +94. Although this differs from the model proposed for the Xenopus TFIIIA-DNA complex, in which the amino-terminal three zinc fingers contact 13 bp of the C-box element (Clemens et al., 1992; Liao et al., 1992) and finger 4 lies over the minor groove, the fact that G^{82} is not protected against methylation in the yeast TF3-DNA complex argues against G^{82} being contacted by finger 3. If finger 3 is in contact with G^{82}, then my data indicate that this contact occurs only in the presence of the finger 4. In this case, TF3, which binds to DNA with an affinity that is similar to that of the full length protein, would have only two fingers in contact
with DNA. I note that using protein synthesized in vitro, Milne and Segall (1993) did not detect an interaction between the 5S RNA gene and a polypeptide containing the first two fingers of yeast TFIIIA.

Further studies are required to allow detailed comparisons between the finger-DNA interactions in the yeast and Xenopus TFIIIA-DNA complexes. Exact positioning of individual fingers awaits deduction of the three-dimensional structure of the protein-DNA complexes. As discussed above, however, my preliminary study suggests that some differences may exist. This is perhaps not unexpected. Yeast TFIIIA does not contain the conserved linker motif TGEK found between fingers 1 and 2 and between fingers 2 and 3 of Xenopus TFIIIA. The His-His spacings, which are likely to affect the structure of the DNA-binding α-helix, also differ between fingers 1, 3, and 5 of the TFIIIA proteins from the two organisms (Ginsberg et al., 1984; Archambault et al., 1992; Woychik and Young, 1992). These differences, in addition to differences in potential DNA-contacting amino acids, could lead to differences in the DNA binding properties of the polypeptides. Deduction of the structures of protein-DNA crystals of Zif268 (Pavletich and Pabo, 1991), GLI (Pavletich and Pabo, 1993) and Tramtrack (Fairall et al., 1993) has revealed that zinc fingers can dock into the major groove of DNA with variations in base and phosphate contacts and in the spacing of adjacent binding sites. Linkers have been shown to have a major influence on the binding affinity of zinc fingers (Choo and Klug, 1993; Clemens et al., 1994; Zang et al., 1995). Furthermore, it is interesting to note that the amino-terminal three fingers of yeast TFIIIA serve not only in DNA binding, but also to recruit TFIIIC to the TFIIIA-DNA complex (Milne and Segall, 1993). The observations that putative human TFIIIA (Arakawa et al., 1995; Drew et al., 1995) and Xenopus TFIIIA share more extensive identity than do Xenopus TFIIIA and yeast TFIIIA (Archambault et al., 1992; Woychik and Young, 1992) and that no similarity has yet been found between the deduced amino acid sequences of subunits of human TFIIIC and yeast TFIIIC (L’Etoile et al., 1994; Lagna et al., 1994; Sinn et al., 1995), are consistent with the notion that a yeast-specific
TFIIIA-DNA interaction is established to direct assembly of yeast TFIIIC into the transcription complex.
CHAPTER 3

A Hydrophobic Segment within the 81-Amino Acid Domain of TFIIIA from *Saccharomyces cerevisiae* Is Essential for Its Transcription Factor Activity

Figures 3-1 to 3-5 of this Chapter are presented essentially as published in:
*Molecular and Cellular Biology* **18**:420-432

Figure 3-6 in the Appendix to this Chapter is unpublished.
ABSTRACT

Transcription factor IIIA (TFIIIA) binds to the internal control region of the 5S RNA gene as the first step in the in vitro assembly of a TFIIIB-TFIIIC-TFIIIA-DNA transcription complex. An 81-amino acid domain that is present between zinc fingers 8 and 9 of TFIIIA from Saccharomyces cerevisiae is essential for the transcription factor activity of this protein (C. A. Milne and J. Segall, J. Biol. Chem. 268:11364-11371, 1993). I have monitored the effect of mutations within this domain on the ability of TFIIIA to support transcription of the 5S RNA gene in vitro and to maintain cell viability. TFIIIA with internal deletions that removed residues 282 to 315, 316 to 334, 328 to 341, or 342 to 351 of the 81-amino acid domain retained activity, whereas TFIIIA with a deletion of the short leucine-rich segment \(^{352}\text{NGLNLLLNN}^{359}\) at the carboxyl-terminal end of this domain was devoid of activity. Analysis of the effects of double and quadruple mutations in the region extending from residue 336 to 364 confirmed that hydrophobic residues in this portion of the 81-amino acid domain, particularly L343, L347, L354, L356, L357, and L358, and to a lesser extent F336 and L337, contributed to the ability of TFIIIA to promote transcription. I propose that these hydrophobic residues play a role in mediating an interaction between TFIIIA and another component of the transcriptional machinery. I also found that TFIIIA remained active if either zinc finger 8 or zinc finger 9 was disrupted by mutation, but that TFIIIA containing a disruption of both zinc finger 8 and zinc finger 9 was inactive. As described in an Appendix to this Chapter, I found that mutations in the A-box-like sequence of the 5S RNA gene suppressed, at least in part, the transcriptional defect of TFIIIA(L354A/L356A/L357A/L358A); from this preliminary experiment, I suggest that one role of the 81-amino acid domain is to prevent an inappropriate interaction between TFIIIC and the A-box-like sequence of the 5S RNA gene.
INTRODUCTION

TFIIIA, which is required only for transcription of the 5S RNA gene, serves to recruit TFIIIA to the TFIIIA-DNA complex (see Chapter 1, section 1.4.2). On tRNA genes, TFIIIC binds directly to the intragenic A- and B-box promoter elements and acts to place TFIIIB upstream of the start site of transcription (Kassavetis et al., 1989). Despite the requirement for TFIIIA in the assembly of a preinitiation complex on the 5S RNA gene, the relative placement of the individual subunits of TFIIIC and TFIIIB in preinitiation complexes formed on a 5S RNA gene and on a tRNA is similar (Bartholomew et al., 1990, 1991; Braun et al., 1992a).

Milne and Segall (1993) previously analyzed a series of truncated forms of yeast TFIIIA for their ability to bind to the 5S RNA gene, incorporate TFIIIC into the TFIIIA-DNA complex, and support transcription of the 5S RNA gene. These authors found that a polypeptide containing the amino-terminal three zinc fingers of yeast TFIIIA binds to the ICR of the 5S RNA gene and that this truncated form of TFIIIA can recruit TFIIIC. However, the resultant TFIIIC-TFIIIA-DNA complex is unable to promote transcription of the 5S RNA gene. It was found that the yeast-specific 81-amino acid domain that is present between zinc fingers 8 and 9 is essential for the transcription factor activity of yeast TFIIIA (Milne and Segall, 1993). As a step towards understanding the role of this novel 81-amino acid domain in establishing an active transcription complex, I carried out a mutational analysis to identify amino acids within this domain that are essential for its transcription factor activity. In addition, I assessed the potential role of zinc fingers 8 and 9 in the transcription factor activity of yeast TFIIIA.
MATERIALS AND METHODS

Plasmids. pXS-TFC2, which served as the parental plasmid for introduction of mutations into the coding sequence for TFIIIA, was constructed as follows. First, an XbaI- and SspI-less derivative of pBluescript II SK(+) was generated. The unique XbaI site of pBluescript II SK(+) was destroyed by digesting the plasmid with XbaI, filling in the overhanging ends with the Klenow form of DNA polymerase I in the presence of deoxynucleoside triphosphates (dNTPs), and religating the DNA. The resultant plasmid was digested with SspI to yield a 2,831-bp fragment and a 130-bp fragment that contains the promoter for the β-lactamase gene. The large fragment was gel purified and ligated with a 55-bp fragment that contains a weak bacterial promoter (kindly provided by D. E. Pulleyblank) to produce pXS, an XbaI- and SspI-less vector. pXS-TFC2 was generated by cloning a KpnI-BamHI fragment, which contains the coding region of TFIIIA obtained from pJA454 (Archambault et al., 1992), between the corresponding sites in the polylinker of pXS. This places the coding sequence for TFIIIA downstream of a promoter for T7 RNA polymerase.

Plasmids coding for versions of TFIIIA with carboxyl-terminal truncations were generated by replacing the XbaI-BamHI fragment of pXS-TFC2 with DNA amplified from pJA454 by the polymerase chain reaction (PCR). The reverse primers for the amplification reactions contained a BamHI restriction site at their 5' ends followed by a stop codon and then sequence from TFC2. The forward primer (primer A) annealed within the coding region of the eighth zinc finger of TFIIIA. The PCR products were gel purified, digested with XbaI and BamHI, repurified, and cloned between the corresponding sites of pXS-TFC2 to generate plasmids coding for versions of TFIIIA that contained amino acids 1 to 378, 1 to 359, 1 to 353, 1 to 347, 1 to 339, and 1 to 311.
Site-directed mutagenesis (alanine-scanning) was achieved by recombinant PCR using the overlap extension procedure described by Ho et al. (1989) and Higuchi (1990). Complementary reverse and forward oligonucleotides containing the desired mutation(s) were used in separate PCRs with pJA454 as the template. The sequences upstream and downstream of the mutant codons were amplified by using the reverse mutant oligonucleotide and upstream primer A (see above) and the forward mutant oligonucleotide and downstream primer B, respectively. Primer B anneals within the coding region of TFIIIA downstream of the ninth zinc finger. The PCR products of the two reactions were gel purified, mixed, and subjected to three PCR cycles in order to allow extension of the heteroduplexes formed between the overlapping mutant sequences. The extended heteroduplexes were then amplified in the presence of primers A and B, and the resulting 502-bp fragment was gel purified, digested with XbaI and SspI, repurified, and used to replace the corresponding XbaI-SspI fragment of pXS-TFC2. Constructs made in this way directed the synthesis of versions of TFIIIA containing the following alanine-scanning mutations: K287A/K289A, L296A/V297A, D299A/H300A, K308A/H309A, D314A/E315A, R324A/K325A, F336A/L337A, D342A/E344A, L343A/L347A, K345A/R346A, E348A/E351A, N352A/N355A, L354A/L356A, L354A, L356A, L357A/L358A, N359A, and R363A/K364A. Versions of pXS-TFC2 coding for TFIIIA containing quadruple alanine-scanning mutations were made by recombinant PCR as described above by using a version of pXS-TFC2 that contained the appropriate double alanine-scanning mutation as the template in the first set of PCR reactions. This approach generated mutant genes encoding versions of TFIIIA with the following quadruple alanine-scanning mutations: F336A/L337A/L343A/L347A, D342A/E344A/E348A/E351A, L343A/L347A/L354A/L356A, L343A/L347A/L357A/L358A, K345A/R346A/L357A/L358A, K345A/R346A/R363A/K364A, L354A/L356A/L357A/L358A, and L357A/L358A/R363A/K364A.
Versions of pXS-TFC2 coding for TFIIIA lacking amino acids 282 to 287, 282 to 315, and 282 to 353 were constructed by taking advantage of the unique EcoRV restriction site at codon 282 and unique restriction sites introduced by alanine-scanning mutations at codons 287, 315, and 353. To construct pXS-TFC2(Δ282-287), pXS-TFC2(K287A/K289A) was digested with NdeI, and after the overhanging ends had been filled in with the Klenow form of DNA polymerase I in the presence of dNTPs, the DNA was digested with EcoRV. The ~5-kbp fragment was gel purified and religated to create pXS-TFC2(Δ282-287), which codes for a version of TFIIIA with an in-frame deletion and which retains the K289A mutation. To construct pXS-TFC2(Δ282-315), pXS-TFC2(D314A/E315A) was digested with PstI, and after the overhanging ends had been blunted by treatment with the Klenow form of DNA polymerase I, first in the absence and then in the presence of dNTPs, the DNA was digested with EcoRV. The ~4.9-kbp fragment was gel purified and religated to create pXS-TFC2(Δ282-315) which codes for a version of TFIIIA with an in-frame deletion. To construct pXS-TFC2(Δ282-353), pXS-TFC2(N352A/N355A) was digested with NheI, and after the overhanging ends had been filled in with the Klenow form of DNA polymerase I in the presence of dNTPs, the DNA was digested with EcoRV. The ~4.8-kbp fragment was gel purified and religated to create pXS-TFC2(Δ282-353), which codes for a version of TFIIIA with an in-frame deletion and which retains the N355A mutation.

Versions of pXS-TFC2 coding for TFIIIA(Δ316-334), TFIIIA(Δ328-341), TFIIIA(Δ342-351), and TFIIIA(Δ352-359) were made by recombinant PCR using a variation of the overlap extension procedure described above (Ho et al., 1989; Higuchi, 1990). Partially overlapping oligonucleotides spanning the deletion junction were used as reverse and forward primers in separate PCRs with primers A and B (see above), respectively. The partially overlapping PCR products were gel purified, mixed, and subjected to three PCR cycles to allow extension of heteroduplexes, which were then amplified by the addition of primers A and B. The amplified DNA, which contained a deletion of the coding region of TFIIIA, was gel purified, digested with XbaI and SspI, repurified, and cloned between the corresponding sites of pXS-
TFC2. This recombinant PCR approach was used previously to construct a gene encoding TFIII-AΔ81, referred to as TFIII(A284-364) in this thesis, and is described in detail in Milne and Segall (1993).

All PCR amplifications were performed by using the high-fidelity Vent DNA polymerase (New England Biolabs) as per the manufacturer's standard conditions. The sequence of all amplified DNA was verified by DNA sequencing. The sequences of the oligonucleotides used to generate the mutations are available upon request.

Mutation of a zinc-coordinating residue in each of fingers 8 and 9 was obtained in a pilot experiment using Taq DNA polymerase under conditions of reduced fidelity (Leung et al., 1989) to introduce random mutations during PCR amplification of the sequence of TFIII-A from codons 266 to 397. A 100-μl reaction mixture contained 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 6.7 μM EDTA, 0.17 mg of bovine serum albumin/ml, 10 mM β-mercaptoethanol, 1 mM of each dNTP, 6.1 mM MgCl₂, 0.5 mM MnCl₂, 20 pmol each of primers A and B (see above), 6 fmol of pJA454 as the template, and 2.5 U of Taq DNA polymerase. The amplified DNA was gel purified, digested with XbaI and SspI, repurified, and ligated between the corresponding sites of pXS-TFC2. The ligated products were recovered by transformation into Escherichia coli and the DNAs of several plasmids were sequenced from the XbaI site to the SspI site. This led to the identification of a plasmid that contained mutations in both codons 272 and 367. A unique EcoRV restriction site located between these codons was used to separate the two mutations: an NcoI-EcoRV fragment containing the mutation of codon 272 and an EcoRV-BamHI fragment containing the mutation of codon 367 were separately subcloned between the corresponding sites of pXS-TFC2 to generate pXS-TFC2(H272R) and pXS-TFC2(C367Y), respectively. DNA sequencing confirmed that these plasmids contained the single mutations H272R and C367Y.

The yeast shuttle vector pG3 (Schena et al., 1991), a pUC18-derived plasmid that contains a 2μm origin of replication and the selectable marker TRP1, was used for in vivo
expression of wild-type and mutant forms of TFIIIA. *KpnI-BamHI* fragments containing the open reading frames of the wild-type and mutant versions of TFIIIA were purified from pXS-TFC2 plasmids and inserted between the *KpnI* and *SalI* sites of pG3 after the *BamHI* - and *SalI*-generated ends had been filled in by the Klenow form of DNA polymerase I in the presence of dNTPs. This placed the coding region of TFIIIA between the constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene and the transcription terminator of the phosphoglycerate kinase gene.

**In vitro synthesis of TFIIIA.** Wild-type and mutant versions of TFIIIA were synthesized *in vitro* by using the TnT coupled transcription-translation system (Promega), in which a rabbit reticulocyte lysate supports translation of transcripts synthesized by T7 RNA polymerase. The reactions were carried out according to the manufacturer's instructions and with the addition of ZnSO₄ to 0.1 mM. pXS-TFC2 or its variants were used as the template unless otherwise indicated. TFIIIA(1-397) and TFIIIA(1-365) were synthesized *in vitro* from pJA454 that had been linearized with *SspI* and from pJA454-1 that had been linearized with *BamHI*, respectively (Milne and Segall, 1993). Wild-type and mutant proteins that had been synthesized in the presence of [³⁵S]methionine were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to confirm that protein of the appropriate size was synthesized (data not shown). *In vitro*-synthesized proteins used in gel mobility shift assays and transcription assays were not radiolabeled.

**Electrophoretic mobility shift assays and transcription assays.** Electrophoretic mobility shift assays (EMSAs) were performed as described elsewhere (Rowland and Segall, 1996 or Chapter 2 of this thesis) except that 0.25 μg of pBluescript II SK(+) was included as a competitor DNA in the reactions.

