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Structural and functional studies of *Xenopus laevis* transcription factor IIIA zinc finger mutants

Del Río, Samuel, Ph.D.

Case Western Reserve University (Health Sciences), 1992

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STRUCTURAL AND FUNCTIONAL STUDIES OF *Xenopus laevis* 
TRANSCRIPTION FACTOR IIIA ZINC FINGER MUTANTS 

by 
SAMUEL DEL RIO 

Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy 

Thesis Advisor:  David R. Setzer, Ph.D. 

Department of Molecular Biology and Microbiology 
CASE WESTERN RESERVE UNIVERSITY 
May 1992
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GRADUATE STUDIES

We hereby approve the thesis of

Samuel Del Rio

candidate for the Ph.D. degree.*

Signed: Russell Mann

(chairman)

[Signatures of other members of the committee]

Date April 6, 1992

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STRUCTURAL AND FUNCTIONAL STUDIES OF *Xenopus laevis*
TRANScription FACTOR IIIA ZINC FINGER MUTANTS

ABSTRACT

by

Samuel Del Río

Transcription factor IIIA (TFIIMA), a sequence-specific DNA-binding protein from *Xenopus laevis*, is a zinc finger protein required for transcription of 5S rRNA genes by RNA polymerase III. We describe the purification and characterization of recombinant TFIIMA and TFIIMA zinc finger mutants expressed in *E. coli*.

We have analyzed a set of TFIIMA mutants in which each mutant protein contains a single amino acid substitution in one of the putative zinc-binding histidine residues. We were able to assign specific functions, 5S DNA interactions and kinetic and equilibrium parameters to the individual zinc fingers of TFIIMA. The ability to analyze TFIIMA zinc finger mutants with an intact C-terminal domain made it possible to determine which TFIIMA zinc fingers were important for transcriptional activation.

Chromatographic properties of the mutant proteins and the results of partial proteolysis studies indicate that the mutation in each case results in structural disruption of the zinc finger domain containing the mutation with no
apparent effect on adjacent fingers. While all fingers contribute to the binding energy when TFIIIA interacts with the intragenic control region (ICR) of the 5S rRNA gene, some fingers are more important than others, with mutations in fingers 3 and 4 resulting in the largest decreases in binding affinity. Though the first six N-terminal fingers of TFIIIA contribute greater than 70% of the relative binding energy to the 5S DNA, mutations in any one of these six N-terminal fingers did not affect the transcriptional activity of TFIIIA.

The three C-terminal fingers of TFIIIA, fingers 7-9, though contributing less than 30% of the relative binding energy to the 5S DNA, are essential for the transcriptional activity of TFIIIA. This demonstrates that the C-terminal fingers of TFIIIA are important not only in binding to the 5' end of the 5S gene ICR, but also in mediating transcriptional activation through interactions with other components of the RNA polymerase III transcription machinery.
DEDICATION

I dedicate this thesis to my wife and friend, Olga Lydia, for her constant love, steadfast support and unceasing prayers.
Acknowledgements

Consider it pure joy, my brothers, whenever you face trials of many kinds, because you know that the testing of your faith develops perseverance. Perseverance must finish its work so that you may be mature and complete, not lacking anything. James 1:2-4 (NIV)

I express my most sincere appreciation to Dr. David R. Setzer for his supervision, guidance, and patience during the course of this work.

I thank the members of my thesis committee, Drs. Russell Maurer, Fritz Rottman, Terry Magnuson, and Pamela Davis, for their encouragement, guidance and input. Especially for their persistent exhortation to analyze the complete set of zinc finger mutants.

I thank Dr. Leslie Webster, past director of the Medical Scientist Training Program, and his staff for their support.

I also thank the members of Dr. Setzer's laboratory, past and present, for providing a motivating and friendly work environment. Specifically, Frank Campbell for his friendship and insightful discussions; and Sandra Menezes for her pleasant smile, friendship and her contribution to this work.

Finally, I thank my family - my wife, Olga, my parents, Abraham and Francisca Del Rio, my in-laws,
Nathaniel and Lydia Del Toro, my brothers and sisters, Abraham, Maritsa, David, Steven, Richard, and Julie - for their constant love, support and encouragement throughout the course of my graduate career. "And we know that in all things God works for the good of those who love him."
(Romans 8:28; NIV)
NOTE

Chapter II of this thesis has previously appeared in the literature and is presented in its entirety (See Literature Cited; Del Rio and Setzer, 1991).

The contribution by Sandra Menezes to portions of the work presented in Chapter III is hereby acknowledged. Ms. Menezes was responsible for the thermolysin analyses of the nine TFIIIA zinc finger mutants. These experiments culminated in the data presented in Table IV and Figures 19 and 20.
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LIST OF ABBREVIATIONS

ASN asparagine
ATP adenosine triphosphate
bp base pair
BPB bromophenol blue
BSA bovine serum albumin
CPM counts per minute
CTP cytosine triphosphate
DNA deoxyribonucleic acid
DTT dithiothreitol
EDTA ethylenediamine-tetraacetic acid
eq equivalents
EXAFS extended X-ray absorption fine structure
GTP guanine triphosphate
HEPES N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HIS histidine
ICR intragenic control region
IPTG isopropyl β-D-thiogalactopyranoside
kcal kilocalorie
kdal kilodalton
NTP ribonucleotide triphosphate
ova ovarian
PAGE polyacrylamide gel electrophoresis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>PAR</td>
<td>4-(2-pyridylazo)resorcinol</td>
</tr>
<tr>
<td>PMPS</td>
<td>p-hydroxymercuriphenylsulfonate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>rec</td>
<td>recombinant</td>
</tr>
<tr>
<td>RIS</td>
<td>radioanalytic imaging system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>UTP</td>
<td>uracil triphosphate</td>
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CHAPTER I
INTRODUCTION

The study of the developmental process is intimately associated with the study of differential gene expression since differential gene expression determines many of the differences between cell types. Differential gene expression is controlled at various levels throughout development including temporal and spatial regulation of transcription. That is, genes are activated or inactivated at specific times during development and the expression of genes can be restricted or limited to specific cell types during development. Within specific cell types, interactions between a myriad of trans-acting factors and cis-acting elements regulate expression of specific genes. Hence, the study of macromolecular interactions between various components of the transcriptional apparatus can provide clues as to how regulation of transcription results in cell differentiation. Therefore, the study of the interactions between the *Xenopus laevis* transcription factor IIIA (TFIIIA) and the 5S rRNA genes transcribed by RNA polymerase III may help to elucidate the mechanism by which the various classes of 5S RNA genes are developmentally regulated.

The 5S rRNA genes of *Xenopus laevis*, the South African
clawed toad, are transcribed by RNA polymerase III. 5S RNA is a molecule of 120 nucleotides (10,30,31,58,80) which is present in every eukaryotic organism and is associated with the large subunit of ribosomes. The major cis-acting control element of 5S rRNA genes is the intragenic control region (ICR) (7,97) which functions as the promoter for expression by RNA polymerase III. In the genome of *Xenopus laevis* there are two major classes of 5S RNA genes, somatic-type and oocyte-type (10,23,30,80,88). The haploid genome of *Xenopus laevis* contains approximately 400 copies of somatic 5S RNA genes that are expressed constitutively in all cells and approximately 24,000 copies of oocyte 5S RNA genes that are transcribed only in oocytes (80). Although Xenopus 5S RNA gene sequences are highly conserved in general, the oocyte-type genes differ from somatic-type genes at four positions: nucleotides 30, 53, 55 and 79 (30,31,58,70,80,128). Three of these sequence differences are located within the intragenic control region. The mechanism by which these related genes are differentially expressed in somatic cells is not completely understood. TFIIIA binds the somatic and major oocyte 5S RNA genes with equal affinity (68). Sequence differences preceding the minimal promoter most likely affect TFIIB and TFIIC interactions (89).
A. Transcription of 5S rRNA genes

In addition to TFIIIA and RNA polymerase III, at least two other factors (TFIIB and TFIIC) are necessary for accurate transcription of 5S DNA (21,42,77,99,104,125,130,131). The activities in chromatographic fractions TFIIB and TFIIC have only been partially characterized and there is evidence that TFIIC represents more than one essential factor (57,74,130,131). Through formation of stable transcription complexes on 5S rRNA genes, these transcription factors remain associated with the 5S DNA during multiple rounds of transcription (60).

Pathways for complex assembly and the roles of various factors in complex formation and stability have been elucidated (105). These studies have confirmed the importance of TFIIII as a central and primary factor in establishing sequence-specific transcription initiation on 5S rRNA genes.

The ICR (nucleotides +45 to +96 (6,7,97,99) of the 5S RNA gene is necessary and sufficient to direct accurate initiation of transcription. This region controls transcription through its interaction with TFIIII. In the absence of 5' flanking sequence and the 5' end of the coding sequence, transcription initiates in the substituted DNA approximately 50 bp upstream of the proximal end of the
ICR (97). Stable complex formation can be considered a step-wise process in which the first step, binding of TFIIIA, is reversible. Several lines of evidence suggest that the binding of TFIIIC may be the second step in stable complex assembly, the step required for template commitment (3,60,103,105). Thus, TFIIIC must recognize and bind to a preexisting TFIIIA-5S DNA complex in order to stabilize the complex. Finally, TFIIIB binds the complex in the rate limiting step (Figure 1).

Systematic oligonucleotide-directed mutagenesis within the 5S gene ICR (82-84) shows that the promoter extends from bp 50-97 of the 5S gene. The analysis of the effects of mutations on in vitro transcription and on the individual steps in the formation of initiation complexes identifies three separate elements that determine promoter strength: A box, the intermediate element, and C box. The A box element (bp 50-64) has a relatively low affinity for TFIIIA and is directly involved in the binding of TFIIIC; the intermediate element (bp 67-72) and C box (bp 80-97) are the main determinants of affinity for TFIIIA.

B. TFIIIA

Xenopus laevis TFIIIA has been purified to homogeneity and shown to be a Zn-containing, single polypeptide of 344 amino acids in length with a molecular weight of
Figure 1. Kinetic scheme for 5S RNA synthesis. Simplified scheme for the steps leading to the transcription of 5S rRNA genes, where $k_1, k_3 >> k_2$ indicating that binding of TFIIB is the rate limiting event.
approximately 40,000 daltons (42). The haploid genome of *Xenopus laevis* contains only one gene for TFIIIA (42,117).

TFIIIA binds specifically to the ICR with a stoichiometry of 1:1 (4,108) and an equilibrium binding constant of 0.4-1.0 nM (22,46,94). The binding of TFIIIA to the 5S rRNA gene protects nucleotides +45 to +96 from DNaseI digestion in footprinting assays (20,26,28,98,122). The Stokes radius for TFIIIA has been calculated to be 34 Å (4). The sedimentation coefficient of TFIIIA was determined to be 2.8S (4). Using the Stokes radius and molecular weight values for TFIIIA, the frictional coefficient was calculated to be 1.53 (4), a value which indicates a highly asymmetric molecule, with estimated size of 135 x 18 Å. These data suggest that TFIIIA may be sufficiently elongated to interact with the entire length of the ICR (150 Å, assuming B-form DNA (52)). Schroth et al. (101,102) have shown that TFIIIA induces a bend at the ICR of both the *Xenopus borealis* somatic-type (101) and *Xenopus laevis* oocyte-type 5S rRNA genes (102).

TFIIIA also functions as a sequence-specific RNA-binding protein, binding to the 5S RNA transcripts in the cytoplasm of immature oocytes, forming a storage ribonucleoprotein particle (7S RNP) that stabilizes the 5S RNA until it is required for ribosome assembly (79,81). Thus, TFIIIA possesses the unusual ability to interact
specifically with two different nucleic acids. This property suggests that 5S RNA gene transcription might be regulated by feedback mechanisms, since a required factor for transcription (TFIIMA) binds to the gene product as well as to the gene itself (79). The interaction between TFIIMA and *Xenopus laevis* oocyte 5S rRNA has been investigated by several groups. It has been shown that TFIIMA binds to 5S rRNA with a dissociation constant of 1 nM, the complex having a half-life of 45 minutes (93,95).

Analysis of TFIIMA proteolytic fragments (108) and TFIIMA deletion mutants (124) suggests that the protein is composed of separate transcription and DNA-binding domains. The carboxyl terminal domain (≈8 kd) is required for full transcriptional activity but not for the sequence-specific binding to the ICR. These data suggested that the amino terminal domain (≈30 kd) corresponds to the DNA-binding domain and can be aligned over the ICR with its amino terminus toward the 3' end of the ICR and its carboxyl terminus toward the 5' end of the ICR (117). Two lines of evidence suggest that binding of TFIIMA to the ICR is not uniform; deletion mutants of the ICR show that TFIIMA binding to the 3' end of the ICR is required for binding at the 5' end but not the reverse (99); and protection by TFIIMA of G residue methylation is strongest at the 3' end of the ICR and weakest at the 5' end (28).
All of the analyses contained within this thesis were done with the *Xenopus borealis* somatic 5S rRNA gene whereas the coding sequence used for the expression and mutagenesis of TFIIIA came from a cDNA clone (42) from *Xenopus laevis*. The *Xenopus laevis* and *Xenopus borealis* TFIIIAAs have 344 and 339 (39) amino acids respectively. Overall, the proteins share 84% amino acid sequence homology (39). The most conserved and diverged regions are the N-terminal two fingers and the C-terminal tail respectively. A comparison between DNaseI protection by *Xenopus laevis* or *Xenopus borealis* TFIIIA on the coding and non-coding strands of the *Xenopus borealis* somatic 5S RNA gene revealed nearly identical patterns (38).

C. Zinc Finger Structure

The 5S rRNA gene specific transcription factor TFIIIA is the prototype for zinc-finger proteins. The repeating amino acid sequence motif which defines this class of proteins was first recognized by analysis of sequence information derived from a cDNA clone of the TFIIIA gene (42,69) (Figure 2).

The amino terminal domain of TFIIIA consist of nine repeating units called 'zinc fingers' (11,69). 'Zinc fingers', as proposed by Miller et al., (69) are independent structural domains of ≈30 amino acids. Each
Figure 2. Amino acid sequence of TFIIB, aligned to show the repeated units. The repeat units are numbered 1-9 on the left side of the diagram. Conserved Cys and His residues are in bold print. A dash (−) indicates an alignment gap. (Figure adopted from Miller et al. (69))
(MGEKALPVVYKR)

1  YICSFDACGAYNKWNWKLQ-AHLC-KH 37
2  TGEK-PFPCKKEEGCCEKEKGFTSLLHT-RHSL-TH 67
3  TGEK-NFTCDSDGCDLDLRTTKANMK-KHFNRFH 98
4  NIKICVYVCHFENCGBKAFKKNQLK-VHQF-SH 129
5  TQQL-PYECPHEGCDKRFSLPSRLK-RHEK-VH 159
6  AG----YPCKKDDSCSFVGTKWTLYLKHVAECH 188
7  QD----LAVC----DVCNKFRHRKYLRDHWK-TTH 214
8  EKERTVYLCPRDGCDSYTTAFNLR-SHIQSFH 246
9  EEQR-PFVCEHAGCGKCFAMKKSE-RHHSV-VH 276

277  DPEKRKLKEKCPRPKRSALASRLTGYIIPPKSKEKN 311
     ASVSGTEKTDSDLVKNKPSGTETNGSLSVLKDKTIQ 344
zinc finger contains a zinc ion and a consensus sequence of
\((*)_5-(F,Y)-*-(*)_2\) or \(4-C-(*)_3-F-(*)_2-L-(*)_2-H-(*)_2-H\), where
"*" is any amino acid. The repeat unit contains two
invariant cysteine and two invariant histidine residues
which are proposed to act as ligands for the zinc ion
(Figure 3). These zinc fingers have been postulated to
interact with a repeated sequence element within the ICR
(90). This putative DNA repeat is characterized by one or
more G residues (on the non-coding strand) repeated on
average every 5.5 base pairs along the ICR.

The zinc finger model was initially based on: (i) the
high zinc content of the 7S ribonucleoprotein particle (7S
particle); (ii) proteolytic digestion of 7S particles
yielded periodic intermediates and small persistent
fragments consistent with small, \(\approx 30\) amino acid structural
domains; and (iii) the analysis of the amino acid sequence
of TFIIIA demonstrating nine tandem repeats of \(\approx 30\) amino
acids in length.(69)

Additional evidence consistent with the proposed 'zinc
finger' model includes: (i) TFIIIA's dependence on zinc(II)
for both DNA-binding and transcriptional activation (48);
(ii) the genomic structure of the TFIIIA gene which shows
that six of the eight exon/intron boundaries fall between
the putative domains (121) suggesting that these units have
structural significance; (iii) EXAFS spectroscopic data
Figure 3. Schematic representation of zinc fingers. The tetrahedral arrangement of the zinc ligands, cysteine (C) and histidine (H), is depicted. The consensus sequence for a TFIID finger (69) is shown, where the invariant C and H residues are boxed (□), asterisks (*) mark positions where an insertion sometimes occurs in the normal pattern and dots (.) mark variable positions in the sequence.
demonstrating that there are approximately nine zinc ions per TFIIIA molecule, all of which have a similar environment and are tetrahedrally coordinated by two cysteinyl sulfhydryl groups and two histidyl imidazole nitrogens (24); (iv) the work by Frankel et al., (34) which demonstrated the metal-dependent folding of a single zinc finger of TFIIIA; (v) spectroscopic studies of wild-type and mutant zinc finger peptides by Parraga et al., (85) which indicated that the ligand spacing and both thiol and imidazole participation in zinc binding are specific and necessary requirements for zinc finger folding; (vi) SP1's, a three zinc finger containing protein, requirement of zinc for DNA binding (55); (vii) genetic and mutational analyses of zinc-finger containing proteins ADR1 (5,118,119) and Krüppel (40,86) demonstrating the importance of the invariant cysteine (5,86,118,119) and histidine (5,118,119) residues present in the zinc finger motif as well as the importance of the zinc finger repeat (5,40,118,119) in the function of these proteins; and (viii) PMPS-induced Zn$^{2+}$ release measured by (PAR)$_2$·Zn$^{2+}$ complex formation indicated that TFIIIA contains approximately 9 equivalents of Zn$^{2+}$ (45).

The first three-dimensional configuration for a C$_2$H$_2$ finger domain was proposed by Berg (2), based on the crystal structures of other metalloproteins (Figure 4). The
Figure 4. Proposed tertiary structure for a zinc finger repeat. (Figure taken from Berg (2))
N terminus forms an anti-parallel \( \beta \)-sheet and a reverse turn from which the two cysteines bind to the zinc ion, whereas the C-terminal residues form an \( \alpha \)-helix from which the histidines also bind the zinc. This organization allows the three conserved hydrophobic residues to interact, thus forming a hydrophobic core which stabilizes the "finger tip". Using two-dimensional NMR (62,63,73,75,93) various groups have solved the structure of other zinc finger peptides ADR1 (75,93), Xfin (62,63) and SWI5 (73). These structures confirm the essential elements of the Berg model, although a few of the details are different. Recently the crystal structure of a complex containing the three zinc fingers from the mouse immediate early protein Zif268 (19) and a consensus DNA binding site (18) has been determined (78). The structure of the three zinc fingers of this polypeptide in the co-crystal is consistent with the structure proposed by Berg. In the Zif268-DNA complex, each finger is structurally similar, binds in the major groove of B-DNA, and wrap partway around the DNA helix. Each finger was shown to interact with a three-base pair subsite, allowing the three zinc fingers to span the 9 base pair target sequence. Table I contains a partial list of the ever increasing number of zinc finger proteins of the \( \text{C}_{2}\text{H}_{2} \) class.