*In vitro* transcription assays were performed as described elsewhere (Taylor and Segall, 1985) with the yeast 5S RNA gene (p19-5S) as a template, with the exception of the *in vitro* transcription reactions described in the Appendix to this Chapter which contained p19-5S-
derived templates with linker scanning mutations in the 5S RNA gene (Challice and Segall, 1989). A 50-μl reaction mixture contained 4.5 μl of an in vitro transcription-translation reaction mixture that had been programmed to produce the indicated version of TFIIIA and 12 μl of a yeast-derived heparin-agarose fraction (fraction h) that contained TFIIIC, TFIIIB, and RNA polymerase III (Taylor and Segall, 1985).

**Yeast media, culture conditions, and transformations.** Rich medium (yeast extract-peptone-dextrose [YPD]) and minimal medium (synthetic dextrose [SD]) were as previously described (Hepworth et al., 1995). All yeast cultures were grown at 30°C. Transformation of yeast cells was performed by the lithium acetate method of Geitz et al. (1992).

**In vivo analysis of the mutant versions of TFIIIA.** The haploid yeast strain YRW1 (MATα can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 ade2-1 tfc2::LEU2, harboring pJA230) was constructed to test the ability of the variant forms of TFIIIA to support cell viability (Milne, 1994). As the first step in construction of YRW1, the plasmid pRKO (Milne, 1994) was digested with BssHII and BamHI to release a DNA fragment that contains the yeast LEU2 gene flanked by 213 and 300 bp of noncoding sequence from the regions upstream and downstream, respectively, of the TFC2 gene. This fragment was used to replace the entire coding region of the chromosomal TFC2 gene with the LEU2 gene by integrative transformation as follows. The diploid strain LP112 (Petko and Lindquist, 1986) was transformed with the gel-purified BssHII-BamHI fragment and replacement of one chromosomal copy of the TFC2 gene by LEU2 was confirmed by Southern blot analysis of a Leu+ transformant. Plasmid pJA230, a CEN/ARS-based plasmid with a URA3 selectable marker and a 10-kbp insert of yeast DNA containing RPO26 and TFC2 (Archambault et al., 1990), was then introduced into the TFC2/tfc2::LEU2 strain. Sporulation of a Ura+ transformant generated the haploid strain YRW1. Since TFC2 is an essential gene (Archambault et al., 1992), viability of YRW1 depends on the presence of pJA230.
YRW1 was transformed with derivatives of pG3 directing expression of wild-type or mutant forms of TFIIIA. After transformants had been selected on SD medium lacking uracil and tryptophan, the pG3-containing strains were grown on SD medium lacking tryptophan and containing uracil to allow for loss of pJA230. Cells were then streaked on SD medium containing 5-fluoro-orotic acid (5-FOA) and uracil and lacking tryptophan. Because 5-FOA kills cells containing the URA3 gene, only those cells that have lost pJA230 and that contain a pG3 derivative encoding a functional version of TFIIIA will grow on the 5-FOA-containing plates.

**Preparation of anti-TFIIIA and Western blot analysis.** I confirmed that the various forms of pG3-encoded TFIIIA were expressed *in vivo* by standard Western blot analysis. Polyclonal antibodies were generated by injection of rabbits with 500 µg of bacterially expressed and purified full-length yeast TFIIIA emulsified with an equal volume of complete Freund's adjuvant. Purification of yeast TFIIIA was carried out as described previously (Rowland and Segall, 1996) except that as a final step the protein band corresponding to TFIIIA was excised from an SDS-polyacrylamide gel to achieve further purification. Rabbits were boosted every 4 weeks with 100 µg of yeast TFIIIA emulsified with an equal volume of incomplete Freund's adjuvant. Blood was collected from the rabbits 2 weeks after each boost.

YRW1 and strains of YRW1 containing pG3-derived plasmids that directed expression of mutant versions of TFIIIA were grown overnight in SD medium lacking uracil and tryptophan to an optical density at 600 nm of ~3.0. The cells from 1 ml of culture at this density, or the appropriate volume of culture if the cell density was different, were harvested by centrifugation. The pellets of cells were resuspended in 1 ml of YPD medium, and extracts of proteins were prepared from the yeast cells as described (Yaffe and Schatz, 1984). The proteins were separated on a 10% SDS-polyacrylamide gel and then electroblotted onto nitrocellulose filters at 4°C in transfer buffer (25 mM Tris-HCl, 194 mM glycine, 20%
methanol, 0.05% SDS). The filters were blocked in phosphate-buffered saline (PBS)-milk (5% powdered skim milk in PBS containing 0.05% Tween) for 1 h to overnight and were then incubated for 1 h in PBS-milk containing a 1:2,000 dilution of crude serum containing polyclonal antibodies against yeast TFIIIA (see above). The filters were washed three times for 5 to 15 min each in PBS-milk and were then incubated in PBS-milk containing a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody. The filters were washed four times for 5 to 15 min each in TTBS (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl, 0.05% Tween), and the secondary antibody was detected by the ECL chemiluminescence system (Amersham).

RESULTS

The nine zinc fingers of yeast TFIIA occur in succession except for zinc fingers 8 and 9, which are separated by an 81-amino acid domain (Fig. 3-1A). Milne and Segall (1993) previously showed that TFIIA lacking the 81-amino acid domain recruits TFIIIC to a TFIIA-5S RNA gene complex; the resultant complex, however, is unable to promote transcription. It was also found that the ninth zinc finger of TFIIA, although not essential, contributes to efficient transcription (Milne and Segall, 1993). In this study, I have defined the region of the 81-amino acid domain that is essential for its function, which I refer to as its transcription factor activity, and I have further assessed the requirement for the adjacent zinc fingers in supporting efficient transcription.
Alanine-scanning mutagenesis through charged regions of the 81-amino acid domain. As a first approach towards identification of amino acids within the 81-amino acid domain that are involved in the transcription factor activity of TFIIIA, I performed alanine-scanning mutagenesis of charged residues. A charged region of a protein is likely to be solvent exposed and a charged amino acid(s) on this surface may contribute to interprotein contacts in a multiprotein complex. Mutations of surface residues are also expected to minimally perturb the overall structure of the domain. It should be noted, however, that although charged and polar side chains can be important for protein-protein interactions, hydrophobic side chains are often more critical (see below and Discussion). The choice of alanine for substitutions within a target region allows for a consistent series of mutations with a small amino acid that is unable to provide a side chain interaction involving atoms beyond the β carbon. This approach has been used extensively for identification of amino acids that are critical for protein-protein interactions (Wells, 1991; Clackson and Wells, 1995).

Seventeen of the 30 charged residues (assuming that the histidines are positively charged) found within the 81-amino acid domain of TFIIIA are clustered within three regions that extend from residues 312 to 317, residues 321 to 327, and residues 340 to 348 (Fig. 3-1B). The first and third of these regions are predominately acidic, whereas the second region consists entirely of basic residues. Using a PCR-based approach for site-directed mutagenesis (see Materials and Methods), I changed nine pairs of adjacent, or nearby, charged amino acids to alanine and assessed the effects of these double mutations on the ability of TFIIIA to direct in vitro transcription of the 5S RNA gene.

For these studies, the mutant forms of TFIIIA were synthesized in vitro (see Materials and Methods). I first confirmed that approximately equivalent amounts of TFIIIA of the appropriate sizes were produced, as monitored by SDS-polyacrylamide gel electrophoretic analysis of proteins synthesized in the presence of [35S]methionine (data not shown). As a preliminary test for the functional integrity of the mutant proteins, I used an electrophoretic
Figure 3-1. Effects of alanine replacement of charged residues within the 81-amino acid domain on activity of TFIIIA.

(A) Schematic representation of yeast TFIIIA. Yeast TFIIIA is 429 amino acids in length and contains nine zinc finger motifs with an 81-amino acid region interrupting the repeating zinc finger motifs between fingers 8 and 9. The numbered boxes represent the nine zinc fingers of yeast TFIIIA; the stippled region between fingers 8 and 9 represents the 81-amino acid domain; the diagonally striped boxes represent the 48-amino acid amino-terminal region and the 35-amino acid carboxyl-terminal region.

(B) Amino acid sequence of yeast TFIIIA from residues 253 to 397. The sequences for zinc fingers 8 and 9, with the zinc-coordinating cysteines and histidines in boldface, and for the intervening 81-amino acid domain are given. The residue number, relative to the initiator methionine, of the last amino acid on each line is given on the right. Minus and plus symbols above residues in the 81-amino acid domain indicate negatively and positively charged amino acids, respectively. Double arrowheads indicate pairs of charged residues that were mutated to alanine; the positions of the residues are given below the lines connecting the arrowheads.

(C) Abilities of mutant versions of TFIIIA to bind to the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex, as assessed by EMSA. A radioactively labeled DNA fragment containing the yeast 5S RNA gene was incubated with \textit{in vitro}-synthesized versions of TFIIIA in the absence (odd-numbered lanes) or presence (even-numbered lanes) of partially purified yeast TFIIIC prior to electrophoresis on a nondenaturing polyacrylamide gel. Lanes 1 and 2, \textit{in vitro} transcription-translation reactions that were not programmed to synthesize TFIIIA; lanes 3 and 4, wild-type TFIIIA; lanes 5 to 22, TFIIIA with the following mutations: K287A/K289A (lanes 5 and 6); D299A/H300A (lanes 7 and 8); K308A/H309A (lanes 9 and 10); D314A/E315A (lanes 11 and 12); K324A/R325A (lanes 13 and 14); D342A/E344A (lanes 15 and 16); K345A/R346A (lanes 17 and 18); E348A/E351A (lanes 19 and 20); and R363A/K364A (lanes 21 and 22). The positions of free DNA (minus sign), TFIIIA-DNA complexes (open arrowhead), and TFIIIC-TFIIIA-DNA complexes (solid arrowhead) are indicated on the right.

(D) Abilities of mutant versions of TFIIIA to support \textit{in vitro} transcription of the 5S RNA gene. \textit{In vitro} transcription reaction mixtures contained the yeast 5S RNA gene as a template; partially purified yeast TFIIIC, TFIIIB, and RNA polymerase III; and the version of \textit{in vitro}-synthesized TFIIIA indicated above the lane. RL, reticulocyte lysate (\textit{in vitro} transcription-translation reaction not programmed to synthesize TFIIIA); WT, wild-type. The RNAs synthesized \textit{in vitro} were analyzed on a 7 M urea-10% polyacrylamide gel. The autoradiogram shows the portion of the gel containing 5S RNA.
mobility shift assay (EMSA) to assess formation of protein-DNA complexes. Because the three amino-terminal zinc fingers of TFIIIA suffice for high-affinity binding of TFIIIA to the ICR of the 5S RNA gene and for incorporation of TFIIIC into the TFIIIA-DNA complex (Milne and Segall 1993; Rowland and Segall, 1996), I anticipated that mutations within the 81-amino acid domain would not impair formation of a TFIIIC-TFIIIA-DNA complex. Indeed, each of the in vitro-synthesized mutant proteins bound to the 5S RNA gene (Fig. 3-1C, odd-numbered lanes from 5 to 21) and recruited TFIIIC (Fig. 3-1C, even-numbered lanes from 6 to 22) in a manner similar to wild-type TFIIIA (Fig. 3-1C, lanes 3 and 4). Each form of TFIIIA had about the same activity, as measured by the ability to form a TFIIIC-TFIIA-DNA complex in this qualitative EMSA. The in vitro-synthesized proteins were then tested for their ability to support in vitro transcription of the 5S RNA gene in the presence of a yeast fraction containing TFIIIC, TFIIIB, and RNA polymerase III (see Materials and Methods). All nine of the mutant forms of TFIIIA containing pairwise substitutions of charged residues (K287A/K289A, D299A/H300A, K308A/H309A, D314A/E315A, K324A/R325A, D342A/E344A, K345A/R346A, E348A/E351A, and R363A/K364A) directed transcription of the 5S RNA gene as efficiently as did wild-type TFIIIA (Fig. 3-1D, lanes 2 to 11). This analysis eliminated the possibility that any of 18 charged residues within the 81-amino acid domain was essential for the transcription factor activity of TFIIIA. Because of the qualitative nature of this assay, I cannot exclude the possibility that minor deficiencies in the activity of TFIIIA escaped detection.

Effects of carboxyl-terminal truncations on the transcription factor activity of TFIIIA. Because site-directed mutagenesis of charged amino acids did not identify any residues as critical for the transcription factor activity of TFIIIA, I next analyzed a series of TFIIIA that contained carboxyl-terminal truncations that extended into the 81-amino acid domain. It was shown previously that a form of TFIIIA that is truncated at the end of the eighth zinc finger, and therefore lacks the entire 81-amino acid domain, is unable to support transcription of the 5S RNA gene, whereas a form of TFIIIA that is truncated at the
beginning of the ninth zinc finger, and therefore contains the 81-amino acid domain, supports transcription of the 5S RNA gene, albeit less efficiently than does wild-type TFIIIA (Milne and Segall, 1993).

For this study, I constructed a series of truncated TFIIIAAs that terminated at various positions within the ninth zinc finger and the 81-amino acid domain (Fig. 3-2A). I refer to these carboxyl-terminal truncated forms of TFIIIA as TFIIIA(1-n), where n identifies the carboxyl-terminal residue of the protein. After confirming that the in vitro-synthesized forms of these TFIIIAAs were of the appropriate size (data not shown) and were active in forming a TFIIIC-TFIIIA-5S RNA gene complex (Fig. 3-2B), I tested their ability to support in vitro transcription of the 5S RNA gene (Fig. 3-2C). As expected from a previous study (Milne and Segall, 1993), TFIIIA(1-397), which contained the ninth zinc finger but lacked the last 35 amino acids of the protein, was as active as wild-type TFIIIA in supporting accurate transcription (Fig. 3-2C, lanes 3 and 4). TFIIIA(1-378), which terminated within the ninth zinc finger, had modestly reduced activity and TFIIIA(1-365), which lacked the ninth zinc finger, had further reduced activity (Fig. 3-2C, lanes 5 and 6, respectively). In some experiments, 5S RNA transcripts could be readily detected in reactions containing TFIIIA(1-365) (Milne and Segall, 1993; and data not shown) and in other experiments the level of 5S RNA transcripts obtained with this form of TFIIIA was just above background (Fig. 3-2C, compare lane 6 with lane 2). TFIIIAAs with carboxyl-terminal deletions that extended into the 81-amino acid domain [TFIIIA(1-359), TFIIIA(1-353), TFIIIA(1-347), TFIIIA(1-339), and TFIIIA(1-311)], appeared unable to support transcription of the 5S RNA gene (Fig. 3-2C, lanes 7 to 11). I note that because control reactions lacking TFIIIA gave rise to a trace amount of 5S RNA, due to contaminating TFIIIA present in the yeast fraction containing TFIIIC, TFIIIB, and RNA polymerase III (Fig. 3-2C, lanes 1 and 2, and data not shown), I could not confidently distinguish mutant forms of TFIIIA that had very low levels of activity from forms of TFIIIA that were inactive. Therefore, as an alternative approach for monitoring the activity of TFIIIA, I tested the ability of these mutant forms of TFIIIA to
Figure 3-2. Effects of carboxyl-terminal deletions on activity of TFIIIA.

(A) Amino acid sequence of zinc finger 8, the 81-amino acid domain, and zinc finger 9 of yeast TFIIIA as shown in Fig. 3-1B. Arrowheads indicate the positions of the carboxyl termini of truncated forms of TFIIIA; the number of the last residue of each truncated protein is given below the bent tail.

(B) Abilities of carboxyl-terminal-truncated versions of TFIIIA to bind to the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex, as outlined in the legend for Fig. 3-1C. Lanes 1 and 2, no transcription-translation mixture; lanes 3 and 4, in vitro transcription-translation reactions that were not programmed to synthesize TFIIIA; lanes 5 and 6, wild-type TFIIIA synthesized in vitro; lanes 7 to 22, in vitro-synthesized, truncated versions of TFIIIA extending from the initiator methionine to residue 397 (lanes 7 and 8), 378 (lanes 9 and 10), 365 (lanes 11 and 12), 359 (lanes 13 and 14), 353 (lanes 15 and 16), 347 (lanes 17 and 18), 339 (lanes 19 and 20), or 311 (lanes 21 and 22).

(C) Abilities of carboxyl-terminal-truncated versions of TFIIIA to support in vitro transcription of the 5S RNA gene. For details, see the legend for Fig. 3-1D.

(D) Abilities of carboxyl-terminal-truncated versions of TFIIIA to support cell viability. A plasmid shuffle system was used to test the abilities of mutant versions of TFIIIA to replace wild-type TFIIIA in vivo. pG3-derived plasmids that expressed truncated versions of TFIIIA were transformed into the yeast strain YRW1 (see the text for details), and the abilities of these cells to grow on medium containing 5-FOA were monitored. Vector, pG3 not expressing TFIIIA; WT, pG3 expressing wild-type TFIIIA; aa1-n, pG3 expressing a version of TFIIIA that extends from the initiator methionine to residue n.