Two models for the TFIIIA-DNA interaction have been
Table I. A partial list of the C$_2$H$_2$ class of zinc finger proteins (or cDNA sequences)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein/sequence</th>
<th>Repeats</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Xenopus</td>
<td>TFI IIIA</td>
<td>9</td>
<td>(42,69)</td>
</tr>
<tr>
<td></td>
<td>p43</td>
<td>9</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td>Xfin</td>
<td>37</td>
<td>(91)</td>
</tr>
<tr>
<td></td>
<td>XFG 5-1</td>
<td>7</td>
<td>(54)</td>
</tr>
<tr>
<td>Mouse</td>
<td>mk2</td>
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<td>7</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>Zif268</td>
<td>3</td>
<td>(19,78)</td>
</tr>
<tr>
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<td>brlA</td>
<td>2</td>
<td>(1)</td>
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<tr>
<td>Human</td>
<td>SP1</td>
<td>3</td>
<td>(55)</td>
</tr>
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<td></td>
<td>PRDII-BF1</td>
<td>4</td>
<td>(29)</td>
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<tr>
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<td>GLI</td>
<td>5</td>
<td>(56)</td>
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<td>(12,41)</td>
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<tr>
<td>Drosophila</td>
<td>Krüppel</td>
<td>4</td>
<td>(96)</td>
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<td>hunchback</td>
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<td>(114)</td>
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<td>(71)</td>
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<td>sry</td>
<td>5</td>
<td>(123)</td>
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<tr>
<td>Yeast</td>
<td>ADR1</td>
<td>2</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>SWIS</td>
<td>3</td>
<td>(72,73)</td>
</tr>
</tbody>
</table>
proposed (28): (i) the "wrapping around" model, where successive zinc fingers make structurally equivalent contacts following the helical path of the major groove without crossing the minor groove (2) and (ii) the "alternating" model, where alternate fingers bind on one face of the DNA in an equivalent manner to the major groove, so that successive minor grooves must be crossed (28). Based on the quantitative analysis of high-resolution hydroxyl radical footprints, Churchill et al. (20) have proposed a model which is a modification of the "alternating" model. In the Churchill model, the protein as a whole lies on one face of the DNA with the zinc fingers in the major groove, all with the same polarity but with linkers alternately located in the major groove or crossing over the minor groove.

D. Mapping of TFIIIA onto the 5S RNA gene ICR

Using a DNaseI protection assay and 3' end (nucleotides 80-97) and 5' end (nucleotides 41-66) deletions of the 5S DNA ICR, Sakonju et al., (99) demonstrated that TFIIIA bound more tightly to the 3' end of the 5S DNA ICR. Phosphate protection and base methylation studies (98) indicated that TFIIIA is in close proximity to nucleotides +69--+71 and +79++89 on the non-coding strand of the 5S RNA gene. Taylor et al., (117)
subsequently mapped the N-terminus of TFIIIA to the 3' end of the ICR and the C-terminus of TFIIIA to the 5' end of the ICR (Figure 5).

By using DNaseI and hydroxyl radical footprinting techniques and a collection of either C-terminally or N-terminally truncated TFIIIA molecules, Vrana et al. (124) were able to map zinc fingers to the ICR. They proposed that adjacent zinc fingers in TFIIIA do not bind to precisely adjacent nucleotides along the ICR as predicted by the model of Rhodes and Klug (90). Instead, clusters of zinc fingers bind to three regions of the ICR, corresponding to the three regions delimited by Pieler (82-84). Their results suggested that TFIIIA interacts with at least one full turn of the helix at each end of the ICR (boxes A and C) and binds across the minor groove of the DNA at the center of the ICR, the intermediate box. They proposed that fingers 8 and 9 interact with the box A sequences, fingers 7, 6 and 5 interact with the intermediate box sequences and that fingers 1 and 2 interact with the box C sequences. Their results also suggested that each carboxyl-terminal zinc finger (fingers 5-9) made a major local contribution to the footprint and a minor contribution to the overall binding energy of TFIIIA to the ICR. The largest contribution to the binding energy was due to the interaction of the two amino terminal zinc fingers.
Figure 5. Orientation of TFIIIA with respect to the 5S RNA gene. A schematic interpretation of the structural features of TFIIIA and its interaction with the 5S RNA gene. The intragenic control region (ICR) is indicated above the 5S RNA gene, with the 5' end of the non-coding strand at the left of the figure. The protein sequence runs from right to left. The amino end of TFIIIA is followed by the nine repeating zinc fingers (residues 13-276) in contact with the control region. The C-terminal portion of TFIIIA is situated toward the 5' end of the ICR.
(fingers 1 and 2) with a sequence of about 17 bp (+80--97). Quantitative hydroxyl radical footprinting of the TFIIB-DNA complex has revealed a strong 10-11 bp periodicity of protection over the region from +40--90 (20).

By cross-linking TFIIB to its cognate binding site in the 5S rRNA gene using a highly photosensitive analogue of thymidine, 5-azidodeoxyuridine 5'-triphosphate, Lee et al. (61) were able to show that >90% of the cross-linking occurred from two sites in the 5S RNA gene corresponding to T residues at positions 84 and 88 in the non-coding and coding strands, respectively. Digestion with V8 protease of the cross-linked TFIIB-5S DNA non-coding strand complex indicated that the region including zinc-finger 2 plus the finger 2-3 linker was in contact with position 84 (61).

Recently, using a peptide fragment containing the N-terminal three fingers of TFIIB (fingers 1 2 and 3), two separate groups (17,64) mapped the N-terminal three fingers of TFIIB to the 3' end of the ICR (+96--77), whereas peptide fragments containing fingers 1 and 2, or fingers 2,3 and 4 of TFIIB did not exhibit sequence-specific DNA binding (64).

E. Rationale and Research Aims

The goal of this project was to further the understanding of the structure and function of TFIIB as a
sequence-specific DNA binding protein. In particular, its interaction with the *Xenopus borealis* somatic-type 5S rRNA gene was studied.

The specific aims of the work presented in this thesis were to address the following questions:

(1) How important are the conserved Zn-binding amino acids in mediating structural stability of an individual zinc finger within the context of the entire protein?

(2) Do the various zinc fingers in TFIIIA contribute equally to the binding energy when TFIIIA binds to the intragenic control region (ICR), or are some more important than others? In the latter case, which fingers are the most important?

(3) Can we map specific fingers to particular regions of the ICR by analyzing TFIIIA mutants containing 8 wild-type fingers and one mutated finger?

(4) Is the ability of TFIIIA to support transcription of 5S rRNA genes correlated with its affinity for the gene in a simple binary complex? Can the mutant forms of TFIIIA affect the formation of higher order transcription complexes by altering the interactions with other components of the transcriptional apparatus?

The work presented in this thesis addresses these proposed questions. I have attempted to follow a logical course in the analyses of the zinc finger TFIIIA mutants,
relying on results and observations from initial experiments to guide me in the preparation and execution of later experiments. In the course of this work the following steps were completed:

(1) TFIIIA mutants were generated using site-directed mutagenesis. Initially, histidine residues involved in coordinating the zinc ion were targeted.

(2) An E. coli expression and purification method was developed which allowed for purification of milligram quantities of TFIIIA and TFIIIA zinc finger mutants from bacterial extracts.

(3) The structural integrity of individual fingers containing a mutation in a conserved zinc ligand was determined by use of limited proteolysis using thermolysin as a probe for structural perturbations.

(4) The sequence-specific DNA-binding properties of the mutant TFIIIA proteins were analyzed: (i) qualitatively using footprint assays; (ii) quantitatively using gel retardation assays to determine equilibrium binding constants and kinetic dissociation rates.

(5) The ability of the TFIIIA zinc finger mutants to support 5S rRNA gene transcription was assayed using a TFIIIA dependent in vitro transcription assay.
CHAPTER II

HIGH YIELD PURIFICATION OF ACTIVE TRANSCRIPTION FACTOR

IIIA EXPRESSED IN E. coli

A. Abstract

Transcription factor IIIA (TFIIIA), a sequence-specific DNA-binding protein from *Xenopus laevis*, is a zinc finger protein required for transcription of 5S rRNA genes by RNA polymerase III. We describe the purification and characterization of recombinant TFIIIA (recTFIIIA) expressed in *E. coli*. RecTFIIIA was purified to greater than 95% homogeneity at a yield of 2-3 milligrams per liter of bacterial culture. This purified protein protects the internal control region of a 5S rRNA gene from DNase I digestion, yielding footprints on both strands identical to those produced by the ovarian protein (ovaTFIIIA). Quantitative analysis of binding data from gel retardation assays yielded a $K_d$ of about 0.4 nM for TFIIIA from both sources. Using a quantitative TFIIIA-dependent *in vitro* transcription assay, we found that recTFIIIA is equivalent to ovaTFIIIA in supporting transcription of 5S rRNA genes. We conclude that recTFIIIA is functionally indistinguishable from the protein purified from *Xenopus* ovaries, and can be readily obtained in pure form and large quantity.
B. Introduction

Transcription Factor IIIA (TFIIIA) is a sequence-specific DNA binding protein required for transcription initiation by RNA polymerase III on 5S rRNA genes (26,104,107). TFIIIA binds specifically to the internal control region of the 5S rRNA gene, protecting nucleotides +45 to +96 from DNase I digestion (26,28,99). Although two or more other factors are required for accurate and efficient transcription of 5S rRNA genes (33,74,104,107,133), TFIIIA is likely to be the primary protein acting to establish sequence specificity in transcription initiation. TFIIIA is a zinc metalloprotein (48), containing 344 amino acids (42) and as many as 9 Zn$^{2+}$ ions (69). In fact, it is the prototype "zinc finger" protein, containing nine tandemly repeated zinc finger motifs (69). In addition to its DNA-binding and transcription factor activities, TFIIIA also acts as an RNA-binding protein, associating with 5S RNA to form a 7S ribonucleoprotein particle (79), believed to function as a storage particle for 5S rRNA in immature oocytes of *Xenopus* (81).

Because of its key role in 5S rRNA gene transcription, its status as the first Zn$^{2+}$ finger protein identified, and its unusual function as both a sequence-specific DNA and RNA-binding protein, detailed analysis of the structure and
function of TFIIIA is of considerable interest. Availability of large quantities of purified protein for biophysical studies and the ability to produce mutant forms of TFIIIA will be necessary for structure-function analyses. Previously described methods for expression of TFIIIA in E. coli result in the production of rather small quantities of the protein, fail to yield protein of high purity, or produce a polypeptide containing additional foreign amino acids at the N-terminus (32,49,76,120). While these methods may yield material suitable for some analyses, published procedures are not readily adaptable for the production of wild-type or mutant forms of TFIIIA that can be used in quantitative biochemical and/or biophysical studies.

We describe here the application of the in vivo T7 RNA polymerase expression system developed by Studier and colleagues (111) to the production and purification of functional TFIIIA in E. coli. We obtain 2-3 mg of TFIIIA at greater than 95% purity from 1 liter of bacterial culture, or even higher quantities with a small sacrifice in purity. This purified protein is functionally indistinguishable from TFIIIA purified from Xenopus ovaries.

C. Methods and Materials

1. Bacterial Strains and Vectors
pGA11 (106) is a phagemid derived from the vector pGP12 (106) and contains the EcoRI fragment of the *Xenopus laevis* TFIIIA cDNA clone pUC3a1.b of Ginsberg et al. (44) cloned into the unique EcoRI site of pGP11 in the orientation permitting rescue of phage containing the sense strand of TFIIIA DNA. Using site-directed mutagenesis (106) with an oligonucleotide of sequence 5'-TCTCTCCCATATGCTCTTCAAGCA-3', we altered the sequence at and around the initiator methionine codon of TFIIIA to include an NdeI site. This plasmid was designated pGA11-NdeI. The NdeI/BamHI fragment of pGA11-NdeI containing the TFIIIA sequence was cloned into pET-11b (111), similarly digested with NdeI and BamHI, to obtain pTA101. In separate experiments, the *Xenopus laevis* TFIIIA coding sequence in phagemid pGA1 (106) at codons 300-304 was changed to 5'-GGT TAC ATC CCG CCG-3' using oligonucleotide-directed mutagenesis as previously described (106). The sequence of the oligonucleotide used was 5'-CTTTGCTCTTCCGGATGTAACCAGTGAGGC-3'. The new sequence in this region conserves the amino acid sequence of TFIIIA, but changes the codons used for residues 300 and 302-304 from rare usage codons to high usage codons in *E. coli* (44,92). We have called the resulting phagemid pGA1:EP(300-304). From pGA1:EP(300-304), we isolated an XhoI/BamHI fragment containing the 3' end of the TFIIIA cDNA sequence,
Figure 6. A. pTA102, expression vector for TFIIIA.
T7lac: Fusion promoter containing the T7 promoter and lac operator sequence; S10: Ribosome binding site and translation control region from the gene 10 protein of phage T7; Tø: Transcription termination signal from phage T7; bla: β-lactamase gene; ori: origin of replication. Unique recognition sites for BamHI and NdeI are indicated.

B. TFIIIA sequence from amino acids 297 to 307.
The conservative nucleotide sequence changes incorporated into the TFIIIA sequence in pTA102 are underlined. The amino acids encoded by the relevant codons are also underlined.
...297 298 299 300 301 302 303 304 305 306 307... TFIIIA Amino Acid number
...Arg Leu Thr Gly Tyr Ile Pro Pro Lys Ser Lys... TFIIIA Amino Acid sequence
5'-CGC CTC ACT GGA TAC ATA CCC CCC AAG AGC AAA-3' TFIIIA Wild-type DNA sequence
5'-CGC CTC ACT GCT TAC ATG CCG CCG AAG AGC AAA-3' pTA102 TFIIIA DNA sequence
including the sequence changes at codons 300-304. This fragment was exchanged for the corresponding XhoI/BamHI fragment in pTA101 to generate pTA102 (Figure 6).

pC9Xbs201 contains the *Xenopus borealis* somatic-type 5S rRNA gene from the EcoRI/BamHI fragment of plasmid pXbs201 (7) cloned into pUC9 similarly digested with EcoRI and BamHI. Prior to digestion with EcoRI and BamHI, the unique HindIII site of pUC9 had been removed by digestion, fill-in and re-ligation. pST5RD-XhoI was derived from pST5RD (106) by introducing an XhoI site immediately downstream of the 5S rRNA gene termination signal using the oligonucleotide 5'-GGCTTTTGCCTGAGTGCCATTCTG-3' in a site-directed mutagenesis experiment. Digestion of pST5RD-XhoI with XhoI produces a 199-bp DNA fragment containing the entire 5S rRNA gene from -49 to +124 with some flanking polylinker sequence at the 5' end.

In general, *E. coli* K-12 strain NM522 (43) was used for growth, isolation, and analysis of the DNA vectors. *E. coli* B strain BL21(DE3) (F' ompT r' s m' s) (110,111) was used to express the cloned TFIIIA gene.

2. SDS-Polyacrylamide Gel Electrophoresis

To assess the composition and purity of various fractions, proteins were separated on 12% polyacrylamide SDS gels as described (59).
3. Western Blot Analysis

Transfer of proteins from SDS polyacrylamide gels was performed with a Bio-Rad Trans-blot cell to 0.45 μM nitrocellulose (BioTrace-NT, Gelman Sciences) in 0.096 M glycine/0.0125 M Tris base at 70 volts for 3 hours at 4°C. Following transfer, the filter was preblocked in 5% (w/v) nonfat dry milk/0.02% NaN₃ in TBS/Tween (10 mM Tris-HCl, pH 7.4/140 mM NaCl/0.1% Tween 20) for 1-3 hours at room temperature with gentle shaking. The blot was washed two times with TBS/Tween and then incubated with rabbit anti-serum raised against purified ovarian TFIIIA in 50 ml TBS/Tween for 2 hours at 37°C with gentle rocking. Unreacted antibody was removed by washing two times in TBS/Tween, one time in HSTBS/Tween (10 mM Tris-HCl, pH 7.4/1.0 M NaCl/0.5% Tween 20), and three times in TBS/Tween. The blot was then incubated with ¹²⁵I-protein A (ICN) (10-15 μCi) in 50 ml's TBS/Tween for 2 hours at 37°C with gentle rocking. Excess ¹²⁵I-protein A was removed by washing two times in TBS/Tween, one time in HSTBS/Tween, and three times in TBS/Tween. The blot was dried and exposed to Kodak XAR film at -70°C.

4. Ovarian TFIIIA Purification

TFIIIA was purified from immature Xenopus laevis ovaries as described by Smith et al. (108) and as modified
by Campbell and Setzer (13).

5. Recombinant TFIIIA Purification

One liter of rich medium (25 g Bactotryptone/15 g yeast extract/5 g NaCl/pH 7.0) containing 100 μg/ml ampicillin and 4 g/liter glucose was inoculated with 9 ml of an overnight culture of BL21(DE3)/pTA102. The culture was grown at 37°C with shaking at 250 rpm to an OD₆₆₀ of 0.4 to 0.6, and then supplemented with ZnSO₄ (100 μM) prior to induction with 1 mM IPTG at 37°C for 3-4 hrs. Cells were harvested by centrifugation at 6,000 x g in a Sorvall GS3 or GSA rotor and were washed once with 15 ml buffer A (20 mM Na'HEPES, pH 7.4/5 mM MgCl₂/5 mM DTT/50 μM ZnSO₄/10% glycerol) containing 250 mM NaCl. The washed cellular pellet was resuspended in 15 ml buffer A/250 mM NaCl/1 mM PMSF and sonicated for a total of 2-6 minutes with frequent cooling in ice. The sonicated extract was spun for 10 minutes at 6,000 x g in a SS-34 rotor, and the resulting pellet resuspended in 15 ml buffer A/250 mM NaCl/5 M urea/1 mM PMSF. The resuspended pellet was sonicated again for a total of 2 minutes with frequent cooling on ice. An additional 15 ml of buffer A/250 mM NaCl/5 M urea was added to the resuspended extract and mixed by inversion for 24 hours at 4°C. The extract was spun for 20 minutes at 11,000 x g. The supernatant (crude TFIIIA extract) was carefully
decanted. We refer to this fraction as S2.

Buffer A/250 mM NaCl/5 M urea saturated with (NH₄)₂SO₄ at 4°C was added to the S2 fraction to bring it to 40% saturation with (NH₄)₂SO₄ and mixed at 4°C for 1 hour. The 40% (NH₄)₂SO₄ precipitate was pelleted by centrifuging for 20 minutes at 11,000 x g. Additional buffer A/250 mM NaCl/5 M urea saturated with (NH₄)₂SO₄ was added to the 40% (NH₄)₂SO₄ supernatant to achieve 80% (NH₄)₂SO₄ saturation and mixed at 4°C for 1 hour. After the sample was centrifuged for 20 minutes at 11,000 x g, the 80% (NH₄)₂SO₄ supernatant was decanted and the pellet was redissolved in buffer A/5 M urea, using 3.4 ml of buffer per 0.1 ml of pellet.

The 40-80% (NH₄)₂SO₄ fraction was applied to a 3 ml BioRex 70 column (BioRad) pre-equilibrated in buffer A/250 mM NaCl/5 M urea. After washing the column with the same buffer, bound protein was eluted with a linear 18 ml gradient of NaCl (250 mM to 1 M in buffer A/5 M urea). Following analysis of column fractions on silver-stained SDS-polyacrylamide gels (129), fractions enriched for TFIIIA were pooled and dialyzed (Spectra/Por molecularporous membrane #3) overnight against buffer A/1.2 M (NH₄)₂SO₄.