(E) Assessment by Western blot analysis of in vivo expression of truncated versions of TFIIIA. Protein extracted from YRW1 yeast cells containing pG3-derived plasmids was separated on an SDS-10% polyacrylamide gel and electrotransferred to a nitrocellulose filter, and the filter was probed with anti-TFIIIA antibody. Lane 1, TFIIIA partially purified from yeast; lane 2, yeast TFIIIA purified from bacteria; lane 3, protein extract of YRW1 cells; lanes 4 to 9, protein extract of YRW1 cells containing pG3 expressing no TFIIIA (lane 4), wild-type TFIIIA (lane 5), TFIIIA(1-365) (lane 6), TFIIIA(1-359) (lane 7), TFIIIA(1-353) (lane 8), and TFIIIA(1-347) (lane 9). The asterisk on the left indicates a cross-reacting molecule not related to TFIIIA. The positions and sizes (in kilodaltons) of molecular mass markers are shown on the right.
support cell growth. I note that the only essential function of TFIIIA in vivo is in promoting transcription of the 5S RNA gene (Camier et al., 1995).

To assess the ability of mutant forms of TFIIIA to function in vivo, I used a plasmid-shuffling protocol (Sikorski and Boeke, 1991) to replace the wild-type gene encoding TFIIIA with a mutated version of the gene. This analysis was done with the yeast strain YRW1 (Milne, 1994), in which the chromosomal copy of TFC2, the gene encoding TFIIIA, has been deleted and cell viability is maintained by the presence of pJA230, a plasmid that contains a wild-type copy of TFC2 and URA3 as the selectable marker (Archambault et al., 1992). Plasmids that express mutant forms of TFIIIA can then be introduced into YRW1, and the ability of these strains to survive in the absence of pJA230 can be monitored by assessing growth on medium containing 5-FOA. Because cells that grow on this medium must have lost the URA3-containing pJA230, which also contains the wild-type TFC2 gene, growth indicates that the mutant TFIIIA is active in directing transcription of the 5S RNA gene in vivo.

Using this plasmid shuffling protocol, I found that pG3-derived, high-copy number plasmids (see Materials and Methods) that expressed TFIIIA(1-397), TFIIIA(1-365), or TFIIIA(1-359) supported cell viability, whereas plasmids that expressed TFIIIA(1-353), TFIIIA(1-347), TFIIIA(1-339), or TFIIIA(1-311) did not (Fig. 3-2D and data not shown). Western blot analysis of cells containing pJA230 and pG3-derived plasmids confirmed that the truncated versions of TFIIIA, including those that did not support cell viability, were stable in vivo (Fig. 3-2E). I note that for the development time used for the blot shown in Fig. 3-2E, TFIIIA expressed from the low-copy number plasmid pJA230 in strain YRW1 could not be readily detected (Fig. 3-2E, lanes 3 and 4), whereas TFIIIA expressed from the glyceraldehyde-3-phosphate dehydrogenase promoter in a high-copy number plasmid was readily detectable (Fig. 3-2E, lane 5). Expression of TFIIIA from the glyceraldehyde-3-phosphate dehydrogenase promoter on a low-copy plasmid did not significantly reduce
TFIIIA protein levels relative to that observed using the same promoter on the high-copy pG3-derived plasmid (O. Rowland, data not shown). Although transcripts of the 5S RNA gene could not be detected in an *in vitro* transcription reaction containing TFIIIA(1-359), this form of TFIIIA nonetheless supported cell viability. It is possible that the *in vitro* system lacked the sensitivity to detect a low level of TFIIIA activity and that this low level of activity was sufficient to support cell viability; it is also possible that the high level of expression of the mutant TFIIIA *in vivo*, as observed by Western blot analysis, compensated for its reduced activity in promoting transcription of the 5S RNA gene.

In summary, I found that TFIIIA(1-365), which lacked the ninth zinc finger and had reduced activity *in vitro*, and TFIIIA(1-359), which lacked an additional 6 amino acids and had no detectable activity *in vitro*, each supported cell viability. Deletion of the next 6 amino acids, which removed a leucine-rich segment of the 81-amino acid domain, abolished the ability of TFIIIA to support cell viability. I therefore concluded that an amino acid(s) within the region from residue 354 to residue 359 was essential for the transcription factor activity of TFIIIA.

**Effects of internal deletions within the 81-amino acid domain of TFIIIA.** As described above, analysis of the effects of carboxyl-terminal deletions suggested that a residue(s) within the leucine-rich sequence adjacent to amino acid 359 of TFIIIA was essential for the activity of the protein. Because the hydrophobic, nonpolar nature of this segment suggested the possibility that it might play a role in folding of the 81-amino acid domain, I assessed the effects of a series of internal deletions within the 81-amino acid domain (Fig. 3-3A) on the activity of TFIIIA. I anticipated that if the leucine-rich segment did contribute to folding of the 81-amino acid domain rather than being directly involved in its function, this approach might identify another region(s) that contributed to the function of TFIIIA.
Figure 3-3. Effects of deletions within the 81-amino acid domain on activity of TFIIIA.

(A) Amino acid sequences of zinc finger 8, the 81-amino acid domain, and zinc finger 9 of yeast TFIIIA, as shown in Fig. 3-1B. Rectangles and numbers above the amino acid sequence represent regions deleted from the 81-amino acid domain. The deletions in TFIIIA(Δ284-364), which lacks the entire 81-amino-acid domain, and in TFIIIA(Δ282-353) are not represented.

(B) Abilities of versions of TFIIIA with deletions within the 81-amino acid domain to bind the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex, as outlined in the legend for Fig. 3-1C. Lanes 1 to 4 were as described for Fig. 3-1C. Lanes 5 to 20, in vitro-synthesized versions of TFIIIA lacking residues 284 to 364 (lanes 5 and 6), 282 to 287 (lanes 7 and 8), 282 to 315 (lanes 9 and 10), 282 to 353 (lanes 11 and 12), 316 to 334 (lanes 13 and 14), 328 to 341 (lanes 15 and 16), 342 to 351 (lanes 17 and 18), or 352 to 359 (lanes 19 and 20).

(C) Abilities of versions of TFIIIA with deletions in the 81-amino acid domain to support in vitro transcription of the 5S RNA gene. For details, see the legend to Fig. 3-1D.

(D) Abilities of versions of TFIIIA with deletions in the 81-amino acid domain to support cell viability. A plasmid shuffle system was used to test the abilities of mutant versions of TFIIIA to support cell viability as described in the legend to Fig. 3-2D. Growth of cells containing pG3-derived plasmids on medium containing 5-FOA is shown. vector, pG3 not expressing TFIIIA; WT, pG3 expressing wild-type TFIIIA; Δn-n pG3 expressing a version of TFIIIA that lacks amino acids n to n.

(E) Assessment by Western blot analysis of in vivo expression of TFIIIA containing deletions in the 81-amino acid domain. The analysis was carried out as described in the legend to Fig. 3-2E. Protein in lanes 1 to 9 was extracted from YRW1 cells containing pG3-derived plasmids expressing no TFIIIA, wild-type TFIIIA, TFIIIA(Δ284-364), TFIIIA(Δ282-315), TFIIIA(Δ282-353), TFIIIA(Δ316-334), TFIIIA(Δ328-341), TFIIIA(Δ342-351), and TFIIIA(Δ352-359), respectively. The asterisk on the left indicates a cross-reacting molecule not related to TFIIIA. Only the portion of the blot that contains TFIIIA is shown.
As expected, I found that TFIIIA(A352-359), which lacked the leucine-rich segment of the 81-amino acid domain, was unable to support \textit{in vitro} transcription of the 5S RNA gene (Fig. 3-3C, lane 10). Of the other internally deleted forms of TFIIIA that were tested, only TFIIIA(A284-364), which lacked the entire 81-amino acid domain (Milne and Segall, 1993), and TFIIIA(A282-353), which lacked all but the 11 carboxyl-terminal residues of the 81-amino acid domain, failed to support \textit{in vitro} transcription of the 5S RNA gene (Fig. 3-3C, lanes 3 and 6). In contrast, TFIIIA(A282-287) and TFIIIA(A282-315), which lacked 6 and 34 amino acids, respectively, at the amino-terminal end of the 81-amino acid domain, supported \textit{in vitro} transcription (Fig. 3-3C, lanes 4 and 5). TFIIIA(A282-315), however, was not as active as was wild-type TFIIIA (Fig. 3-3C; compare lanes 2 and 5). TFIIIA(A316-334) and TFIIIA(A328-341), which contained overlapping deletions in the central portion of the 81-amino acid domain of TFIIIA, also supported transcription, but less efficiently than did wild-type TFIIIA (Fig. 3-3C, lanes 7 and 8). Finally, TFIIIA(A342-351), which lacked 10 amino acids just upstream of the leucine-rich region was as active as wild-type TFIIIA in supporting \textit{in vitro} transcription (Fig. 3-3C, lane 9).

I also tested these TFIIIA\textsubscript{s} for their ability to support cell viability, using the plasmid-shuffling protocol described above. TFIIIA(A284-364), TFIIIA(A282-353), and TFIIIA(A352-359), which failed to support \textit{in vitro} transcription of the 5S RNA gene, also failed to support cell viability (Fig. 3-3D). These mutant forms of TFIIIA, although inactive, were nonetheless stable \textit{in vivo} and accumulated to a level higher than that obtained with pJA230-borne \textit{TFC2} (Fig. 3-3E; compare lanes 3, 5, and 9 with lane 1). As expected, those forms of TFIIIA which supported \textit{in vitro} transcription, TFIIIA(A282-315), TFIIIA(A316-334), TFIIIA(A328-341), and TFIIIA(A342-351), supported cell viability (Fig. 3-3D). The highly basic region from residue 321 to 327 is a putative nuclear localization signal (Osborne and Silver, 1993). However, the ability of TFIIIA(A316-334) to support cell viability implies
that this basic region is not necessary for nuclear localization, at least when TFIIIA is overexpressed.

In summary, I found that forms of TFIIIA with internal deletions that spanned the sequence from residue 282 to 351 of the 81-amino acid domain [TFIIIA(Δ282-315), TFIIIA(Δ316-334), TFIIIA(Δ328-341), and TFIIIA(Δ342-351)] retained activity. TFIIIA(Δ352-359), which lacked 8 amino acids spanning the leucine-rich segment at the carboxyl-terminal end of the 81-amino acid domain, however, was inactive. Because I found that TFIIIA tolerated deletions throughout most regions of the 81-amino acid domain without loss of activity, I speculated that the leucine-rich segment was the only essential region of the 81-amino acid domain and that this region was directly involved in the transcription factor activity of TFIIIA.

**Effects of double alanine-scanning mutations of hydrophobic and nonpolar amino acids in the carboxyl-terminal portion of the 81-amino acid domain.** I next subjected the region from residue 343 to 359, which spans the leucine-rich segment at the carboxyl-terminal end of the 81-amino acid domain, to alanine-scanning mutagenesis (Fig. 3-4A). For ease of reference, forms of TFIIIA with double mutations were assigned code names beginning with the letter D. I found that TFIIIA(N352A/N355A) (D3), TFIIIA(L354A), TFIIIA(L356A), and TFIIIA(N359A) supported efficient *in vitro* transcription of the 5S RNA gene (Fig. 3-4C, lanes 6, 8, 9, and 11). TFIIIA(L343A/L347A) (D2), TFIIIA(L354A/L356A) (D4), and TFIIIA(L357A/L358A) (D5) had reduced, but readily detectable, activity (Fig. 3-4C, lanes 5, 7 and 10). Of these mutants, I consistently found that TFIIIA(L343A/L347A) (D2) was more active than TFIIIA(L357A/L358A) (D5) and that TFIIIA(L354A/L356A) (D4) was the most compromised for activity (Fig. 3-4C, lanes 5, 7, and 10). For comparison, I also monitored the effect of replacement by alanine of the hydrophobic residues at positions 296 and 297 and at positions 336 and 337. I found that both TFIIIA(L296A/V297A) and TFIIIA(F336A/L337A) (D1) were as active as wild-type TFIIIA (Fig. 3-4C, lanes 3 and 4).
Figure 3-4. Effects of double and quadruple alanine-scanning mutations within the carboxyl-terminal portion of the 81-amino acid domain on activity of TFIIIA.

(A) Schematic representation of TFIIIA, showing the positions of mutated residues within the amino acid sequence from residue 335 to 365. The series of mutations that were introduced into the sequence from amino acid 335 to amino acid 365 are listed on the left, preceded by code names for the double (D1 to D5) and the quadruple (Q1 to Q8) alanine substitutions, and are represented by A's aligned below the amino acid sequence. Dashes represent wild-type amino acids. For ease of alignment, Phe336 and Leu residues are shaded on all lines. A circled or boxed A indicates that alanine has replaced a negatively or positively charged residue, respectively. The two columns at the right summarize the transcription factor activity of each mutant TFIIIA with respect to its ability to support in vitro transcription of the 5S RNA gene (as shown in panels C and E) and cell viability (as shown in panels F and G). Left column: ++, the ability to support in vitro transcription was similar to that of wild-type TFIIIA; -, only a background level of in vitro transcription was observed. Right column: +, the mutant was able to support cell viability; -, the mutant was not able to support cell viability.

(B and D) Abilities of versions of TFIIIA with single or double alanine-scanning mutations (B) or with quadruple alanine-scanning mutations (D) within the 81-amino acid domain to bind to the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex as outlined in the legend of Fig. 3-1C. Lanes 1 to 4 were as described for Fig. 3-1C. (B) Lanes 5 to 22, TFIIIA with the following mutation(s): L296A/V297A (lanes 5 and 6), F336A/L337A (lanes 7 and 8), L343A/L347A (lanes 9 and 10), N352A/N355A (lanes 11 and 12), L354A/L356A (lanes 13 and 14), L354A (lanes 15 and 16), L356A (lanes 17 and 18), L357A/L358A (lanes 19 and 20), or N359A (lanes 21 and 22). (D) Lanes 5 to 20, TFIIIA with the following mutations: F336A/L337A/L343A/L347A (lanes 5 and 6), D342A/E344A/E348A/E351A (lanes 7 and 8), L343A/L347A/L354A/L356A (lanes 9 and 10), L343A/L347A/L357A/L358A (lanes 11 and 12), K345A/R346A/L357A/L358A (lanes 13 and 14), K345A/R346A/R363A/K364A (lanes 15 and 16), L354A/L356A/L357A/L358A (lanes 17 and 18), or L357A/L358A/R363A/K364A (lanes 19 and 20).

(C and E) Abilities of versions of TFIIIA with single and double (C) or quadruple (E) alanine-scanning mutations in the carboxyl-terminal portion of the 81-amino acid domain to support in vitro transcription of the 5S RNA gene. For details, see the legend to Fig. 3-1D. Where appropriate, the D and Q codes from panel A are given above the lanes.

FIGURE 3-4 LEGEND CONTINUED TWO PAGES OVER.
Figure 3-4 continued.

(F and G) Abilities of versions of TFIIIA with double and quadruple alanine-scanning mutations to support cell viability. A plasmid shuffle system was used to test the abilities of mutant versions of TFIIIA to support cell viability, as described in the legend to Fig. 3-2D. Cells containing pG3-derived plasmids that did not express TFIIIA (vector) or that expressed wild-type TFIIIA (WT) or versions of TFIIIA with the double or quadruple alanine-scanning mutations (D and Q codes, respectively) diagrammed in panel A were streaked onto 5-FOA-containing medium.

(H) Assessment by Western blot analysis of in vivo expression of mutant forms of TFIIIA. Protein extracted from YRW1 yeast cells containing pG3-derived plasmids was analyzed as described in the legend to Fig. 3-2E. Protein in lanes 1 to 12 was extracted from cells containing pG3 expressing no TFIIIA (lane 1), wild-type TFIIIA (lane 2), TFIIIA(L343A/L347A) (lane 3), TFIIIA(L354A/L356A) (lane 4), TFIIIA(L357A/L358A) (lane 5), TFIIIA(L343A/L347A/L354A/L356A) (lane 6), TFIIIA(L343A/L347A/L357A/L358A) (lane 7), TFIIIA(F336A/L337A/L343A/L347A) (lane 8), TFIIIA(K345A/R346A/L357A/L358A) (lane 9), TFIIIA(K345A/R346A/R363A/K364A) (lane 10), TFIIIA(L354A/L356A/L357A/L358A) (lane 11), and TFIIIA(L357A/L358A/R363A/K364A) (lane 12). Asterisk, a cross-reacting molecule not related to TFIIIA; arrow, full-length TFIIIA.
This analysis indicated that none of the hydrophobic and nonpolar residues analyzed was essential for transcription factor activity. However, the double mutation L357A/L358A (Fig. 3-4C, lane 10) led to a modest reduction in the activity of TFIIIA, and the double mutation L354A/L356A led to an even more severe reduction in the activity of TFIIIA (Fig. 3-4C, lane 7); neither of the mutations L354A and L356A affected activity (Fig. 3-4C, lanes 8 and 9).