Following dialysis, the pooled BioRex 70 fractions were filtered through a 0.45 micron Millex-HV filter (Millipore) to remove insoluble material. The resulting
solution was applied to a 1 ml phenyl-Superose column (Pharmacia) pre-equilibrated with buffer A/1.2 M (NH₄)₂SO₄.
Proteins were eluted with a reverse linear 18 ml gradient of (NH₄)₂SO₄ (1.2 M to 0.5 M in buffer A), followed by a step gradient (0.5 M to 0 M (NH₄)₂SO₄). Column fractions were analyzed on silver-stained SDS-polyacrylamide gels to identify fractions containing TFIIIA substantially free of other contaminants. These fractions were used as the source of recombinant TFIIIA in functional assays.

6. Protein Concentration Determination

The method of Bradford (9) was used to determine the concentration of TFIIIA in the recombinant and ovarian preparations. BSA was used as the standard, and the final TFIIIA concentration was determined by multiplying the apparent concentration by 0.62 to account for differential binding of dye by TFIIIA and BSA (134).

7. Gel Retardation Assay

A 199-bp DNA fragment (XhoI fragment of pST5RD-XhoI) containing the *Xenopus borealis* somatic-type 5S rRNA gene was labeled using [α-³²P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I. TFIIIA was incubated at a concentration of 8 nM with variable concentrations (1–9.1 nM) of the labeled fragment in 20 μl reactions containing
buffer B (20 mM Tris-Cl, pH 7.5/7 mM MgCl₂/10 μM ZnCl₂/1 mM DTT/10% glycerol/70 mM KCl) supplemented with 10 μg/ml poly(dI·dC) and 100 μg/ml BSA. After incubating the reactions at 25°C for 30 minutes the samples were quickly cooled on ice and then immediately loaded onto a non-denaturing 6% (0.12 % bis-acrylamide) polyacrylamide gel containing 5% glycerol. The gel was precooled to 4°C and pre-run at 300 volts for 30-60 minutes. The gel and running buffer consisted of 0.025 M Tris base/0.2 M glycine. Electrophoresis was for 3 hours at 4°C. Wet gels were dried on cellophane (Bio-Rad) prior to autoradiography. Cerenkov radiation in gel slices, corresponding to the "free" and "bound" bands on the autoradiographs, was measured (Beckman, LS 1801) to determine the amount of DNA which was free or complexed with TFIIIA. Scatchard analysis (100) of these data was used to estimate equilibrium dissociation constants (K₀'s) and the concentration of active TFIIIA in our preparation, assuming a binding stoichiometry of 1:1 (4,108).

8. DNase I Protection

100 nanograms of DNA template (pC9Xbs201) containing the 5S RNA gene was labeled on the 5' end of the non-coding strand (HindIII site) using [γ-³²P]ATP and T4 polynucleotide kinase after having dephosphorylated the 5' ends with calf
intestine alkaline phosphatase. Alternatively, the DNA was labeled on the 3’ end of the coding strand (HindIII site) using [α-32P]dCTP and modified T7 DNA polymerase (112,113) (Sequenase, United States Biochemical). Secondary digestion with EcoRI removed one of the labeled ends onto a small DNA fragment that did not interfere with the subsequent footprints. The labeled DNA (1 nM) was preincubated at 25°C for 30 minutes with increasing amounts TFIIIA (1 nM, 5 nM, 10 nM and 20 nM) in buffer B supplemented with 100 μg/ml BSA. DNase I (18 ng) was added and the reactions incubated at room temperature for 90 seconds. The reactions were stopped by the addition of an equal volume of 150 mM NaCl/50 mM Tris-Cl, pH 8.0/5 mM EDTA, pH 8.0/0.5% SDS/50 μg/ml glycogen (SETS/glycogen). Following extraction with phenol and precipitation in ethanol, products were run on 8% polyacrylamide/8 M urea sequencing gels. Wet gels were transferred to Whatman 3M paper prior to autoradiography.

9. Transcription Assay

A TFIIIA-dependent in vitro transcription assay was used to determine the transcriptional activity of the recombinant TFIIIA. Template DNA (pc9Xbs201) (2.5 nM) was preincubated at 25°C for 60 minutes in a 20 μl reaction containing buffer B, 5 μl of a Dignam nuclear extract (25) prepared from a Xenopus laevis kidney cell line, 20 units
RNAsin (Promega), and variable concentrations of TFIIB (0 nM, 0.05 nM, 0.2 nM, 0.5 nM, 0.8 nM, 1.5 nM, and 3.0 nM). Following the preincubation period, 10 μCi [α-32P]UTP (800 Ci/mmole) was added along with ATP, GTP, and CTP to a final concentration of 500 μM and UTP to a final concentration of 50 μM. Transcription proceeded for an additional 60 minutes, and was then stopped by the addition of 150 μl of SETS/glycogen. Following extraction with phenol and precipitation in ethanol, transcription products were resolved on a 10% polyacrylamide/8.33 M urea gel. Wet gels were dried on cellophane prior to autoradiography. The amount of 5S RNA produced in each reaction was determined by measuring Cerenkov radiation in gel slices.

B. Results
1. Expression and Purification of TFIIB

We have made use of the lac-controlled, plasmid-based T7 RNA polymerase expression system of Studier and colleagues (111) for production of Xenopus TFIIB in E. coli. This system is far superior to several others we or others (32,49,76,120) have tested, producing approximately 5-10 fold more TFIIB in crude form than we have obtained using any other expression method. Following IPTG induction for 4 hours, TFIIB constitutes 15%-20% of the total cellular protein (See Figure 7 and Table II); this
Figure 7. Coomassie blue-stained SDS-polyacrylamide gel of fractions from the purification of recTFIIIA.
Lane a: whole cell extract, without IPTG induction (100 μl of culture); lane b: whole cell extract, with IPTG induction (100 μl of culture); lane c: S2 fraction (equivalent to 120 μl of culture); lane d: 40%-80% (NH₄)₂SO₄ cut (20 μg total protein); lane e: pooled BioRex 70 fraction (20 μg total protein); lane f: phenyl-Superose fraction (20 μg total protein).
Table II. Purification of Recombinant TFIIIA from *Escherichia coli*

Values represent yields from 1 liter of culture.

<table>
<thead>
<tr>
<th>Step</th>
<th>Purity a</th>
<th>TFIIIA b</th>
<th>Step yield</th>
<th>Over all yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>mg</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Whole cell extract</td>
<td>19</td>
<td>67 c</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>S2 fraction</td>
<td>26</td>
<td>39</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>40-80% (NH₄)₂SO₄</td>
<td>42</td>
<td>30</td>
<td>77</td>
<td>45</td>
</tr>
<tr>
<td>BioRex 70</td>
<td>76</td>
<td>11.4</td>
<td>38</td>
<td>17</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>&gt;95</td>
<td>2.4</td>
<td>21</td>
<td>4</td>
</tr>
</tbody>
</table>

a Protein purity determined from densitometric scanning of the SDS-polyacrylamide gel of Figure 7.

b Concentration of TFIIIA calculated as follows:

\[
\text{Concentration} = \frac{\text{Sample's protein concentration} \times \text{Sample volume} \times (\% \text{ purity})}{(0.62)}
\]

The method of Bradford (9) was used to determine the protein concentration of the samples. BSA was used as the standard, and the final concentration of TFIIIA was determined by multiplying the apparent concentration by 0.62 to account for differential binding of dye by TFIIIA and BSA (134).

c Estimated from the SDS-polyacrylamide gel in Figure 7 where the TFIIIA in the phenyl-Superose fraction (lane f) was used as a standard.
corresponds to about 60 to 70 milligrams of recTFIIIA per liter of culture. Based on sedimentation analysis, however, the recTFIIIA is in a highly aggregated and largely insoluble form in the initial crude extract (data not shown). We found that induction of cultures at 19°-25°C rather than at 37°C results in much higher solubility of the recTFIIIA; unfortunately, analysis of this protein by sedimentation on glycerol gradients suggests that, although soluble, the recTFIIIA remains aggregated in particles exhibiting a heterogeneous size distribution (data not shown). We also found that the aggregated and insoluble TFIIIA produced at 37°C can be partially solubilized by treatment with 5 M urea. These solubilized molecules are reduced to monomer form. Since yields of recTFIIIA are much higher at 37°C (data not shown), since 5 M urea treatment is known not to adversely affect activity of TFIIIA obtained from ovaries (108), and since protein produced at the lower temperatures of induction would nonetheless require urea treatment to promote disaggregation, we have elected to induce TFIIIA production at 37°C and solubilize, using 5 M urea, the initially highly aggregated protein found in the insoluble cell fraction following sonication and centrifugation. Subsequent purification has been carried out on this material. Approximately 60% of the recTFIIIA is solubilized in the initial 5 M urea extraction
step, and recTFIIIA comprises about 25% of the protein in the resulting fraction. Overall, the 5 M urea treatment yields a 1.5 fold enrichment of recTFIIIA as compared to an untreated whole cell extract.

We tested the solubility of TFIIIA relative to other polypeptides in the 5 M urea extract at a variety of (NH₄)₂SO₄ concentrations. Using a 40%-80% (NH₄)₂SO₄ cut, we can achieve an additional 1.5 fold enrichment of recTFIIIA. In most cases (Table II, for example), the yield of recTFIIIA is quite high (greater than 75%). We have found some variability in recoveries at this step, however, and it can probably be eliminated with little or no effect on the ultimate purity of the final recTFIIIA preparation (data not shown). This may be advisable if high yield is particularly important.

We analyzed the behavior of recTFIIIA on a variety of ion exchange and affinity columns, and found that substantial and roughly equivalent purification can be achieved on a number of resins, including hydroxyapatite, phosphocellulose, BioRex 70, and MonoS (data not shown). We detected no binding, and minimal purification, on a MonoQ column under the conditions tested. For our purposes, we elected to further purify the 40%-80% (NH₄)₂SO₄ fraction by chromatography on BioRex 70 in the presence of 5 M urea. Using a 250 mM to 1 M linear NaCl gradient, recTFIIIA
elutes from the column with a peak at about 400 mM NaCl. Total recovery from the column is high, and recovery in the pooled fractions may be as high as 40-60% (Table II). The major contaminant following BioRex 70 chromatography is a polypeptide of about 37,000 daltons that co-chromatographs with recTFIIDA on a variety of columns and reacts with mono-specific TFIIDA antibodies raised against purified ovarian TFIIDA (Figure 8A). We therefore believe that this polypeptide is a truncated form of TFIIDA. Furthermore, the absence of an analogous shorter form of TFIIDA in preparations of a mutant form of protein lacking C-terminal sequences suggests to us that this shorter polypeptide may be truncated at the C-terminus (data not shown).

Prior to chromatography on phenyl-Superose, urea was removed from the TFIIDA preparation by dialysis while simultaneously introducing a high concentration of salt. The high salt concentration is necessary to ensure binding to phenyl-Superose and may also be necessary to guard against aggregation of the protein, since it exhibits limited solubility in the absence of urea and in the presence of low concentrations of salt. We have found considerable variation in the amount of recTFIIDA that precipitates and is lost during this dialysis step. In the example of Table II, recTFIIDA recovery following dialysis and filtration was only 60%, but yields are generally
Figure 8. Western blot analysis using anti-TFIIIA antibodies.

A. lane 1: BioRex 70 pooled fraction; lane 2: phenyl-Superose fraction. Each lane contains approximately 500 ng total protein.

B. lane 1: phenyl-Superose fraction of recTFIIIA; lane 2: ovarian TFIIIA. Each lane contains approximately 500 ng total protein.
somewhat higher than this. We found that phenyl-Superose chromatography is necessary to separate full length recTFI III A from the 37,000 dalton contaminant. RecTFI III A elutes in a reverse linear salt gradient with a peak at about 0.95 M (NH₄)₂SO₄, while the 37,000 dalton truncated form elutes with a peak at about 0.86 M (NH₄)₂SO₄. Only the fractions at the leading edge of the recTFI III A peak were pooled in order to minimize contamination with the 37,000 dalton polypeptide. These initial column fractions yielded 2-3 milligrams recTFI III A at greater than 95% purity, based on densitometric scanning of coomassie blue stained SDS-polyacrylamide gels. Again, the total yield of recTFI III A from the column is high, but only about 20-30% of the recTFI III A is recovered free of the contaminating 37,000 dalton polypeptide (Figure 8A). In some cases, we have pooled the remaining TFI III A-containing fractions, supplemented the pooled fractions with additional salt, and re-chromatographed the pooled fractions on phenyl-Superose to obtain additional protein free of the 37,000 dalton contaminant. Such a procedure was not used to derive the data of Figure 7.

These results are summarized in Figures 7 and 8 and Table II. RecTFI III A can be obtained at greater than 95% purity at a yield of 2-3 mg per liter of bacterial culture, representing an overall yield of about 4%. The mobility of
recTFIIIA on SDS-PAGE was slightly greater than that of the ovaTFIIIA control (Figure 8B). We have not determined the reason for this slight difference in electrophoretic mobility, but it does not appear to affect TFIIIA function, as we show below.

2. Functional Assays

We tested the activity of recTFIIIA relative to that isolated from immature ovaries of *Xenopus laevis* (108) (ovaTFIIIA) using a variety of *in vitro* assays. We measured the equilibrium dissociation constants (K₀) for binding of the two preparations of TFIIIA to a 199-bp DNA fragment containing the *Xenopus borealis* somatic-type 5S rRNA gene. This was accomplished using a gel retardation assay to separate DNA fragments bound to TFIIIA from those that are unbound and analyzing the resulting data according to Scatchard (100). The data of Figure 9B demonstrate that recTFIIIA and ovaTFIIIA bind to the 5S rRNA gene-containing DNA fragment with K₀ values of 0.43 ± 0.06 nM and 0.41 ± 0.03 nM, respectively. These errors reflect the precision obtained within a single experiment leading to the generation of one Scatchard curve. We have found that there is considerably greater variability in the K₀ values obtained in separate experiments; nonetheless, estimates of the K₀ generally vary by no more than a factor of two. The
Figure 9. TFIIIA binding to the 5S rRNA gene.

A. Autoradiograph from a gel retardation experiment. lane 1: no TFIIIA; lane 2: 6 nM recTFIIIA; lane 3: 6 nM ovaTFIIIA. The binding reaction in each case contained 8 nM 5S DNA (XhoI fragment from pST5RD-XhoI). "Bound" indicates the position of the TFIIIA/DNA complex, and "Free" indicates the position of the free DNA.

B. Scatchard analysis of gel retardation assays performed with fixed amounts of recTFIIIA (*) or ovaTFIIIA (C) and variable amounts of 5S DNA. Binding reactions contained 8 nM TFIIIA and 1-9 nM DNA (XhoI fragment from pST5RD-XhoI). Data were fit to a straight line using a linear least squares analysis. The calculated equilibrium dissociation constants ($K_0$, equal to (-slope$^{-1}$)) are 0.43 ± 0.06 nM and 0.41 ± 0.03 nM for recTFIIIA and ovaTFIIIA, respectively. Errors given are derived from the standard error of the regression coefficient ($K_a$).
mobility of the recTFIIIA/DNA complexes in nondenaturing polyacrylamide gels is slightly greater than that of the ovaTFIIIA/DNA complexes (Figure 9A), paralleling the difference in mobility seen with the purified proteins in SDS-PAGE analysis. Based on the x-intercepts from the Scatchard curves, we estimate the recTFIIIA and ovaTFIIIA preparations to be 56% and 52% active, respectively, assuming a binding stoichiometry of 1:1 (4,108). Of course, these values are subject to both experimental and systematic errors in our determination of TFIIIA concentration, and may be underestimates.

DNase I protection (footprinting) experiments provide qualitative information concerning the interaction of TFIIIA with the internal control region of 5S rRNA genes. RecTFIIIA produced a protection pattern indistinguishable from that produced by ovaTFIIIA on both the coding and non-coding strands of the 5S rRNA gene (Figure 10), with identical boundaries of protection and including the presence of hypersensitive sites at various positions. Complete protection of the DNA template at a concentration of 5 nM was also afforded by roughly equivalent amounts of the two protein preparations, further corroborating that recTFIIIA and ovaTFIIIA bind with approximately equal affinities.

We also tested the ability of recTFIIIA to support
Figure 10. DNase I protection assay.

1 nM end-labeled DNA (pC9Xbs201) was incubated with increasing amounts of recTFIIDA or ovaTFIIDA prior to digestion with DNase I.

A. Non-coding strand footprint. lane 1: no DNase I added; lanes 2-10: 18 ng DNase I added for 90 seconds; lanes 3-6: 1 nM, 5 nM, 10 nM, or 20 nM recTFIIDA added, respectively; lanes 7-10: 1 nM, 5 nM, 10 nM, or 20 nM ovaTFIIDA added, respectively.

B. Coding strand footprint. lane 1: no DNase I added; lanes 2-10: 18 ng DNase I added for 90 seconds; lanes 3-6: 1 nM, 5 nM, 10 nM, or 20 nM recTFIIDA added, respectively; lanes 7-10: 1 nM, 5 nM, 10 nM, or 20 nM ovaTFIIDA added, respectively.
transcription of a 5S rRNA gene in vitro. Transcriptional efficiency was measured in a nuclear extract prepared from a *Xenopus laevis* kidney cell line (25) following supplementation with variable amounts of TFIIIA isolated from *E. coli* or *Xenopus* ovaries. As can be seen in Figure 11, transcription of a 5S rRNA gene in this extract is substantially dependent on added TFIIIA. Furthermore, quantitative analysis of the in vitro transcription data demonstrates that recTFIIIA and ovaTFIIIA support transcription at equivalent levels, measured either as a transcriptional response at low, subsaturating levels of added TFIIIA, or as the maximal level of transcription attained with saturating amounts of TFIIIA. It is also worth noting that the linear range of response to added TFIIIA occurs at a considerably lower concentration of TFIIIA than would be predicted on the basis of the binding affinity of TFIIIA to the 5S rRNA gene in the absence of other factors. This is consistent with earlier data suggesting that the affinity of TFIIIA for a 5S rRNA gene in a complete transcription complex is much higher than that exhibited by TFIIIA alone (8,51,60,105,127).
Figure 11. TFIIIA-dependent in vitro transcription assay. As described in the text, 2.5 nM DNA (pC9Xbs201) was incubated with variable amounts (0 nM, 0.5 nM, 0.2 nM, 0.5 nM, 0.8 nM, 1.5 nM, 3.0 nM) of recTFIIIA (*) or ovaTFIIIA (□) and a fixed amount of a TFIIIA-deficient nuclear extract from a *Xenopus laevis* kidney cell line to permit the synthesis of $^{32}$P-labeled 5S RNA. Transcription products were separated on a denaturing polyacrylamide gel, cut out following autoradiography, and Cerenkov radiation measured to determine the level of transcriptional activation by the added TFIIIA.
C. Discussion

Previous attempts to express *Xenopus* TFIIIA in *E. coli* using various methods (32,49,76,120) resulted in rather low yields of protein, relatively impure preparations, and/or proteins with foreign amino acids at the N-terminus. Precise quantitative measures of TFIIIA activity, which may prove to be particularly important in assessing the effects of various mutations on TFIIIA function, require pure preparations of protein. Biophysical studies of TFIIIA will require large quantities of the protein that are difficult to obtain directly from *Xenopus* and will benefit greatly from the availability of large quantities of TFIIIA mutants as well as wild-type protein. The method of production and purification we have described here will make these approaches feasible. We have shown that TFIIIA produced in and purified from *E. coli*, while having a slight difference in mobility on SDS-polyacrylamide gels, is functionally indistinguishable, both qualitatively and quantitatively, from the protein isolated from *Xenopus* ovaries. It should therefore be possible to measure even small effects of specific mutations on protein function and to use recTFIIIA to obtain structural data which we are confident reflects the structure and function of the normal frog protein.
CHAPTER III

STRUCTURAL, DNA-BINDING AND KINETIC ANALYSES OF *Xenopus laevis* TRANSCRIPTION FACTOR IIIA ZINC FINGER MUTANTS

A. Introduction

Until recently, the analysis of TFIIIA mutants was hampered by methods of expression and purification which resulted in low yields, low purity and/or preparations of TFIIIA mutant protein containing full length as well as truncated TFIIIA mutant polypeptides. With the new expression and purification method described by Del Rio and Setzer (22), it is now possible to obtain high yields of TFIIIA mutant protein at greater than 95% purity, making it possible to perform qualitative as well as quantitative biochemical and structural analyses on TFIIIA mutant proteins.