**Effects of quadruple alanine-scanning mutations within the carboxyl-terminal portion of the 81-amino acid domain.** Although I found that TFIIIA(Δ352-359) was inactive both in vitro and in vivo, double substitutions in this region (N352A/N355A, L354A/L356A, and L357A/L358A) did not abolish the transcription factor activity of TFIIIA (see above). I therefore next assessed the effects of quadruple mutations in this region, anticipating that replacement of four leucines by alanine would be sufficiently deleterious to abolish function. For ease of reference, forms of TFIIIA with quadruple mutations were assigned code names beginning with the letter Q. Indeed, combining the double mutations L354A/L356A and L357A/L358A, both of which compromised the activity of TFIIIA (Fig. 3-4C, lanes 7 and 10, respectively), with each other or with the double mutation L343A/L347A, which on its own led to a modest decrease in activity of TFIIIA (Fig. 3-4C, lane 5), completely inactivated TFIIIA. I found that TFIIIA(L354A/L356A/L357A/L358A) (Q7), TFIIIA(L343A/L347A/L354A/L356A) (Q3), and TFIIIA(L343A/L347A/L357A/L358A) (Q4) failed to support in vitro transcription of the 5S RNA gene (Fig. 3-4E, lanes 5, 6, and 9) and failed to support cell viability (Fig. 3-4F and G).

For comparison, I combined the double mutation L343A/L347A, which led to a modest decrease in the activity of TFIIIA (Fig. 3-4C, lane 5), with mutation of the hydrophobic residues F336 and L337, which as a double mutation had no effect on the activity of TFIIIA (Fig. 3-4C, lane 4). The resultant TFIIIA(F336A/L337A/L343A/L347A) (Q1) supported a very low level of 5S RNA gene transcription in vitro (Fig. 3-4E, lane 3) and supported cell viability (Fig. 3-4G). I also monitored the effect of mutation of four negatively charged residues (D342, E344, E348, and E351) in this region.
TFIIIA(D342A/E344A/E348A/E351A) (Q2) was as active as wild-type TFIIIA in supporting
in vitro transcription of the 5S RNA gene (Fig. 3-4E, lane 4).

Analysis of the effects of carboxyl-terminal deletions (Fig. 3-2) implicated the region just
carboxyl-terminal to L358 as contributing to, although not being essential for, the
transcription factor activity of TFIIIA. I therefore examined the effect of combining
mutation of the basic residues R363 and K364, which as a double mutation did not affect the
in vitro activity of TFIIIA (Fig. 3-1C, lane 11), with the double mutation L357A/L358A,
which reduced the activity of TFIIIA (Fig. 3-4C, lane 10). I found that
TFIIIA(L357A/L358A/R363A/K364A) (Q8) was unable to support transcription of the 5S
RNA gene in vitro (Fig. 3-4E, lane 10) and failed to support cell viability (Fig. 3-4G). For
comparison, I also combined mutation of the pair of basic residues K345 and R346, which as
a double mutation did not affect the in vitro activity of TFIIIA (Fig. 3-1C, lane 9), with the
double mutation L357A/L358A. Although the in vitro transcription factor activity of
TFIIIA(K345A/R346A/L357A/L358A) (Q5) was just above background (Fig. 3-4E, lane 7),
this form of TFIIIA was nonetheless able to support cell viability (Fig. 3-4G). Growth of this
strain, however, appeared to be slightly impaired. Finally, I tested the effect of combining
the double mutation K345A/R346A and the double mutation R363A/K364A. Substitution of
alanine for these four basic residues had only a minimal effect on the in vitro activity of
TFIIIA (Fig. 3-4E, lane 8), and TFIIIA(K345A/R346A/R363A/K364A) (Q6) supported cell
viability (Fig. 3-4G).

In summary, analysis of the effects of double and quadruple mutations in the region
extending from residue 336 to 364 of TFIIIA confirmed that hydrophobic residues in this
segment are critical for the function of TFIIIA. In particular, L343, L347, L354, L356, L357,
and L358, and to a lesser extent F336 and L337, contribute to the function of this region.
Although charged residues are less important, R363 and K364, and to a lesser extent K345
and R346, also make a contribution to the activity of TFIIIA.
Zinc fingers 8 and 9 contribute to the transcription factor activity of TFIIIA. I fortuitously obtained by error-prone PCR mutagenesis a gene encoding TFIIIA with a mutation in a zinc-coordinating residue of finger 8 (H272R) and in a zinc-coordinating residue of finger 9 (C367Y) (see Materials and Methods) (Fig. 3-5A). This combination of mutations (H272R/C367Y), which would be expected to disrupt the structure of zinc fingers 8 and 9, abolished the ability of TFIIIA to support in vitro transcription of the 5S RNA gene (Fig. 3-5C, lane 3) and to support cell viability (Fig. 3-5D). I found, however, that TFIIIA that contained H272R or C367Y as a single mutation (see Materials and Methods) was only modestly compromised in its ability to support in vitro transcription of the 5S RNA gene (Fig. 3-5C, lanes 4 and 5) and was able to support cell viability (Fig. 3-5D). These data suggest that zinc fingers 8 and 9 make redundant contributions to the transcription factor activity of TFIIIA; the activity of TFIIIA is maintained on disruption of finger 8 or finger 9, but its activity is abolished on disruption of both fingers. I note that the assessment of protein-DNA complex formation by the EMSA shown in Fig. 3-5B suggests that TFIIIA(H272R/C367Y) was less active than wild-type TFIIIA in the formation of TFIIIA-DNA and TFIIC-TFIIIA-DNA complexes. Because of the qualitative nature of this assay, however, I do not know if this apparent difference is significant.
Figure 3-5. Effects of mutations that disrupt the structure of zinc fingers 8 and 9 on activity of TFIIIA.

(A) Schematic representation of yeast TFIIIA as described for Fig. 3-1A. The approximate positions of mutations H272R and C367Y within zinc fingers 8 and 9, respectively, are indicated by arrows.

(B) Abilities of versions of TFIIIA with disruptions of zinc fingers 8 and 9 to bind to the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex, as outlined in the legend of Fig. 3-1C. Lanes 1 to 4 were as described for Fig. 3-1C. Lanes 5 to 10, in vitro-synthesized versions of TFIIIA containing the following mutation(s): H272R/C367Y (lanes 5 and 6), H272R (lanes 7 and 8), or C367Y (lanes 9 and 10).

(C) Abilities of versions of TFIIIA with disruptions of zinc fingers 8 and 9 to support in vitro transcription of the 5S RNA gene. For details, see the legend to Fig. 3-1D.

(D) Abilities of versions of TFIIIA with disruptions of zinc fingers 8 and 9 to support cell viability. A plasmid shuffle system was used to test the abilities of mutant versions of TFIIIA to support cell viability, as described in the legend to Fig. 3-2D.

(E) Assessment by Western blot analysis of in vivo expression of versions of TFIIIA with disruptions in zinc fingers 8 and 9. The analysis was carried out as described in the legend to Fig. 3-2E. Protein was extracted from YRW1 cells containing pG3-derived plasmids expressing no TFIIIA (lane 1), wild-type TFIIIA (lane 2), TFIIIA(H272R/C367Y) (lane 3), TFIIIA(H272R) (lane 4), or TFIIIA(C367Y) (lane 5).
Milne and Segall (1993) previously found that an 81-amino acid domain that is present between zinc fingers 8 and 9 of yeast TFIIIA is essential for the transcription factor activity of the protein. TFIIIA lacking this domain binds efficiently to the 5S RNA gene and recruits TFIIIC, but the resultant TFIIIC-TFIIIA-DNA complex is unable to promote transcription. As a step towards understanding the role of this region in assembly of a functional preinitiation complex, I have defined specific residues within the 81-amino acid domain that are critical for TFIIIA-mediated transcription. I tested versions of TFIIIA containing carboxyl-terminal deletions, internal deletions, and substitutions within the 81-amino acid domain for their ability to support transcription of the 5S RNA gene in vitro and in vivo. I note that the only essential function of TFIIIA in vivo is as a transcription factor for the 5S RNA gene (Camier et al., 1995). In this study, I found that a short hydrophobic segment within the 81-amino acid domain, from residue 352 to 359, was crucial for TFIIIA-mediated transcription.

**Essential role of a hydrophobic patch within the 81-amino acid domain.** I found that the 81-amino acid domain of yeast TFIIIA was surprisingly tolerant to mutation. I was unable to identify any charged residue within this domain that was essential for its function (Fig. 3-1). Moreover, forms of TFIIIA with extensive deletions within the 81-amino acid domain retained the ability to support transcription of the 5S RNA gene (Fig. 3-3). I found that TFIIIA that lacked the sequence from residue 282 to 315, from residue 316 to 334, from residue 328 to 341, or from residue 342 to 351 supported in vitro transcription of the 5S RNA gene, although in some instances to a reduced level, and supported cell viability (Fig. 3-3). However, deletion of an asparagine- and leucine-rich segment (NGLNLNLNN; residue 352 to 359) within the carboxyl-terminal portion of the 81-amino acid domain destroyed the activity of TFIIIA. My data suggest that no other segment within the 81-amino acid domain can
substitute for this asparagine- and leucine-rich segment: TFIIIA lacking the carboxyl-terminal portion of the protein beyond residue 359 supported cell viability, whereas TFIIIA lacking the carboxyl-terminal portion beyond residue 353 did not support viability (Fig. 3-2).

I note that TFIIIA that had been truncated at the beginning of the ninth zinc finger was much less active in its ability to support \textit{in vitro} transcription of the 5S RNA gene than was TFIIIA that had been truncated in the middle of the ninth zinc finger (Fig. 3-2). This suggests that a carboxyl-terminal extension to TFIIIA, even if unstructured, as would be expected for a partial zinc finger, enhances the activity of the asparagine- and leucine-rich segment.

\textbf{Requirement for hydrophobic residues within the carboxyl-terminal portion of the 81-amino acid domain.} Alanine-scanning mutagenesis indicated that no single amino acid in the region from residue 354 to 359 (LNLLLN) was essential for the activity of TFIIIA (Fig. 3-4). Analysis of the effect of double mutations in the segment $^{352}$NGLNLLLN$^{359}$ indicated that the leucine residues were more important for function than the asparagine residues. TFIIIA containing the double mutation N352A/N355A appeared to be as active as wild-type TFIIIA, whereas TFIIIA containing the double mutation L354A/L356A or L357A/L358A had reduced activity. TFIIIA in which L354, L356, L357, and L358 were all mutated was unable to support cell viability. The double mutation L343A/L347A, which consists of residues amino-terminal to the hydrophobic $^{352}$NGLNLLLN$^{359}$ segment, had only a very minor effect on the \textit{in vitro} activity of TFIIIA. Interestingly, combining this double mutation with either the double mutation L354A/L356A or the double mutation L357A/L358A led to forms of TFIIIA that were unable to support cell viability. This observation suggests that the overall hydrophobicity of the region that encompasses $^{352}$NGLNLLLN$^{359}$ is more important for its function than is any single residue. In support of this notion, I found that combining the double mutations F336A/L337A and L343A/L347A, which are in residues upstream of the $^{352}$NGLNLLLN$^{359}$ hydrophobic
segment and which by themselves had little effect on the activity of TFIIIA, led to a dramatic decrease in the activity of TFIIIA. It is interesting that TFIIIA with an internal deletion that eliminated residues 342 to 351 was just as active in vitro as wild-type TFIIIA. In this case, however, L337 occupied the same position, at least at the primary sequence level, as L347 normally does relative to residue 352, and consequently L337 may have fulfilled the role of L347.

In contrast to the importance of hydrophobic residues in the carboxyl-terminal portion of the 81-amino acid domain, the charged nature of this region appeared to be less important for its function. For example, TFIIIA in which the acidic residues D342, E344, E348, and E351 had all been replaced by alanine was as active as wild-type TFIIIA (Fig. 3-4). Similarly, TFIIIA in which the basic residues K345, R346, R363, and K364 had all been replaced by alanine was almost as active as wild-type TFIIIA (Fig. 3-4). Nonetheless, these charged residues appeared to play a role in the activity of TFIIIA; combining mutation of the basic residues K345 and R346 with mutation of L357 and L358 dramatically reduced the ability of TFIIIA to support in vitro transcription of the 5S RNA gene and combining mutation of the basic residues R363 and K364 with mutation of L357 and L358 abolished the ability of TFIIIA to support cell viability.

I note that my alanine-scanning mutagenesis was not exhaustive and that residues that contributed to function may have escaped detection. Additionally, I would not have detected residues outside of the $^{352}$NGLNLLLN$^{359}$ segment that contributed to function in a redundant manner.

Role of the leucine-rich segment of the 81-amino acid domain in TFIIIA-mediated transcription. Milne and Segall (1993) previously presented a model for the role of yeast TFIIIA in the assembly of a transcription complex on the yeast 5S RNA gene in which they took into consideration the fact that TFIIIIC (or τ) consists of two subcomplexes, $\tau_A$ and $\tau_B$, which interact with the A box and B box, respectively, of tRNA genes (Gabrielsen and
It is the interaction of the $\tau_A$ subcomplex with the A box of a tRNA gene that is responsible for appropriate positioning of TFIIIB upstream of the start site of transcription (Joazeiro et al., 1996; also reviewed in White, 1994). In this model, it was proposed that the amino-terminal zinc fingers of yeast TFIIIA interact with the $\tau_B$ subcomplex of TFIIIC and that once TFIIIC had been recruited to a TFIIIA-DNA complex by this interaction, the 81-amino acid domain of TFIIIA interacted with the $\tau_A$ subcomplex of TFIIIC (Milne and Segall, 1993). The latter interaction was predicted to be responsible for docking the multisubunit TFIIIC on the TFIIIA-DNA complex with the appropriate topography so that TFIIIB could be properly positioned to fulfill its role as an initiation factor. My study implicates a hydrophobic segment in the carboxyl-terminal portion of the 81-amino acid domain as essential for its function and, according to the above model, leads to the suggestion that this segment interacts with a region of TFIIIC.

In this context, it is interesting that hydrophobic side chains are a key characteristic of many protein-protein interfaces (Young et al., 1994; Jones and Thornton, 1996). One of the best studied examples of the role of hydrophobic surfaces in mediating a protein-protein interaction is the binding of human growth hormone to its receptor (Cunningham and Wells, 1989; de Vos et al., 1992; Clackson and Wells, 1995; reviewed in Wells, 1996). As assessed by mutational and structural analyses, the critical interactions are between well-packed hydrophobic and sterically complementary surfaces. Although peripheral contacts mediated by electrostatic interactions do contribute to the binding of human growth hormone to its receptor, they are less important than the core hydrophobic interactions.

Although it was initially postulated that acidic residues of some transcriptional regulators were key to their activity, more detailed studies have indicated that the function of these activation surfaces in fact depends on hydrophobic residues. For example, mutational analyses have revealed that it is a specific pattern of aromatic and large hydrophobic amino acids that plays a critical role in activation by such transactivators as VP16 (Cress and
Triezenberg, 1991; Regier et al., 1993), Rta (Hardwick et al., 1992), RelA (Blair et al., 1994), Sp1 (Gill et al., 1994), Gcn4 (Drysdale et al., 1995; Jackson et al., 1996), p53 (Lin et al., 1994), and the glucocorticoid receptor (Almof et al., 1997). Similarly, a leucine-rich motif, LXXLL, present in several coactivators is responsible for mediating their interaction with nuclear receptors (Heery et al., 1997; Torchia et al., 1997).

Various studies have led to the conclusion that many transcriptional activators function by contacting a component(s) of the basal transcriptional machinery that is associated with RNA polymerase II (reviewed in Ptashne and Gann, 1997). In several cases, the extent to which a mutation in an activator reduces transactivation has been shown to correlate with the extent to which it weakens an interaction with a component(s) of the basal transcriptional machinery (for examples, see Cress and Triezenberg, 1991, Ingles et al., 1991, and Xiao et al., 1994). An interesting example of the role of hydrophobic surfaces in mediating protein-protein interactions by transcriptional regulators is provided by studies of the tumor suppressor transactivator p53. The same hydrophobic residues that are exposed on one face of an amphipathic α helix of p53 (Kussie et al., 1996) and that are required for activation of transcription (Lin et al., 1994; Chang et al., 1995) are also required for interaction of p53 with TBP and TBP-associated factors (Chang et al., 1995; Thut et al., 1995; Lu and Levine, 1995). Moreover, a recent structural analysis of a peptide of p53 bound to a domain of Mdm-2, a cellular oncogene, revealed that a deep hydrophobic cleft in Mdm-2 provides steric complementarity for the hydrophobic face of the transactivation helix of p53 (Kussie et al., 1996). The fact that hydrophobic residues of p53 that are known to be crucial for its ability to transactivate are buried in the interface with Mdm-2 provides an explanation for inactivation of p53 by Mdm-2 (Lin et al., 1994; Chang et al., 1995; Picksley et al., 1994; Kussie et al., 1996).