Several questions which were posed in the Introduction (Chapter I) are now addressed in this chapter: (i) How important are the conserved Zn-binding amino acids present in the zinc fingers of TFIIIA in mediating structural stability in an individual zinc finger in the context of the entire protein?, (ii) Do the various zinc fingers in TFIIIA contribute equally to the binding energy when TFIIIA binds to the intragenic control region (ICR), or are some more important than others? In
the latter case, which fingers are the most important?, and (iii) Can we map specific fingers to particular regions of the ICR by analyzing TFIIIA mutants containing 8 wild-type fingers and one mutated finger?, are addressed in this chapter. Questions relating to the transcriptional activity of TFIIIA are addressed in Chapter IV.

With the use of oligonucleotide-directed mutagenesis and the ability to express and purify TFIIIA mutants, we had the necessary tools and resources to generate and analyze any TFIIIA mutant proteins. Instead of generating TFIIIA mutants at random, we decided to take a more systematic approach to the mutational analysis of TFIIIA.

Using the established zinc finger motif as a starting reference, we decided to target mutations to the conserved zinc ion ligands which had been postulated to be essential for the structural integrity and function of the zinc finger (2,69). Histidine was selected as the target for mutagenesis. We reasoned that the amino acid to be substituted for the histidine residue should not be a potential zinc ligand, but the substitution should be otherwise conservative on the basis of charge, hydrophobicity and size. Amino acid side chains are obvious candidates as ligands to the zinc in metalloproteins. These chemical groups include: (i) the carboxyl groups of aspartyl and glutamyl residues; (ii) the
N-terminal $\alpha$-amino groups of the peptide chain; (iii) the $\varepsilon$-amino groups of lysyl residues; (iv) the imidazole nitrogens of histidyl residues; (v) the phenolic hydroxyl groups of tyrosyl residues; (vi) the sulfhydryl groups of cysteinyl residues; and (vii) the guanidinium groups of arginyl residues. (15) Of these, only three have been identified as zinc ligands in zinc metalloproteins. These include the imidazole nitrogen of histidine, the $\tau$-carboxyl group of glutamic acid, and the sulfhydryl group of cysteine (15). Therefore, an appropriate choice for the histidine substitution is asparagine since it is a relatively conservative substitution that nonetheless prevents zinc ion coordination at that particular position in the polypeptide chain. In fact, the side chain of asparagine may be superimposed on that of histidine so that the nitrogen atom in the amide group of asparagine is in an equivalent position to one of the nitrogens in the imidazole ring of histidine (65) (Figure 12). An additional benefit in choosing asparagine was that mutagenesis from histidine, coded by CAT or CAC, to asparagine, coded by AAT or AAC, required only a single base pair substitution.

As an initial set of TFIIB mutants, we have generated TFIIB zinc finger mutations which contain a histidine to asparagine substitution in the third zinc ligand, the first of the two conserved histidine residues (Figure 13),
Figure 12. Comparison of side chains of histidine (His) and asparagine (Asn), illustrating the approximate equivalence of the nitrogen atoms.
resulting in a protein with one mutated zinc finger and eight wild-type fingers (Figure 14).

The analyses consisted of (i) limited proteolysis using thermolysin as a probe for structural perturbations; (ii) DNaseI protection assays; and (iii) gel retardation assays to determine equilibrium binding constants and kinetic dissociation rates:

B. Methods and Materials
1. Bacterial Strains and Vectors

pXLTF3AR contains the EcoRI fragment of pUC3a1.b (42), a Xenopus laevis TFIIIA cDNA clone, inserted into the EcoRI site of pIBI20, a commercially available phagemid (International Biotechnologies, Inc.). pXLTF3AR, pGA1:EP(300-304) (22) and pGA11-NdeI (22) were used for the oligonucleotide-directed mutagenesis of the TFIIIA sequence. pT7-TF (120) and pTA102 (22) contain a Xenopus laevis TFIIIA sequence. pET11b (111) was used for the expression of the mutant TFIIIA proteins in E. coli (22).

pC9Xbs201 (22) contains the Xenopus borealis somatic-type 5S rRNA gene from the EcoRI/BamHI fragment of plasmid pXbs201 (7). pST5RD-XhoI (22) was derived from pST5RD (106) by introducing an XhoI site immediately downstream of the 5S rRNA gene termination signal using the oligonucleotide 5'-GGCTTTTGCTCGAGTGCCATTCTG-3' in a site-directed
Figure 13. Location of the histidine to asparagine mutations. The invariant cysteines (C) and histidines (H) are shown tetrahedrally coordinated by the zinc ion. The arrows denote the position of the histidine (H) to asparagine (N) substitution.
Figure 14. Schematic representation of the set of nine TFIIIA zinc finger mutants. The mutant proteins were named H"#"N, where "#" denotes the amino acid position in the TFIIIA protein sequence containing the histidine (H) to asparagine (N) substitution. The location and the number of the zinc finger mutated for a specific TFIIIA mutant are indicated by the filled-in oval and repeat number, respectively.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Location</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>H33N</td>
<td>NH₂⁻</td>
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mutagenesis experiment. Digestion of pST5RD-XhoI with XhoI produces a 199-bp DNA fragment containing the entire 5S rRNA gene from -49 to +124 with some flanking polylinker sequence at the 5' end.

In general, *E. coli* K-12 strain NM522 (43) was used for growth, isolation, and analysis of the DNA vectors. *E. coli* B strain BL21(DE3) (F' ompT r_{53}'m_{15}) (110,111) was used to express the cloned TFIIIA gene.

2. Mutagenesis and Cloning

Table III lists the various oligos used for oligonucleotide-directed mutagenesis of the TFIIIA sequence. The various TFIIIA zinc finger mutants were named H""N, where "#" denotes the amino acid position in the TFIIIA protein sequence containing the histidine to asparagine substitution. Most oligos were degenerate in the first position of the triplet coding for the histidine amino acid. The mutations for TFIIIA zinc fingers 1-7 were generated using a modified version of the site-directed mutagenesis method of Taylor, *et al.* (115,116) which uses alpha-phosphorothioate analogs (dNTPaS) of dNTPs. The remaining two TFIIIA zinc finger mutants, fingers 8 and 9, were generated by the oligonucleotide-directed mutagenesis method described by Setzer *et al.* (106) based on a chromogenic plate assay for bacterial colonies producing
Table III. Oligonucleotides used for site-directed mutagenesis

<table>
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<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Mutation</th>
</tr>
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<tbody>
<tr>
<td>A138(GAT)-C</td>
<td>5' GCACAGAT CAT CGCCTGCAG 3'</td>
<td>H33N</td>
</tr>
<tr>
<td>A228(GAT)-C</td>
<td>5' TGAGTGAG CAT GCGGTATA 3'</td>
<td>H63N</td>
</tr>
<tr>
<td>A318(GAT)-C</td>
<td>5' GTTAAAGT CAT CTTCTTCAT 3'</td>
<td>H93N</td>
</tr>
<tr>
<td>A414(GAT)-C</td>
<td>5' GAACGTGAT CAT AACCTTTAA 3'</td>
<td>H125N</td>
</tr>
<tr>
<td>A504(GAT)-C</td>
<td>5' TTTTTTCA CAT AGTTTTAAA 3'</td>
<td>H155N</td>
</tr>
<tr>
<td>A588(GAT)-C</td>
<td>5' TGCCACGT CAT TTCAAGTA 3'</td>
<td>H183N</td>
</tr>
<tr>
<td>A669(GAT)-C</td>
<td>5' TTTCTGAT CAT ATCCCTCAA 3'</td>
<td>H210N</td>
</tr>
<tr>
<td>A762(GAT)-C</td>
<td>5' TTGTATAT CAT GCTTCTAAG 3'</td>
<td>H241N</td>
</tr>
<tr>
<td>H272N/EcoRI</td>
<td>5' CAACCTGAATTCCTTCTAGG 3'</td>
<td>H272N</td>
</tr>
<tr>
<td>Oligo 802</td>
<td>5' CGGATGCTTTTTTCTTTCCTT 3'</td>
<td></td>
</tr>
</tbody>
</table>
functional β-galactosidase.

The mutagenesis and cloning schemes used to obtain the mutations in the nine TFIIIA zinc fingers are outlined in Figures 15-17. The final TFIIIA zinc finger mutant sequences were cloned into pET11b for expression in E. coli.