In view of the increasing number of examples that demonstrate a role for a hydrophobic surface in mediating a protein-protein interaction, my finding that hydrophobic residues
within the carboxyl terminus of the 81-amino acid domain are of primary importance for its transcription factor activity is consistent with the notion that this region of TFIIIA interacts with TFIIIC.

**Comparison of yeast and Xenopus TFIIIA.** A 14-amino acid segment (KRSLASRLTGYIPP) that is present in the portion of *Xenopus* TFIIIA that extends beyond the ninth zinc finger is essential for the transcription factor activity of this TFIIIA (Mao and Darby, 1993). Zinc fingers 8 and 9 also contribute to the transcription factor activity of *Xenopus* TFIIIA (Del Rio and Setzer, 1993; Rollins *et al.*, 1993); these fingers may serve a direct role in assembly of a functional transcription complex or they may act indirectly, through their interaction with the A box of the ICR, to appropriately position the 14-amino acid segment to fulfill its role in promoting transcription. I find no sequence similarity between this 14-amino acid segment of *Xenopus* TFIIIA and the leucine-rich region of the 81-amino acid domain of yeast TFIIIA that I have defined as being important for its transcription factor activity. This lack of sequence similarity is not surprising, as components of the RNA polymerase III transcriptional machinery appear to be quite divergent among species (see the Introduction to this Thesis and Wang and Roeder, 1997). It is also possible that the molecular details of assembly of a functional transcription complex differ between yeast and *Xenopus*. For example, the activity of the transcription-activating region of *Xenopus* TFIIIA appears to be more position dependent (Mao and Darby, 1993) than the activity of the transcription-activating region of yeast TFIIIA (this study), as assessed by monitoring the effects of deletion of adjacent sequences. Zinc fingers 8 and 9 of both *Xenopus* TFIIIA (Del Rio and Setzer, 1993; Rollins *et al.*, 1993) and yeast TFIIIA (this study) appear to contribute to the transcription factor activity of TFIIIA. However, I found that yeast TFIIIA remained active if either finger 8 or finger 9 was disrupted by mutation; disruption of both these fingers was required in order to abolish the ability of TFIIIA to promote transcription of the 5S RNA gene. This suggests that these zinc fingers play a redundant role in promoting transcription. These fingers may help establish the overall
topography of the preinitiation complex either by extending the interaction of TFIIIA with DNA (Braun et al., 1992a), by interacting with another component of the complex, or by contributing to the function of the 81-amino acid domain.

Further studies will elucidate the role of the hydrophobic segment of the 81-amino acid domain of yeast TFIIIA in generating an active transcription complex on the yeast 5S RNA gene. Potential roles include the previously proposed function of locking TFIIIC into position in the TFIIIA-DNA complex subsequent to its recruitment by the amino-terminal zinc fingers of TFIIIA (Geiduschek and Kassavetis, 1992; Milne and Segall, 1993), a contribution to the changes in architecture and properties of the transcription complex that occur on incorporation of TFIIIB (Kassavetis et al., 1990; Braun et al., 1992a), and, perhaps, an interaction with TFIIIC or with DNA that prevents TFIIIC from making inappropriate contacts with the 5S RNA gene (see the Appendix to this Chapter). These roles need not be mutually exclusive.
APPENDIX TO CHAPTER 3

A Role for the 81-Amino Acid Domain of Yeast TFIIIA in Preventing TFIIIC From Gaining Access to the A-box-like Sequence of the 5S RNA Gene
RESULTS AND DISCUSSION

TFIIIA(L354A/L356A/L357A/L358A) can support \textit{in vitro} transcription of a 5S RNA gene that is mutated in its A-box-like sequence. An A-box-like sequence, which is present at nt +50 to +61 in the yeast 5S RNA gene, is not required for efficient \textit{in vitro} transcription of this gene (Challice and Segall, 1989). This sequence derives its name from its similarity with the A-box promoter element of tRNA genes. For comparison, the consensus sequence of the A-box from tRNA genes is TRGCNNAGY(N)GG (nucleotides +8 to +19) (where R stands for purine, Y for pyrimidine, and N for any nucleotide) (Allison et al., 1983; Geiduschek and Kassavetis, 1992) and the sequence of the A-box from the \textit{S. cerevisiae} 5S RNA gene is TAGTTAAGCTGG (Challice and Segall, 1989). TFIIIC is initially recruited to a tRNA gene by the interaction of the 138-kDa subunit of the $\tau_B$ subcomplex of TFIIIC with the B-box promoter element of the gene; a subsequent weaker interaction of the 95- and 55-kDa subunits of the $\tau_A$ subcomplex of TFIIIC with the A-box element of the tRNA gene docks TFIIIC so that its 131-kDa subunit is positioned to direct TFIIIB to its upstream DNA-binding site (Bartholomew et al., 1990, 1991; Joazeiro et al., 1996; also see section 1.4.1 and Fig. 1-3A of the INTRODUCTION to this thesis). In contrast, on the 5S RNA gene, TFIIIA is in close proximity to the A-box-like sequence. (Braun et al., 1992a; also see section 1.4.2 and Fig. 1-3B of the INTRODUCTION to this thesis). During the assembly of a functional transcription complex on the 5S RNA gene it may, therefore, be necessary for TFIIIA not only to recruit TFIIIC but also to prevent the $\tau_A$ subcomplex of TFIIIC from gaining access to the A-box-like sequence (Braun et al., 1992a).

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1 See the Materials and Methods section of the main body of Chapter 3 for details on \textit{in vitro} synthesis of TFIIIA and the \textit{in vitro} transcription assay.
To test the possibility that the hydrophobic segment of the 81-amino acid domain of TFIIIA acts to prevent an inappropriate interaction of TFIIIC with the A-box-like sequence of the 5S RNA gene, I tested whether mutations in this sequence could suppress the transcriptional defect of TFIIIA(L354A/L356A/L357A/L358A). For this experiment, I used the previously described templates LS49/58 (-2) and LS52/58 (+2), which contain linker scanning mutations in the A-box-like sequence of the 5S RNA gene (Challice and Segall, 1989). Wild-type TFIIIA supports *in vitro* transcription of the wild-type 5S RNA gene and these two mutant templates, LS49/58 (-2) and LS52/58 (+2) (Challice and Segall, 1989; Fig. 3-6B-D, lane 2). TFIIIA(L354A/L356A/L357A/L358A), which did not support *in vitro* transcription of the wild-type 5S RNA gene (Fig. 3-4E, lane 9; Fig 3-6B, lane 3) and did not support cell viability (Fig. 3-4G), did support *in vitro* transcription from the mutant templates LS49/58 (-2) and LS52/58 (+2) (Fig. 3-6C and D, lane 3). Synthesis of 5S RNA from these templates, however, appeared to be less efficient in the presence of TFIIIA(L354A/L356A/L357A/L358A) than in the presence of wild-type TFIIIA (Fig 3-6C and D, compare lanes 2 and 3). Also the transcripts synthesized from the mutant templates in the presence of TFIIIA(L354A/L356A/L357A/L358A) appeared to be larger than those synthesized in the presence of wild-type TFIIIA.

As a control, I monitored transcription of a series of additional 5S RNA genes that contain linker scanning mutations throughout the coding region and the 5' flanking sequence of the 5S RNA gene (Challice and Segall, 1989). I found that none of these templates suppressed the defect of TFIIIA(L354A/L356A/L357A/L358A) (Fig. 3-6E and data not shown). For example, LS62/71, a version of the 5S RNA gene that contains a linker scanning mutation downstream of the A-box-like sequence, was, as expected, efficiently transcribed in the presence of wild-type TFIIIA (Challice and Segall, 1989; Fig. 3-6E, lane 2), but was not transcribed in the presence of TFIIIA(L354A/L356A/L357A/L358A) (Fig. 3-6E, lane 3).
Figure 3-6. Abilities of wild-type and mutant versions of TFIIIA to support in vitro transcription from 5S RNA gene templates that contain deletion-substitution mutations.

(A) Schematic representations of wild-type and mutant versions of the 5S RNA gene of *S. cerevisiae*. The wild-type (WT) 5S RNA gene of *S. cerevisiae* contains a 14-bp ICR between nucleotides +81 and +94 (Challice and Segall, 1989) that is similar in location and sequence to the C-box of the 50-bp ICR of the *Xenopus* 5S RNA gene (Pieler et al., 1987). The *S. cerevisiae* 5S RNA gene also contains a sequence between nucleotides +50 and +61 that is similar in location and sequence to the A-box of the ICR of the *Xenopus* 5S RNA gene. The conserved A-box sequence is not an essential promoter element for transcription in vitro of the yeast 5S RNA gene (Challice and Segall, 1989). LS49/58 (-2) and LS52/58 (+2) are mutant versions of the yeast 5S RNA gene that contain linker-scanning mutations (deletion-substitution mutations) in the A-box-like sequence of the yeast 5S RNA gene (Challice and Segall, 1989). LS62/71 is a mutant version of the yeast 5S RNA gene that contains a linker scanning mutation downstream of the A-box-like sequence (Challice and Segall, 1989). The linker scanning mutations are coded LSX/Y (N), with X and Y referring to the nucleotide positions that flank the mutation and N referring to the alteration in spacing by N nucleotides caused by an inexact deletion-substitution mutation.

(B, C, D, and E) Abilities of wild-type and mutant versions of TFIIIA to support in vitro transcription of the wild-type 5S RNA gene (B), LS49/58 (-2) (C), LS52/58 (+2) (D), or LS62/71 (E). In vitro transcription reaction mixtures contained the indicated 5S DNA template; partially purified yeast TFIIIC, TFIIIB, and RNA polymerase III; and the version of in vitro-synthesized TFIIIA indicated above the lane. RL, rabbit reticulocyte (in vitro transcription-translation reaction not programmed to synthesize TFIIIA); WT, wild-type TFIIIA; 81*, TFIIIA(L354A/L356A/L357A/L358A); zf8*9*, TFIIIA(H272R/C367Y). The RNAs synthesized in vitro were analyzed on a 7 M urea-10% polyacrylamide gel. The autoradiograms show the portion of the gels containing 5S RNA. The solid arrowhead to the left of each autoradiogram denotes the position of the major 5S RNA species synthesized in vitro in the presence of wild-type TFIIIA.
A

WT 5S RNA gene:

LS49/58 (-2):

or

LS52/58 (+2):

LS62/71:

B

WT 5S RNA gene

C

LS49/58 (-2)

D

LS52/58 (+2)

E

LS62/71
I also tested TFIIIA(H272R/C367Y), an inactive version of TFIIIA in which zinc fingers 8 and 9 were disrupted (see Fig. 3-5), for its ability to support transcription of LS49/58 (-2), LS52/58 (+2), and LS62/71. This version of TFIIIA remained inactive on these templates (Fig. 3-6C and D, lanes 4).

These data are consistent with the notion that the hydrophobic segment of the 81-amino acid domain of TFIIIA serves to exclude TFIIIC from the vicinity of the A-box-like sequence in the 5S RNA gene. Mutations in the A-box-like sequence, but not elsewhere in the 5S RNA gene, restored activity to TFIIIA(L354A/L356A/L357A/L358A) but not to TFIIIA(H272R/C367Y). In the absence of an A-box-like sequence that can provide a binding site for the $\tau_A$ subcomplex of TFIIIC, there is no need for TFIIIA to be able to prevent TFIIIC from gaining access to this site.

Milne and Segall (1993) proposed that TFIIIC is recruited to a yeast TFIIIA-5S DNA complex by an interaction of the amino-terminal zinc fingers of TFIIIA with the $\tau_B$ subcomplex of TFIIIC. The 81-amino acid domain would then interact with the $\tau_A$ subcomplex of TFIIIC and this second interaction would serve to dock TFIIIC on the TFIIIA-5S DNA complex with the appropriate topography so that TFIIIB could be properly positioned upstream of the gene (also see Discussion of Chapter 3). An additional, or alternative role, for the 81-amino acid domain could be to prevent the $\tau_A$ subcomplex of TFIIIC from gaining access to the A-box-like sequence of the 5S RNA gene (Braun et al., 1992a).

In this study, I have presented preliminary data that support the idea that the hydrophobic patch at the carboxyl-end of the 81-amino acid domain of TFIIIA serves to prevent TFIIIC from interacting inappropriately with the A-box-like sequence of the 5S RNA gene. I found that mutations in the A-box-like sequence suppressed the transcriptional defect of TFIIIA(L354A/L356A/L357A/L358A) (Fig. 3-6); that is, the leucine-rich segment of wild-type TFIIIA was no longer required if the 5S RNA gene template lacked the non-essential A-
box-like sequence. The observation that the resultant transcripts appeared larger than those produced in the presence of wild-type TFIIIA remains to be investigated; in particular, it will be interesting to determine the placement of TFIIIB on the mutant 5S RNA genes and the site at which transcription starts in the presence of TFIIIA(L354A/L356A/L357A/L358A).

I speculate that if TFIIIC, once recruited to a TFIIIA-5S DNA complex, was allowed to interact with the A-box-like sequence of the 5S RNA gene, the resultant transcription complex would be inactive. I hypothesize that in such a complex TFIIIC would be constrained in such a manner that it could not interact productively with TFIIIB. In support of this, I note that a polypeptide containing only the three amino-terminal zinc fingers of TFIIIA cannot support transcription; nonetheless, this mutant TFIIIA binds to the 5S RNA gene with high affinity and recruits TFIIIC (Milne and Segall, 1993; Chapter 2 of this thesis). This suggests that any potential interaction of TFIIIC with the A-box-like sequence of the 5S RNA gene, which would presumably be enhanced in such a complex, cannot serve to promote transcription of the 5S RNA gene. This model can be tested by probing for interactions of TFIIIC with the A-box-like region of the wild-type 5S RNA gene in TFIIIC-TFIIIA-5S DNA complexes formed with TFIIIA(L354A/L356A/L357A/L358A) and also with a polypeptide containing the three amino-terminal zinc fingers of yeast TFIIIA. This could be done by a DNA-protein photo-crosslinking analysis of the complexes (Braun et al., 1992a).

The three carboxyl-terminal zinc fingers of Xenopus TFIIIA interact with the A-box promoter element of the Xenopus 5S RNA gene (Clemens et al., 1992; Hayes and Clemens, 1992; Hayes and Tullius, 1992; Fairall and Rhodes, 1992; Hansen et al., 1993). This interaction would make an 81-amino acid-like domain unnecessary; indeed, this domain is unique to yeast TFIIIA. It is interesting to speculate that the 81-amino acid domain of yeast TFIIIA could be made dispensable by creating carboxyl-terminal zinc fingers that made sequence specific contacts with the 5S RNA gene in the region of nt +50 to +61.
It is likely that the carboxyl-terminal region of yeast TFIIIA has discrete functional regions. I found that TFIIIA containing a disruption of either zinc finger 8 or zinc finger 9 was active, but that TFIIIA containing disruptions in both finger 8 and finger 9 was transcriptionally inactive (Fig. 3-5). This defect could not be suppressed by mutation of the A-box-like sequence of the 5S RNA gene (Fig. 3-6). It is possible that zinc fingers 8 and 9 of yeast TFIIIA serve a redundant role in a non-specific interaction with DNA that might be required to keep this portion of TFIIIA in the vicinity of DNA in the TFIIIA-DNA complex. Alternatively, or perhaps additionally, these fingers could serve a role in positioning TFIIIC in the transcription complex. It will be informative to map the specific interactions between TFIIIA and TFIIIC.
CHAPTER 4

Dual Role of the Amino-Terminal Zinc Finger of Yeast TFIIIA in Interacting with the 5S RNA Gene and in Recruiting TFIIIC

John Hwang, a summer student in Dr. Segall’s lab, assisted me in making the plasmid constructs described in this chapter.
ABSTRACT

TFIIIA of *Saccharomyces cerevisiae* contains nine zinc fingers of the Cys$_2$/His$_2$ type. The amino-terminal three zinc fingers of *S. cerevisiae* TFIIIA suffice for binding to the internal control region of the 5S RNA gene and for recruiting TFIIIC to the gene (C. A. Milne and J. Segall, J. Biol. Chem. 268:11364-11371, 1993). I have analyzed the effect of mutating a zinc-coordinating histidine residue in each of zinc fingers 1, 2, 3, and 5 of TFIIIA; these mutations would be expected to disrupt the structure of the individual zinc finger. This chapter describes preliminary experiments testing the mutant proteins for their ability to bind the yeast 5S RNA gene, to recruit TFIIIC into the TFIIIA-DNA complex, to support *in vitro* transcription of the yeast 5S RNA gene, and to support cell viability. TFIIIA containing a disruption of the first zinc finger was compromised for DNA binding, appeared unable to recruit TFIIIC, did not support *in vitro* transcription of the 5S RNA gene, and did not maintain cell viability. TFIIIA proteins containing a disruption of the second or third zinc finger bound 5S DNA less efficiently than did the version of TFIIIA containing a disruption of the first zinc finger, but nonetheless they supported *in vitro* transcription of the 5S RNA gene and supported cell viability. Based on these data, I conclude that the first zinc finger of TFIIIA is the only finger that is essential for recruitment of TFIIIC to the 5S RNA gene.
INTRODUCTION

The zinc finger motif of the Cys$_2$/His$_2$-type, referred to as zinc finger from herein, has an amino acid consensus sequence of (Tyr/Phe)-X-Cys-X$_{2-4}$-Cys-X$_3$-Phe-X$_5$-Leu-X$_2$-His-X$_{3-4}$-His-X$_{2-5}$ (where X is any amino acid) (for reviews, see Berg, 1990 and Kaptein, 1991). This motif was first identified in *Xenopus* TFIIIA (Miller et al., 1985) and has since been identified in a large number of eukaryotic DNA-binding proteins (Jacobs, 1992). In addition to DNA recognition, zinc fingers have also been found to mediate RNA recognition (for examples, see Koster et al., 1991; McBryant et al., 1995; Caricasole et al., 1996; and Arranz et al., 1997) and protein-protein interactions (MacKay and Crossley, 1998). The zinc finger is an independent folding motif, consisting of a small antiparallel $\beta$-sheet packed against an $\alpha$-helix (Párraga et al., 1988; Lee et al., 1989). The two invariant cysteines, which are located in the $\beta$-loop, and the two invariant histidines, which are located in the carboxyl-terminal portion of the $\alpha$-helix, tetrahedrally coordinate a central zinc ion that serves to stabilize the structure of the zinc finger domain (Diakun et al., 1986; Frankel et al., 1987; Párraga et al., 1988). The three conserved hydrophobic residues are also likely to stabilize the zinc finger domain through interactions in a hydrophobic pocket. Crystallographic and NMR studies of zinc finger-DNA complexes have provided detailed insights into the molecular basis of zinc finger-DNA recognition (Pavletich and Pabo, 1991, 1993; Fairall et al., 1993; Houbaviy et al., 1996; Kim and Berg, 1996; Foster et al., 1997; Wuttke et al., 1997). In these structures, residues generally in the amino-terminal portion of the $\alpha$-helix are responsible for specific DNA recognition, largely through DNA major groove contacts. The molecular mechanisms by which zinc fingers interact with RNA or with protein have yet to be determined in detail.

I have analyzed the properties of mutant forms of yeast TFIIIA that contain a structural disruption in each of zinc fingers 1, 2, 3, and 5 of yeast TFIIIA. I found that the zinc finger
disruptions affect DNA binding to various degrees, but only a disruption of zinc finger 1 abolishes transcription factor activity. This is likely due to an essential role for zinc finger 1 in recruiting TFIIIC to the TFIIIA-DNA complex.

MATERIALS AND METHODS

Construction and in vitro expression of mutant versions of TFIIIA. A series of pXS-TFC2-derived plasmids (see Chapter 3) were constructed to code for versions of TFIIIA with the following mutations: H69N in zinc finger 1 [pXS-TFC2(H69N)]; H98N in zinc finger 2 [pXS-TFC2(H98N)]; H126N in zinc finger 3 [pXS-TFC2(H126N)]; and H181N in zinc finger 5 [pXS-TFC2(H181N)]. First, site-directed mutagenesis was performed following the recombinant PCR approach described in Chapter 3 with pJA454 as the initial template. The upstream and downstream primers used for the construction of pXS-TFC2(H69N), pXS-TFC2(H98N), and pXS-TFC2(H126N) annealed to the DNA sequence just upstream of the TFIIIA-coding sequence and to the DNA sequence at the end of the coding region for zinc finger 4 of TFIIIA, respectively. Second, the final PCR product in all three cases was digested with NcoI and HindIII, and the resulting ~510-bp restriction fragments were gel-purified and used to replace the corresponding NcoI-HindIII fragment of pXS-TFC2 (see Chapter 3). The upstream and downstream primers used for the construction of pXS-TFC2(H181N) annealed to the DNA sequence at the beginning of the coding region for zinc finger 4 and to the DNA sequence at the end of the coding region for zinc finger 8, respectively. The final PCR product was digested with HindIII and XbaI, and the resulting
~280-bp restriction fragment was gel-purified and used to replace the corresponding HindIII-XbaI fragment of pXS-TFC2. All PCR amplifications were performed by using the high-fidelity Vent DNA polymerase (New England Biolabs) as per the manufacturer's standard conditions. The sequence of all amplified DNA was verified by DNA sequencing.

The pXS-TFC2 series of plasmids was used for in vitro synthesis of TFIIIA as described in Chapter 3 with the exception that a Modulis™ transcription-translation system (MBI Fermentas) was used. A pET-11d-derived series of plasmids was constructed by cloning the NcoI-BamHI fragments from the pXS-TFC2-derived plasmids (see above) between the corresponding sites of pET-11d. These pET-11-d plasmids were used for bacterial expression of TFIIIA as described in Chapter 2. A pG3-derived series of plasmids was constructed as described in Chapter 3 for expression of mutant forms of TFIIIA in yeast.

Partial purification of yeast TFIIIA from bacteria. TFIIIA proteins, which were expressed from the pET-11d-derived plasmids in Escherichia coli strain BL21(DE3), were extracted from the bacterial cells and solubilized from inclusion bodies with 5M urea as described in Chapter 2. For the gel mobility shift and in vitro transcription assays described in this chapter, an aliquot of this solubilized protein was used following a 1:5 dilution into dilution buffer (20mM HEPES (pH 7.9), 10 mM MgCl2, 50 μM ZnSO4, 100 mM KCl, 20% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mg/ml bovine serum albumin).

Electrophoretic mobility shift assays and in vitro transcription assays. Electrophoretic mobility shift assays (EMSAs) were performed as described in Chapter 2 with the exception that 0.5 μg of pBluescript II SK(+) was included as a competitor DNA in the reactions. TFIIIA included in a 20-μl reaction mixture consisted of 2 μl of an in vitro transcription-translation reaction mixture that had been programmed to produce the indicated version of TFIIIA or 2 μl of diluted protein extract containing the indicated version of bacterially produced TFIIIA (see above). In vitro transcription assays were performed as described elsewhere (Taylor and Segall, 1985) with the yeast 5S RNA gene (p19-5S) as a
template. A 50-μl reaction mixture contained 2 μl of yeast-derived partially purified TFIIIC, 5 μl of yeast-derived partially purified TFIIIB, and 5 μl of yeast-derived partially purified RNA polymerase III, and, as indicated, 4.5 μl of an in vitro transcription-translation reaction mixture that had been programmed to produce the indicated version of TFIIIA or 4.5 μl of diluted protein extract containing the indicated version of bacterially produced TFIIIA (see above). The partially purified TFIIIC- and pol III-containing fractions derived from yeast were prepared as described for fractions j and k, respectively, in Taylor and Segall (1985). The partially purified TFIIIB-containing fraction derived from yeast was prepared as described for fraction i in Taylor and Segall (1985), except that it was further purified by chromatography on hydroxyapatite (Brockhouse, 1994).

**In vivo analysis of the mutant versions of TFIIIA.** The yeast strain YRW1 (MATα can1-100 his3-11 leu2-3,112 trpl-1 ura3-1 ade2-1 tfe2::LEU2, harboring pJA230) was described previously in Chapter 3. The conditions used for growth and transformation of yeast cells, and for Western blot analysis of yeast lysates are described in Chapter 3.

**RESULTS**

It was previously shown that a polypeptide containing zinc fingers 1 through 3 of yeast TFIIIA is able to bind specifically to the yeast 5S RNA gene and that the TFIIIA-DNA complex formed with this polypeptide is able to recruit TFIIIC to the gene (Milne and Segall, 1993). As a first step in determining the relative contribution of each of these fingers to DNA-binding and to recruitment of TFIIIC, I expressed mutant forms of full-length TFIIIA that contained a
Figure 4-1. Mutant versions of yeast TFIIIA that contain structural disruptions of zinc fingers 1, 2, 3, and 5.

(A) The consensus amino acid sequence of the Cys2/His2-type of zinc finger is given in single letter code, with the zinc-coordinating cysteines and histidines in boldface (an X represents any amino acid). The histidine residue that serves as the third zinc-liganding residue is underlined. Asparagine was substituted for this residue to disrupt individual zinc fingers.

(B) Schematic representations of wild-type and mutant versions of yeast TFIIIA. The name of the version of TFIIIA depicted in each schematic is given on the left. The numbered boxes represent the nine zinc fingers of yeast TFIIIA, the stippled region between zinc fingers 8 and 9 represents the 81-amino acid domain, and the diagonally striped boxes represent the 48-amino acid amino-terminal region and the 35-amino acid carboxyl-terminal region. A crossed-out box denotes a disrupted zinc finger; the approximate position of the histidine-to-asparagine mutation responsible for the disruption is given below the finger.
A

\[(Y/F)-X-C-X_{2-4}C-X_{3-5}F-X_{5-6}L-X_{2-3}H-X_{3-4}H-X_{2-8}\]

\[\downarrow\]

N

B

TFIIIA (wild-type) N

\[
\begin{array}{cccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 81 \text{ aa} & 9 & C
\end{array}
\]

TFIIIA (H69N) N

\[
\begin{array}{cccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 81 \text{ aa} & 9 & C
\end{array}
\]

\[\downarrow\]

H69N

TFIIIA (H98N) N

\[
\begin{array}{cccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 81 \text{ aa} & 9 & C
\end{array}
\]

\[\downarrow\]

H98N

TFIIIA (H126N) N

\[
\begin{array}{cccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 81 \text{ aa} & 9 & C
\end{array}
\]

\[\downarrow\]

H126N

TFIIIA (H181N) N

\[
\begin{array}{cccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 81 \text{ aa} & 9 & C
\end{array}
\]

\[\downarrow\]

H181N
histidine-to-asparagine substitution at the first of the two conserved histidine residues of the zinc finger (see Fig. 4-1A). This mutation is expected to disrupt the structure, and presumably the function, of the zinc finger without significantly altering the function of the other zinc fingers of TFIIIA (see Discussion). This is the same mutational approach that was used by Setzer and colleagues in their analysis of the function of individual zinc fingers of *Xenopus* TFIIIA (Del Rio *et al.*, 1993). Studies with these "broken finger" mutants of *Xenopus* TFIIIA were useful in determining the relative contribution of individual zinc fingers of *Xenopus* TFIIIA to 5S DNA-binding and to assembly of a functional transcription complex on the amphibian 5S RNA gene (Del Rio *et al.*, 1993; Del Rio and Setzer, 1993; Rollins *et al.*, 1993).

I expressed versions of TFIIIA containing a histidine-to-asparagine substitution in zinc finger one [TFIIIA(H69N)], in zinc finger two [TFIIIA(H98N)], and in zinc finger three [TFIIIA(H126N)]. For comparison, I also expressed a version of TFIIIA with a histidine-to-asparagine substitution in zinc finger five [TFIIIA(H181N)]. Although zinc finger five is in contact with DNA in the TFIIIA-DNA complex (see Chapter 2), it is not essential for DNA-binding or for recruitment of TFIIIC to a TFIIIA-DNA complex (Milne and Segall, 1993).

I first analyzed mutant forms of TFIIIA that had been synthesized *in vitro* (see Materials and Methods). I previously found that this approach provides a reliable and rapid way of obtaining equivalent amounts of protein for analysis (see Chapter 3). I confirmed here that approximately equivalent amounts of TFIIIA proteins of the appropriate sizes were produced, as monitored by SDS-polyacrylamide gel electrophoretic analysis of proteins synthesized in the presence of $[^{35}S]$methionine (Fig. 4-2A). The ability of the mutant proteins to bind the 5S RNA gene and to recruit TFIIIC was assessed by a gel mobility shift assay. Disruption of zinc finger one led to a large reduction in the ability of TFIIIA to bind the 5S RNA gene relative to the level of DNA-binding obtained with wild-type TFIIIA; nonetheless a low level of TFIIIA(H69N)-5S DNA complexes were detected (Fig. 4-2B, compare lanes 5 and 7). This is similar to the result that was obtained on analysis of an *in vitro*-synthesized version of TFIIIA.
Figure 4-2. Characterization of wild-type and mutant versions of TFIIIA synthesized *in vitro*.

(A) SDS-polyacrylamide gel analysis. Wild-type and mutant forms of TFIIIA synthesized *in vitro* in the presence of [35S]methionine were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. An autoradiogram of the gel is shown. The names above each lane refer to the translation products of *in vitro* transcription-translation reactions programmed to synthesize the indicated version of TFIIIA; RL, reticulocyte lysate (*in vitro* transcription-translation reaction not programmed to synthesize any protein); WT, wild-type TFIIIA; mutant versions of TFIIIA are designated by the amino acid position of the histidine-to-asparagine substitution. The arrow on the left denotes the position of the TFIIIA proteins. The positions and sizes (in kilodaltons) of molecular mass markers are shown on the right.

(B) Abilities of mutant versions of TFIIIA to bind to the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex, as assessed by EMSA. A radioactively labeled DNA fragment containing the yeast 5S RNA gene was incubated with *in vitro*-synthesized versions of TFIIIA in the absence (odd-numbered lanes) or presence (even-numbered lanes) of partially purified yeast TFIIIC prior to electrophoresis on a nondenaturing polyacrylamide gel. The names above pairs of lanes refer to the versions of TFIIIA (nomenclature as in panel A) that were present in the gel shift reaction mixtures; the reaction mixtures of lanes 1 and 2 contained no transcription-translation mixture. The positions of free DNA (minus sign), TFIIIA-DNA complexes (open arrowhead), and TFIIIC-TFIIIA-DNA complexes (solid arrowhead) are indicated on the right.

(C) Abilities of mutant versions of TFIIIA to support *in vitro* transcription of the 5S RNA gene. *In vitro* transcription reaction mixtures contained the yeast 5S RNA gene, partially purified yeast TFIIIC, TFIIIB, and RNA polymerase III, and the version of *in vitro*-synthesized TFIIIA indicated above the lane (nomenclature as in panel A). The RNAs synthesized *in vitro* were analyzed on a 7 M urea-10% polyacrylamide gel. The autoradiogram shows the portion of the gel containing 5S RNA.
that lacked the amino-terminal region and the first zinc finger (Milne and Segall, 1993). DNA-binding could not be detected with TFIIIA containing a disruption of zinc finger two [TFIIIA(H98N)] or a disruption of zinc finger three [TFIIIA(H126N)] (Fig. 4-2B, lanes 9 and 11). TFIIIA with a disruption of zinc finger five [TFIIIA(H181N)] had reduced, but clearly detectable DNA-binding activity (Fig. 4-2B, lane 13). This was the only mutant TFIIIA for which recruitment of TFIIIC to the TFIIIA-DNA complex could be detected (Fig. 4-2B, lane 14). My ability to assess incorporation of TFIIIC into mutant TFIIIA-DNA complexes was limited by the fact that formation of TFIIIA-DNA complexes was low or non-detectable. I next tested the in vitro-synthesized mutant TFIIIA for their ability to support in vitro transcription of the 5S RNA gene (Fig. 4-2C). TFIIIA(H181N) was the only mutant TFIIIA that supported a level of 5S RNA synthesis that was clearly above the background level (Fig. 4-2C, lanes 3 to 6).

I also analyzed wild-type TFIIIA, TFIIIA(H69N), TFIIIA(H98N), and TFIIIA(H126N) proteins that had been expressed in bacteria (see Chapter 2). For this study, proteins were solubilized from inclusion bodies by using a buffer containing 5M urea; this protein solution was diluted 1:5 into buffer containing no urea just prior to use (see Materials and Methods). Aliquots of this protein mixture were then used in a standard gel shift assay and in vitro transcription assay. The use of protein produced in bacteria had the advantage that more protein could be obtained than was the case with the use of in vitro-synthesized protein. For example, in a reaction containing bacterially produced wild-type TFIIIA, all the probe was present in a complex with TFIIIA indicating that TFIIIA could be added in excess over the labeled 5S DNA (Fig. 4-3A, lane 3). I found, however, that there was greater variability in the activity of TFIIIA that was partially purified from bacteria than in TFIIIA that was synthesized in vitro.

Interaction of TFIIIA(H69N) with the 5S RNA gene was more readily visualized with bacterially produced protein than with in vitro synthesized protein (compare Fig. 4-2B, lane 7
Figure 4-3. Characterization of wild-type and mutant versions of TFIIIA produced from bacteria.

(A) Abilities of mutant versions of TFIIIA to bind to the 5S RNA gene and to recruit TFIIIC to the TFIIIA-DNA complex, as assessed by EMSA. A radioactively labeled DNA fragment containing the yeast 5S RNA gene was incubated with protein extract containing the indicated version of bacterially produced TFIIIA (see Materials and Methods) in the absence (odd-numbered lanes) or presence (even-numbered lanes) of partially purified yeast TFIIIC prior to electrophoresis on a nondenaturing polyacrylamide gel. The names above pairs of lanes refer to the versions of TFIIIA that were present in the gel shift reaction mixtures; the reaction mixtures of lanes 1 and 2 contained no added TFIIIA protein extract; WT, wild-type TFIIIA; mutant versions of TFIIIA are designated by the amino acid position of the histidine-to-asparagine substitution. The positions of free DNA (minus sign), TFIIIA-DNA complexes (open arrowhead), and TFIIIC-TFIIIA-DNA complexes (solid arrowhead) are indicated on the right.

(B) Abilities of mutant versions of TFIIIA to support in vitro transcription of the 5S RNA gene. In vitro transcription reaction mixtures contained the yeast 5S RNA gene, partially purified yeast TFIIIC, TFIIIB, and RNA polymerase III; and protein extract containing the version of bacterially produced TFIIIA (see Materials and Methods) indicated above the lane (nomenclature as in panel A). The RNAs synthesized in vitro were analyzed on a 7 M urea-10% polyacrylamide gel. The autoradiogram shows the portion of the gel containing 5S RNA.
and Fig. 4-3A, lane 5). Moreover, TFIII A-5S DNA complexes were detected with bacterially-produced TFIII A(H98N) and TFIII A(H126N) (Fig. 4-3A, lanes 7 and 9); the amount of bound DNA was however much less than with wild-type TFIII A (Fig. 4-3A, lane 3). Given the extent of TFIII A-5S DNA complex formation with TFIII A(H69N), I anticipated that if this mutant form of TFIIIA could recruit TFIIIC, I would readily visualize a TFIIIC-TFIIIA-DNA complex. I did not detect, however, such a complex (Fig. 4-3A, lane 6); similarly I did not detect incorporation of TFIIIC into TFIII A-DNA complexes containing TFIII A(H98N) or TFIII A(H126N) (Fig. 4-3A, lanes 8 and 10). Nonetheless, these latter two forms of TFIII A supported in vitro transcription of the 5S RNA gene (Fig. 4-3B, lanes 4 and 5). In contrast, bacterially produced TFIII A(H69N) did not support in vitro transcription of the 5S RNA gene (Fig. 4-3B, lane 3). My inability to readily detect in vitro transcription of the 5S RNA gene in reactions containing in vitro-synthesized versions of TFIII A(H98N) and TFIII A(H126N) (Fig. 4-2C) was most likely because of the limited amount of TFIII A in the reactions containing in vitro-synthesized protein relative to reactions containing bacterially produced protein.

I also assessed the ability of these mutant forms of TFIIIA to function in vivo, using a plasmid-shuffling protocol to replace the wild-type version of the gene encoding TFIIIA with a mutated version of the gene (see Chapter 3). In this protocol, growth of cells on medium containing 5-FOA indicates that the mutant TFIIIA is active in directing transcription of the 5S RNA gene in vivo. This is a very sensitive assay for TFIIIA function; I found previously that mutant versions of TFIIIA for which I could not detect activity in an in vitro transcription assay could support cell viability (see Chapter 3). I found that TFIIIA with a disruption of zinc finger one [(TFIIIA(H69N)] did not support cell viability (Fig. 4-4A), whereas TFIIIA with a disruption of zinc finger two [TFIIIA(H98N)], three [TFIIIA(H126N)], or five [TFIIIA(H181N)] supported cell viability (Fig. 4-4A). Western blot analysis confirmed that all mutant versions of TFIIIA were stable in vivo (Fig. 4-4B, lanes 3-6).
Figure 4-4. Assessment of the in vivo activity of mutant versions of TFIIIA.

(A) Abilities of mutant versions of TFIIIA to support cell viability. A plasmid shuffle system was used to test the abilities of mutant versions of TFIIIA to replace wild-type TFIIIA in vivo. pG3-derived plasmids that expressed mutant versions of TFIIIA were transformed into the yeast strain YRW1 (see Materials and Methods), and the abilities of these cells to grow on medium containing 5-FOA were monitored. Growth on this medium indicated that the pG3-derived plasmid present in the cells expressed a functional form of TFIIIA (see Chapter 3 for details). vector, pG3 not expressing TFIIIA; WT, pG3 expressing wild-type TFIIIA; mutant versions of TFIIIA are designated by the amino acid position of the histidine-to-asparagine substitution.

(B) Assessment by Western blot analysis of in vivo expression of mutant versions of TFIIIA. Protein extracted from YRW1 yeast cells containing pG3-derived plasmids was separated on an SDS-12% polyacrylamide gel and electrotransferred to a nitrocellulose filter, and the filter was probed with anti-TFIIIA antibody. The names above each lane refer to the version of TFIIIA expressed from the pG3-derived plasmid (nomenclature as described above in panel A). The arrow on the left denotes the position of the TFIIIA proteins. The asterisk on the left indicates a cross-reacting molecule not related to TFIIIA. The positions and sizes (in kilodaltons) of molecular mass markers are shown on the right.
In summary, my data from studies performed in vitro and in vivo indicate that the first zinc finger of yeast TFIIIA is essential for its activity. Although TFIIIA(H69N) appeared to bind the 5S RNA gene more efficiently than did TFIIIA(H98N) and TFIIIA(H126N), TFIIIA(H69N) did not support cell viability whereas TFIIIA(H98N) and TFIIIA(H126N) did. This indicates that TFIIIA(H98N) and TFIIIA(H126N) can recruit TFIIIC to the 5S RNA gene and promote formation of an active transcription complex. I presume that my inability to detect TFIIIA-TFIIIC-5S DNA complexes in vitro in the presence of TFIIIA(H98N) and TFIIIA(H126N) reflected the low level of TFIIIA-5S DNA complexes formed with these TFIIIA. This suggests that the formation of a TFIIIA-5S DNA complex is not the limiting step in assembly of an active transcription complex either in vitro or in vivo. From the results of this study and those described in Chapter 2, I conclude that the first zinc finger of yeast TFIIIA is involved in both protein-DNA and protein-protein interactions. This finger appears to serve an essential role in recruitment of TFIIIC to the TFIIIA-5S DNA complex.

**DISCUSSION**

TFIIIA promotes accurate transcription of the 5S RNA gene by binding to the ICR of the gene and recruiting TFIIIC. In this study, I have tested the effect of substituting asparagine for the third zinc-coordinating residue of the Cys2/His2 motif in zinc finger 1, 2, 3, and 5. A zinc finger containing such a histidine-to-asparagine mutation is expected to be unable to coordinate zinc and therefore unable to fold properly (Diakun et al., 1986; Frankel et al., 1987; Párraga et al., 1988) and unable to participate in DNA-binding (Hanas et al., 1983b; Challice, 1989). Analysis of mutant versions of Xenopus TFIIIA containing the same histidine-to-asparagine
substitution in each of its nine zinc fingers indicated that each mutant finger was unstructured and indicated that disruption of one zinc finger did not affect the function of neighboring zinc fingers (Del Rio et al., 1993). The recent NMR-derived structure of a polypeptide containing the first three zinc fingers of Xenopus TFIIIA bound to its cognate DNA site indicates, however, that substantial packing interactions do occur between these zinc fingers when bound to DNA (Foster et al., 1997; Wuttke et al., 1997). Thus, the disruption of a zinc finger may influence, to a certain degree, the properties of neighboring zinc fingers. Nonetheless, my studies allow preliminary conclusions to be made on the function of individual zinc fingers of yeast TFIIIA.

I found that the first zinc finger of yeast TFIIIA was essential for transcription of the 5S RNA gene in vitro and in vivo, whereas zinc fingers 2, 3, and 5 were dispensable (Figs. 4-3 and 4-4). My data imply that the transcriptional defect of TFIIIA(H69N) resulted from a defect in recruitment of TFIIIC to the TFIIIA-5S DNA complex rather than from its reduced affinity for DNA. This mutant TFIIIA appeared to bind the 5S RNA gene with higher affinity than did TFIIIA(H98N) and TFIIIA(H126N). These latter two mutant forms of TFIIIA directed transcription of the 5S RNA gene despite their reduced affinity for DNA (Figs. 4-3, and 4-4). The extent of TFIIIA-5S DNA complex formation with TFIIIA(H69N) was sufficient to expect that, if TFIIIC could be recruited with normal efficiency, formation of a TFIIIC-TFIIIA-5S DNA complex would be visualized (Fig. 4-3A).

The comparisons presented here are qualitative in nature and confirmation of my conclusion that zinc finger one of yeast TFIIIA is involved in both protein-DNA and protein-protein

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1 TFIIIC incorporation into TFIIIA(H69N)-5S DNA complexes remained undetectable in preliminary in vitro experiments in which I was able to detect a very low level of TFIIIC-TFIIIA(H98N)-5S DNA and TFIIIC-TFIIIA(H126N)-5S DNA complex formation.
interactions requires that a quantitative analysis of DNA-binding be performed and that more sensitive conditions be established to monitor the interaction of TFIIIC with TFIIIA. The current use of a gel-shift assay to detect the TFIIIC-TFIIIA interaction is limited by the requirement for prior formation of the TFIIIA-5S DNA complex. Experimental approaches designed to assess TFIIIC-TFIIIA interactions in the absence of DNA-bound TFIIIA have thus far been unsatisfactory (O. Rowland, data not shown). It is possible that TFIIIC-TFIIIA complexes formed in the absence of 5S DNA are relatively unstable; incorporation of TFIIIC into a wild-type TFIIIA-DNA prevents chemical modification of G residues that are modified in the TFIIIA-DNA complex (Challice and Segall, 1989) and extends the DNase I footprint on the 5S RNA gene by about 20 nucleotides in the 3' direction (Braun et al., 1989). DNA-protein photo-crosslinking experiments indicate that this extended footprint reflects interactions of TFIIIC subunits with 5S DNA (Braun et al., 1992a). Also, truncation of the 5S RNA gene at nucleotide +110 compromises TFIIIC binding (Braun et al., 1992b). Thus, interactions of TFIIIC with DNA in the TFIIIC-TFIIIA-5S DNA complex may stabilize this complex.

Although my data indicate that neither zinc finger two nor zinc finger three of yeast TFIIIA is essential for its activity, these fingers appear to contribute to DNA-binding and it is certainly possible that these fingers also contribute to incorporation of TFIIIC into the TFIIIA-DNA complex. Because TFIIIA(H98N) and TFIIIA(H126N) supported in vitro transcription of the 5S RNA gene more efficiently that would be predicted based on their affinity for DNA relative to wild-type TFIIIA, I infer that formation of TFIIIA-DNA complexes is not a limiting step. This is consistent with results of transcription studies using Xenopus TFIIIA proteins that are compromised in 5S DNA binding; it was found that 5S RNA synthesis activity is independent of the equilibrium binding constant governing formation of TFIIIA-DNA binary complexes (Del Rio et al., 1993; Del Rio and Setzer, 1993; Rollins et al., 1993). These authors argued that later "irreversible" steps in transcription complex assembly deplete the pool of TFIIIA-DNA complexes, and that transcription activity reflects a stoichiometric measure of the TFIIIA concentration and not its affinity for the 5S RNA gene in a binary complex.
In contrast to the essential nature of the first zinc finger of yeast TFIIIA, disruption of the first zinc finger of *Xenopus* TFIIIA, which reduces its DNA-binding affinity by 2.5 fold, has little effect on its ability to promote transcription of the 5S RNA gene *in vitro* or *in vivo* (Del Rio *et al.*, 1993; Del Rio and Setzer, 1993; Rollins *et al.*, 1993). This suggests that the first zinc finger of *Xenopus* TFIIIA serves only in DNA-binding and that a different finger or region of the protein may be the major determinant for recruitment of TFIIIC. The carboxyl-terminal zinc fingers of *Xenopus* TFIIIA might serve this function; disruption of any one of zinc fingers 7, 8, or 9 of *Xenopus* TFIIIA has no significant effect on its DNA-binding affinity but results in moderate to complete loss of transcription of the 5S RNA gene (Del Rio *et al.*, 1993; Del Rio and Setzer, 1993). The 14-amino acid region carboxyl terminal to zinc finger 9 of *Xenopus* TFIIIA, which is essential for its transcription factor activity (Smith *et al.*, 1984; Vrana *et al.*, 1988; Mao and Darby, 1993), is another candidate region for promoting an interaction of *Xenopus* TFIIIA with TFIIIC (Hayes *et al.*, 1989). This sequence is not present in yeast TFIIIA; yeast TFIIIA, however, contains a leucine-rich region within its unique 81-amino acid sequence that serves an essential role in the formation of an active transcription complex on the yeast 5S RNA gene (see Chapter 3). Thus, the extensive differences in the primary amino acid sequences of *Xenopus* and yeast TFIIIA (see Introduction) may reflect fundamental differences in the mechanism of transcription complex formation on the 5S RNA genes of these two organisms.

In general, the Cys2/His2-type of zinc finger has been considered to be a DNA-binding motif. Recent studies, however, implicate this type of zinc finger as serving in protein-protein interactions (reviewed in MacKay and Crossley, 1998). For example, the four zinc finger region of Ying-Yang 1 (YY1) mediates its interaction with the basic-leucine zipper region of ATFα2 (Zhou *et al.*, 1995), with YAF2 (Kalenik *et al.*, 1997), and with a region of Sp1 that includes its three Cys2/His2-type zinc fingers (Lee *et al.*, 1993b); the zinc fingers of Sp1 also interact with the RelA subunit of NF-κB (Perkins *et al.*, 1994) and with the DNA-binding domain of GATA-1 (Merika and Orkin, 1995); the zinc finger domain of ZNF74 is responsible
for its interaction with the hyperphosphorylated form of the largest subunit of RNA polymerase II (Grondin et al., 1997); and Cys2/His2-type zinc fingers can be responsible for homodimer formation, as is the case for the Ikaros and serendipity δ proteins (Sun et al., 1996; Payre et al., 1997). Although most of these zinc finger-mediated protein-protein interactions have been studied in the absence of DNA, several zinc fingers are known to bind DNA and protein simultaneously (Perkins et al., 1994; Merika and Orkin, 1995; Zhou et al., 1995). Although arrays of zinc fingers are usually required for high affinity and sequence-specific binding to DNA, a single zinc finger is often sufficient for a protein-protein interaction (Zhou et al., 1995; Payre et al., 1997; MacKay and Crossley, 1998).

Further studies will be aimed at identifying those residues in the first zinc finger of yeast TFIIIA that are involved in interaction with TFIIIC; at identifying the subunit of TFIIIC that interacts with the first zinc finger; and at assessing the contribution of DNA in the binding of TFIIIC to the TFIIIA-5S DNA complex.
CHAPTER 5

Summary and Future Directions
SUMMARY

I have undertaken a systematic mutational analysis of TFIIIA from *Saccharomyces cerevisiae* to gain insights into the mechanisms by which this protein leads to the formation of a functional transcription complex on the yeast 5S RNA gene. I analyzed the effects of deletions and amino acid substitutions in various regions of TFIIIA on the ability of this factor to bind the yeast 5S RNA gene, to recruit TFIIIC to the TFIIIA-5S DNA complex, and to promote both *in vitro* and *in vivo* transcription of the yeast 5S RNA gene. The experiments presented in Chapter 2 of this thesis provided insights into the interaction of the repeating zinc fingers of yeast TFIIIA with the 5S RNA gene. My data revealed that zinc fingers 1 to 5 of yeast TFIIIA formed a tight complex with up to 35 bp of DNA and that zinc fingers 6 to 9 of yeast TFIIIA were not in tight association with DNA in the TFIIIA-5S DNA complex. I showed that zinc fingers 1 to 3 of yeast TFIIIA spanned most of the internal control region of the 5S RNA gene and made multiple DNA major groove contacts to form a high-affinity complex with DNA. I also showed that zinc finger 5 made contacts in the major groove of DNA about 8 bp upstream of the ICR and that zinc finger 4 may approach DNA in an atypical manner to form an extended interaction with DNA. In Chapter 4, I presented evidence that each of zinc fingers 1, 2, and 3 made a major contribution to the interaction of yeast TFIIIA with the 5S RNA gene, but that the first zinc finger was the only zinc finger that was essential for the recruitment of TFIIIC to the TFIIIA-5S DNA complex. As described in Chapter 3, I performed an extensive mutational analysis of the 81-amino acid domain that is present between zinc fingers 8 and 9 of yeast TFIIIA. I found that a short leucine-rich segment (352NGLNLLL359) at the carboxyl-end of the 81-amino acid sequence was essential for the ability of TFIIIA to promote transcription of the 5S RNA gene. My mutational analysis indicated that the hydrophobic residues of this segment were critical for
transcription activity. I found that zinc fingers 8 and 9, which flank the 81-amino acid domain, were essential for the transcription activity of yeast TFIIIA. Either of these zinc fingers sufficed to maintain the transcription activity of TFIIIA. I also presented preliminary data in an Appendix to Chapter 3 supporting a role for the essential leucine-rich segment of the 81-amino acid domain of TFIIIA in preventing TFIIIC from making inappropriate interactions with the A box of the 5S RNA gene.

The experiments described in this thesis provide insights into the role of yeast TFIIIA in assembling an active transcription complex on the 5S RNA gene and set the stage for further structural and functional analysis of yeast TFIIIA. I present below some future directions that could continue from my thesis research.

**FUTURE DIRECTIONS**

**Interaction of yeast TFIIIA with the 5S RNA gene**

Detailed insights into the interaction of yeast TFIIIA with the 5S RNA gene likely awaits the X-ray crystal structure of the protein-DNA complex. In pursuing such an analysis, I would initially attempt to obtain the structure of the amino-terminal five zinc fingers of TFIIIA (TF5) bound to DNA. Since zinc fingers 6 to 9 of yeast TFIIIA do not appear to be in tight association with DNA (see Chapter 2), these fingers may generate disorder in the complex and be refractory to crystal formation. I would first define the minimal DNA-binding site to which the TF5 polypeptide binds with high affinity. A series of duplex oligonucleotides spanning the DNA contact site, predicted from my DNase I and methylation protection footprinting data (Chapter 2), would be synthesized and incubated with the TF5
polypeptide. The affinity of the interactions relative to that of TF5 bound to the full length 5S RNA gene would then be measured. The minimal DNA site that binds TF5 with high affinity would then be used for cocrystallization trials. If useful crystals could not be obtained, the structure of the TF3-DNA complex could be achieved through NMR analysis. The results of these structural studies would give insights into the mechanism by which yeast TFIIIA recognizes the 5S RNA gene and further our understanding in general of how arrays of zinc fingers interact with DNA. The NMR structure of the first three zinc fingers of Xenopus TFIIIA bound to its cognate DNA sequence and the crystal structure of the first six zinc fingers of Xenopus TFIIIA bound to DNA have been recently reported (Foster et al., 1997; Wuttke et al., 1997; Nolte et al., 1998). It would be of interest to compare the zinc finger-DNA interactions made in the yeast and Xenopus TFIIIA-DNA complexes to see how the differences in the primary structures of the Xenopus and yeast TFIIIA proteins are reflected in the protein-DNA interactions of these two proteins. In particular, it would be interesting to see if zinc finger 4 of yeast TFIIIA spans the minor groove of DNA in the same manner as zinc finger 4 of Xenopus TFIIIA does.

In the absence of an X-ray crystal or NMR structure, alanine-scanning mutagenesis of the amino-terminal three zinc fingers of yeast TFIIIA might identify amino acids important for high affinity DNA binding. This approach was used to identify amino acids in the two zinc fingers of the transcription factor ADR1 that are important for DNA binding (Thukral et al., 1991). The mutant TFIIIA proteins would be tested for reduced DNA-binding affinity by quantitative gel mobility shift assays (see Chapter 2) and/or nitrocellulose filter binding assays (Romaniuk, 1990). I would focus on the amino-terminal three zinc fingers of yeast TFIIIA for mutagenesis, as these zinc fingers appeared most critical for high affinity binding (Chapter 2). Furthermore, I would focus on the amino acids of the α-helix of each finger because these residues are generally involved in DNA contacts by zinc fingers (Pavletich and Pabo, 1991, 1993; Fairall et al., 1993; Houbaviy et al., 1996; Kim and Berg, 1996; Wuttke et al., 1997). This mutational analysis could also be complemented by examining the effect of
sequence-specific changes within the ICR of the yeast 5S RNA gene on the DNA-binding affinity of the wild-type and mutant TFIIIA proteins. To deduce specific amino acid-bp contacts, a "loss-of-contact" assay would be employed (reviewed in Ebright, 1991). In this assay, a mutant protein that has a reduced affinity for the wild-type DNA sequence is tested for binding to a DNA sequence containing a mutation at a suspected DNA contact site. The wild-type protein will have reduced affinity for this site relative to the wild-type DNA site. In contrast, a protein that is mutated for an amino acid contacting this residue will have a similar affinity for the wild-type DNA sequence and the mutated DNA sequence. This experimental design has been used to identify amino acid-bp contacts in a number of protein-DNA complexes, including the Lac repressor-DNA complex (Ebright, 1985, 1986) and the CAP-DNA complex (Zhang and Ebright, 1990). Additionally, chemical modification of a contacted nucleotide should reduce the affinity of wild-type protein, but not the mutant protein (for examples, see Hochschild and Ptashne, 1986 and Brunelle and Schleif, 1989).

Recruitment of TFIIIC to the 5S RNA gene by TFIIIA

I would also use alanine-scanning mutagenesis to identify amino acids of the first zinc finger of TFIIIA that are involved in interactions with TFIIIC. The amino acid sequence of zinc finger 1 of yeast TFIIIA is depicted below:

\[
49^{\text{YFCDYGCDKAFTRPSILTENQSLVHQLRA}}79
\]

This zinc finger corresponds to amino acid residues 49 to 79 of yeast TFIIIA. The conserved cysteines and histidines are in boldface. The residues that are underlined once are predicted to be part of the anti-parallel \(\beta\)-sheet of the zinc finger and the residues that are underlined twice are predicted to be in the \(\alpha\)-helix of the zinc finger. I would initially mutagenize amino acids of the zinc finger that are expected to face away from DNA as predicted from the structures of other zinc finger modules bound to DNA (Pavletich and Pabo, 1991, 1993;
Fairall et al., 1993; Houbaviy et al., 1996; Kim and Berg, 1996; Wuttke et al., 1997). This means the focus would be on the amino acids of the β-sheet and the β-turn connecting the β-strands. Residues of the carboxyl-terminal portion of the α-helix and the zinc finger linker may also participate in protein-protein interactions and mutational analysis of these residues would be performed in turn. The mutant TFIIIA proteins will be synthesized in vitro and/or purified from bacteria and tested for their abilities to bind the 5S RNA gene and to recruit TFIIIC by using gel shift assays. Their abilities to support transcription of the 5S RNA gene will also be tested. It will be important to verify that DNA-binding is not affected by careful measurements of the affinity (K_d) of the protein-DNA interaction; this would be a test for the structural integrity of the zinc finger domain.

Such a mutational analysis of the first zinc finger of TFIIIA has already been initiated in Dr. Segall's lab by Karen Rothfels, a former summer student and now a graduate student in the lab. She has identified residues of the first zinc finger that when mutated to alanine eliminate the ability of TFIIIA to recruit TFIIIC to 5S RNA gene and to support in vitro transcription of the 5S RNA gene, but do not appear to affect the ability of TFIIIA to bind the 5S RNA gene. These are residues F50 and Y53, which are located in the β-sheet region of the zinc finger. These mutational studies, which are ongoing, will provide insights into the mechanism by which a zinc finger can participate simultaneously in protein-DNA interactions and protein-protein interactions.

A future priority will be to identify the subunit of TFIIIC that interacts with the first zinc finger of yeast TFIIIA. The genes for most of the subunits of TFIIIC have been cloned (Swanson et al., 1991; Parsons and Weil, 1992; Lefebvre et al., 1992; Marck et al., 1993; Arrebola et al., 1998). Interactions could be tested pairwise between the amino-terminal zinc finger(s) of TFIIIA and TFIIIC subunits by affinity chromatography or by yeast two hybrid analysis. It is possible, however, that individual subunits of TFIIIC do not bind TFIIIA; that is, the protein-protein interactions may occur only in the context of the entire TFIIIC.
complex. Indeed, the 95-kDa and 138-kDa subunits of TFIIIC, which interact with the promoter elements of tRNA genes when part of the TFIIIC complex, are not able to individually bind to DNA, at least when the proteins are bacterially produced or synthesized \textit{in vitro} (Swanson \textit{et al}., 1991; Parsons and Weil, 1992; Lefebvre \textit{et al}., 1992). Also, it is likely that DNA contributes significantly to the interaction of TFIIIC with the TFIIIA-DNA complex (Braun \textit{et al}., 1989, 1992a, 1992b). Therefore, a better approach may be one that tests for incorporation of TFIIIC into a TFIIIA-DNA complex. A site-specific protein-protein photocrosslinking analysis of the TFIIIC-TFIIIA-DNA complex would be one such approach. A polypeptide comprised of the amino-terminal three zinc fingers of yeast TFIIIA would be derivatized with a photoactivatable heterobifunctional crosslinker. The amino-terminal three zinc fingers of yeast TFIIIA contain no cysteine residues other than those involved in zinc ion coordination. Therefore, a cysteine residue can be engineered into TFIIIA adjacent to the putative TFIIIC-contact site to allow use of a thiol-specific heterobifunctional photo-crosslinking reagent. Based on the results of the mutational analysis of the first zinc finger of TFIIIA described above, the cysteine residue would be positioned near F50 and Y53, residues of the \( \beta \)-hairpin that have been found to be essential for TFIIIC recruitment (see above). It will be important to verify that the cysteine substitution is not deleterious to the activity of wild-type TFIIIA; if it were, then another nearby site would be chosen for mutation. I expect that an engineered cysteine exposed on the surface of the zinc finger would be more reactive to the crosslinking reagent than the cysteine residues that are involved in zinc ion coordination. An example of a thiol-specific heterobifunctional photo-crosslinking reagent that has been used successfully to study protein-protein interactions \textit{in vitro} is maleimide-4-benzophenone, which has an approximate side chain length of 10 Å (Emili and Ingles, 1995; Dormán and Prestwich, 1994). Derivatized TFIIIA bound to the 5S RNA gene will be incubated with TFIIIC and crosslinks introduced between TFIIIA and TFIIIC by photolysis. The TFIIIA-containing complexes will then be analyzed by SDS-PAGE and visualized with anti-TFIIIA antibody or by using
radiolabeled TFIIIA. If TFIIIA becomes crosslinked to a subunit of TFIIIC, the mobility of TFIIIA will be decreased on the SDS polyacrylamide gel proportionately with the size of the crosslinked TFIIIC subunit. Western blotting with antibodies to subunits of TFIIIC would positively identify the crosslinked TFIIIC subunit.

**Role of the 81-amino acid domain of yeast TFIIIA**

Milne and Segall (1993) proposed a model for the mechanism by which yeast TFIIIA directs the assembly of a functional transcription complex on the yeast 5S RNA gene. These authors proposed that the amino-terminal zinc fingers of TFIIIA position the protein on the 5S RNA gene and serve to recruit TFIIIC to the TFIIIA-DNA complex via protein-protein interactions between the zinc fingers of TFIIIA and the τ_B subcomplex of TFIIIC. This TFIIIC-TFIIIA interaction would then facilitate a putative interaction between the 81-amino acid domain of TFIIIA and the τ_A subcomplex of TFIIIC, and it was suggested that this latter interaction is crucial in docking TFIIIC with the correct topography for formation of a functional TFIIIB-TFIIIC-TFIIIA-DNA complex (see figure below). An additional, or alternative, role for the 81-amino acid domain could be to prevent the τ_A subcomplex of TFIIIC from binding to the A-box-like sequence of the 5S RNA gene (see the Appendix to Chapter 3 for further details). This role for the 81-amino acid domain could occur through an interaction with a component of TFIIIC and, therefore, this role need not be mutually exclusive with the model described above.
It will be important to determine whether TFIIIB is incorporated into the TFIIIC-TFIIIA-DNA complexes formed with TFIIIA containing mutations of the leucine-rich segment of the 81-amino acid domain of TFIIIA (Chapter 3). Incorporation of TFIIIB can be monitored by a gel mobility shift assay and by DNase I footprinting (Braun et al., 1989; Kassavetis et al., 1990; Braun et al., 1992b). If TFIIIB is stably incorporated into the transcriptionally inactive complexes, the addition of heparin, which selectively strips TFIIIC and TFIIIA from 5S DNA (Kassavetis et al., 1990), would allow its presence to be confirmed; its position on DNA could also be established unequivocally. Photoactive nucleotides could be incorporated at specific positions on the 5S RNA gene and the positions of subunits of TFIIIB analyzed by DNA-protein photocrosslinking (Braun et al., 1992a). It is possible that 1) TFIIIB fails to be incorporated 2) TFIIIB is incorporated into the complex, but it is inappropriately positioned or fails to bind DNA, or 3) TFIIIB is appropriately positioned, but pol III cannot initiate transcription. This analysis would indicate whether the defect in the 5S RNA gene transcription complex lies in the TFIIIC-TFIIIB interaction, in the positioning of TFIIIB, or perhaps in the conformational state of TFIIIB. Changes in the properties and architecture of TFIIIB are known to occur during transcription complex formation (Kassavetis et al., 1990, 1992b; Huet et al., 1997; Kumar et al., 1997), and it is possible that these are somehow dependent on the activity of the 81-amino acid domain of TFIIIA. If this proved to be the case, conformational changes of TFIIIB could be detected by DNA-protein photocrosslinking analysis (Braun et al., 1992a) or by hydroxyl radical protein footprinting of TFIIIB subunits (Kumar et al., 1997).

I would also test for the incorporation of TFIIIB into a TFIIIC-TFIIIA-DNA complex formed with TFIIIA that contains disruptions of both zinc finger 8 and 9. Zinc fingers 8 and 9 are redundantly essential for the transcriptional activity of TFIIIA (Chapter 3). It is possible that zinc fingers 8 and 9 of TFIIIA contribute to the function of the 81-amino acid domain; alternatively, these fingers could perform a role in transcription complex formation that is distinct from the role of the 81-amino acid domain of TFIIIA.
I would also determine the subunit(s) of TFIIIC that may contact the 81-amino acid domain of TFIIIA. This could be done by photochemical crosslinking of proteins using a similar strategy to the one described above for detecting TFIIIC subunits that contact the amino-terminal zinc fingers of TFIIIA. I would engineer a cysteine residue near to or within the essential leucine-rich segment of the 81-amino acid domain and use a thiol-specific heterobifunctional photocrosslinking reagent for these studies. Alternatively, a photoactivatable heterobifunctional crosslinker could be conjugated to TFIIIA via primary amine groups. A number of lysine residues are found near to the essential leucine-rich segment of the 81-amino acid domain (see Chapter 3). An example of a crosslinker that could be conjugated to TFIIIA via primary amine groups is SASD (sulfo succinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate), which is also radioiodinatable and cleavable by reduction. The identification of TFIIIC subunits that are in contact with the 81-amino acid domain of TFIIIA would be done as described above for the crosslinking analysis with the amino-terminal zinc fingers of TFIIIA. For these studies, it will be important to ensure that the crosslinking of proteins to TFIIIA is dependent on the presence or functionality of the 81-amino acid domain.

It has been proposed that yeast TFIIIA not only recruits TFIIIC to the 5S RNA gene, but also prevents the τA subcomplex of TFIIIC from gaining access to the A-box-like sequence of the 5S RNA gene (Braun et al., 1992a). If such an interaction were to occur, the τA subcomplex of TFIIIC would be shifted downstream of its normal position and a defective transcription complex would likely be formed on the 5S RNA gene. The leucine-rich segment of the 81-amino acid domain may be responsible for preventing TFIIIC from interacting with the A-box-like sequence of the yeast 5S RNA gene. I tested the ability of TFIIIA(L354A/L356A/L357A/L358A), which contains mutations in the 81-amino acid domain that render TFIIIA transcriptionally inactive on a wild-type 5S RNA gene, to support transcription of 5S RNA genes containing linker scanning mutations through its A-box sequence (see the Appendix to Chapter 3). I reasoned that the absence of an A-box sequence
in the 5S RNA gene would alleviate the need for the region of TFIIIA responsible for preventing TFIIIC from interacting with the A box sequence. Interestingly, TFIIIA(L354A/L356A/L357A/L358A) did support weak in vitro transcription of 5S RNA gene templates that contained mutations of the A-box sequence (Chapter 3 Appendix). It should be emphasized that these results are preliminary and further experiments are required to examine the validity of my interpretation of these observations. For example, the start site of transcription needs to be determined to ensure that specific transcription is occurring. It would also be prudent to make site-directed mutations, rather than linker scanning mutations, in the A box of the 5S RNA gene that are known to abolish the ability of the A box to interact with the $\tau_A$ subcomplex of TFIIIC (Allison et al., 1983; Stillman et al., 1985; Camier et al., 1990). This model could be tested by determining whether TFIIIC subunits are in contact with the A-box sequence of the 5S RNA gene in TFIIIC-TFIIIA-DNA complexes formed with TFIIIA(L354A/L356A/L357A/L358A) or with a polypeptide that contains a deletion of the carboxyl-terminal half of TFIIIA. This can be done by a DNA-protein photo-crosslinking analysis of the complexes (Braun et al., 1992a). Braun et al. (1992a) found that TFIIIA in a TFIIB-TFIIIC-TFIIIA-5S DNA complex efficiently crosslinks to photoactive nucleotides situated in the A-box sequence of the 5S RNA gene. I predict that TFIIIA containing mutations in the 81-amino acid domain would no longer crosslink to the A box of the 5S RNA gene and instead TFIIIC subunits would crosslink to the A box.

The results from the experiments proposed in this section will provide insights into the molecular mechanisms by which TFIIIA fulfills its role in the assembly of an active transcription complex on the 5S RNA gene.
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