3. Expression and Purification

Expression and purification of the TFIIIA zinc finger mutants were as described for the wild-type protein (see Chapter II) (22) with the following modifications: (i) as a guard against continued proteolysis, leupeptin (1μg/ml), pepstatin A (1μg/ml) and PMSF (0.5 mM) were used during the sonication and extraction steps; (ii) the 80% (NH₄)₂SO₄ pellets were redissolved in sufficient buffer A (20 mM Na⁺HEPES, pH 7.4/5 mM MgCl₂/5 mM DTT/50 μM ZnSO₄/10% glycerol)/5 M urea to obtain a conductance reading equal to the conductance of a buffer A/250 mM NaCl/5 M urea solution; and (iii) the final protein fractions from the phenyl-superose column were supplemented with leupeptin (1 ug/ml), pepstatin A (1 ug/ml) and PMSF (0.5 mM). The method of Bradford (9) was used to determine the concentration of the various zinc finger mutant preparations. BSA was used as the standard, and the final protein concentrations were determined by multiplying the apparent concentration by
Figure 15. Mutagenesis and cloning scheme for TFIIIA zinc finger mutants 1 through 7. The names of the TFIIIA zinc finger mutants corresponding to mutations in zinc fingers 1-7 are listed in Figure 14. The specific oligonucleotides used for the mutagenesis of TFIIIA zinc fingers 1-7 are listed in Table III.
Figure 16. Mutagenesis and cloning scheme for TFIIIA zinc finger mutant 8. Oligonucleotide A762(GAT)-C (Table III) was used to mutate the histidine (H) residue at position 241 to asparagine (N). Oligonucleotide 802 (Table III) was used to correct a frame-shift deletion of an A residue in the nucleotide coding sequence of TFIIIA.
Figure 17. Mutagenesis and cloning scheme for TFIIB zinc finger mutant 9. Oligonucleotide H272N/EcoRI (Table III) was used to mutate the histidine (H) residue at position 272 to asparagine (N).
0.62 to account for differential binding of dye by TFIIB and BSA (134).

4. Thermolysin Sensitivity

Limited digestion of TFIIB and the TFIIB zinc finger mutants by thermolysin (66,126) was carried out in buffer B (20 mM Tris-Cl, pH 7.5/7 mM MgCl₂/10 μM ZnCl₂/1 mM DTT/10% glycerol/70 mM KCl) supplemented with 2 mM CaCl₂ at an enzyme-to-substrate ratio of 1:100 by weight. The proteolysis was allowed to proceed at either 25°C or 37°C for 30 minutes. Proteolysis was stopped by the addition of an equal volume of 125 mM Tris (pH 6.8)/4% SDS/10% β-mercaptoethanol/20% glycerol/0.025% BPB dye. Samples were analyzed on 12% polyacrylamide SDS gels as described (59).

5. Scatchard Analyses

Either a 199-bp DNA fragment (XhoI fragment of pST5RD-XhoI) or a 248-bp DNA fragment ( HindIII-BamHI fragment of pC9Xbs201) containing the Xenopus borealis somatic-type 5S rRNA gene was labeled using [α-³²P]dCTP and modified T7 DNA polymerase (112,113) (Sequenase, United States Biochemicals). TFIIB zinc finger mutants were incubated at a constant concentration of protein with a constant concentration of the labeled fragment and increasing concentrations of unlabeled DNA fragment in 20 μl reactions
containing buffer B supplemented with 10 μg/ml poly(dI•dC) and 100 μg/ml BSA. After incubating the reactions at 25°C for 30 minutes the samples were quickly cooled on ice and applied immediately to a non-denaturing 6% (0.12% bis-acrylamide) polyacrylamide gel containing 5% glycerol. The gel was precooled to 4°C and pre-run at 300 volts for 30-60 minutes. The gel and running buffer consisted of 0.025 M Tris base/0.2 M glycine. Electrophoresis was for 4-5 hours at 4°C. Wet gels were fixed in 40% methanol/10% acetic acid, transferred to Whatman 3MM filter paper and sealed in a "seal-a-meal" bag prior to scanning on an Ambis Radioanalytic Imaging System (RIS). CPM in 'bound' and 'free' bands was determined using the Ambis RIS software package. Scatchard analysis (100) of these data was used to estimate equilibrium dissociation constants (Kₜ's) of the mutants assuming a binding stoichiometry of 1:1 (4,108).

6. Dissociation Rate Analyses

A 248-bp DNA fragment (HindIII-BamHI fragment of pc9Xbs201) containing the *Xenopus borealis* somatic-type 5S rRNA gene was labeled using [α-³²P]dCTP and modified T7 DNA polymerase (112,113) (Sequenase, USB). Binding reaction conditions and non-denaturing polyacrylamide gel electrophoresis were similar to those used for the Scatchard analyses. Mutant proteins were pre-incubated with
the labeled DNA for 30' at 25°C. To initiate the reaction, excess unlabeled pc9Xbs201, also at 25°C, was added. These reagents were carefully mixed. At the times indicated in the figure legends, an aliquot was removed and applied immediately to a running non-denaturing polyacrylamide gel. After electrophoresis, the fraction of complexes remaining was determined by scanning the gel on an Ambis RIS.

7. DNaseI Protection

DNase I footprinting experiments (36) were performed to assay for qualitative changes between the TFIIIA zinc finger mutants and 5S rRNA gene ICR. Varying amounts of DNA template (pc9Xbs201) containing the 5S RNA gene was labeled on the 5' end of the non-coding strand, T3NC, (HindIII site) using [γ-32P]ATP and T4 polynucleotide kinase after having dephosphorylated the 5' ends with calf intestine alkaline phosphatase. Alternatively, the DNA was labeled on the 5' end of the coding strand, T5C, (BamHI site) using [γ-32P]ATP and T4 polynucleotide kinase after having dephosphorylated the 5' ends with calf intestine alkaline phosphatase. Secondary digestion with EcoRI and PstI removed one of the labeled ends onto a small DNA fragment that did not interfere with the subsequent footprints and resulted in DNA fragments of 264-bp and 279-bp, T3NC and T5C, respectively. TFIIIA zinc finger mutant proteins were
preincubated with the labeled DNA for 30' at 25°C. DnaseI (8 ng) was added to the reaction mix and incubated at RT for 15", and the reaction mixture was applied immediately to a running 6% non-denaturing polyacrylamide gel. After electrophoresis and autoradiography, bands corresponding to 'bound' complexes were cut out from the gel. The DNA in the gel slices was recovered by overnight elution (in 500 mM CH₃COONH₄; 10 mM Mg(C₂H₃O₂)₂; 1 mM EDTA; 0.1% SDS; 4 ug/ml glycogen, as carrier) at 37°C. The DNA was then precipitated from the eluate, resuspended in 100ul 0.3 M sodium acetate (pH 5.2) and reprecipitated with 2½ volumes of ethanol. The DNA was then resuspended in 12ul of 95% formamide/tracking-dye mixture prior to electrophoresing on a 5% polyacrylamide/7 M urea/40% formamide sequencing gel. Wet gels were transferred to Whatman 3M paper and dried down prior to autoradiography or scanning on an Ambis RIS.

C. Results

1. Mutagenesis and Cloning

A set of nine TFIIIA mutants containing only one histidine to asparagine substitution in a single zinc finger per TFIIIA molecule was obtained and confirmed in each case by sequencing the entire TFIIIA coding sequence. The mutations for zinc fingers 1 through 7 of TFIIIA were generated using a modified version of the site-directed
mutagenesis method of Taylor, et al. (115,116) which uses alpha-phosphorothioate analogs (dNTPs) of dNTPs. The remaining two zinc finger mutations of TFIIIA, mutations in fingers 8 and 9, were generated by the chromogenic selection method described by Setzer et al. (106). The various TFIIIA zinc finger mutants were named H"#"N, where "#" denotes the amino acid position in the TFIIIA protein sequence containing the histidine to asparagine substitution.

The set of nine TFIIIA zinc finger mutant genes were subcloned into pET11b (Figures 15-17) generating pTAH"#"N vectors. The pTAH"#"N vectors in conjunction with BL21(DE3) strain of E. coli were used to express to TFIIIA zinc finger mutant proteins.

2. Expression and Purification of TFIIIA Mutants

All nine TFIIIA zinc finger mutant proteins were expressed at high levels and were full length (data not shown). The mutant proteins chromatographed and behaved identically to wild-type protein through dialysis prior to the phenyl-superose column. Unlike the full length TFIIIA protein which eluted from the phenyl-superose column at an approximate ammonium sulfate concentration of 0.95 M (22), all TFIIIA zinc finger mutant proteins eluted at lower ammonium sulphate concentrations (Figure 18), indicating an
increase in hydrophobic character of the mutant proteins when chromatographed on the phenyl-superoxide column. The increased hydrophobic character of the TFIIMA zinc finger mutant proteins may have resulted from the disruption of individual zinc fingers thereby exposing to solvent the normally concealed hydrophobic residues which form the hydrophobic core of the zinc finger. Although this is not direct evidence for disruption of individual fingers, it was an intriguing observation. To assess structural alterations directly, thermolysin was used as a probe for structural instability.

3. Thermolysin Sensitivity

A common method used to probe protein structure involves the use of proteases. Unstructured regions will be more susceptible to proteolysis as compared to those that are highly structured. Since the broken finger mutants should contain regions of localized disruption, each mutated protein is hypothesized to contain additional protease sensitive sites which correlate with the mutated region. Using thermolysin (66,126) the zinc finger mutants were subjected to limited proteolysis and compared the resulting digestion patterns to that of the wild-type protein pattern (Figure 19). The wild-type protein contains a thermolysin sensitive site at its carboxyl end resulting
Figure 18. Phenyl-superose column peak elution. The chart demonstrates the ammonium sulphate concentration at which the various TFIIIA zinc finger mutants eluted from the phenyl-superose column using a reverse (NH$_4$)$_2$SO$_4$ gradient. The (NH$_4$)$_2$SO$_4$ concentrations are based on the conductivity (Yellow Springs Instrument, Model 32 conductance meter) of the various TFIIIA zinc finger mutant phenyl-superose fractions.
in a truncated peptide with an approximate Mr of 35,000 daltons, which most likely corresponds to the 30,000 dalton fragment observed by Smith et al. (108) when they subjected 7S particles to limited proteolysis using trypsin, chymotrypsin, papain or thermolysin and the 33,000 dalton fragment observed by Miller et al. (69) with tryptic digestion of 7S particle.

Analysis of the limited thermolysin digestion patterns for the zinc finger mutants reveals that each mutation (with the possible exception of H210N, see below) generates a new and mutant-specific thermolysin sensitive site. The size of the various bands were determined (Table IV) and compared to the size of bands expected as a result of disruptions caused by histidine to asparagine substitutions in specific mutated fingers. The results in Table IV indicate that the observed proteolytic bands map the sensitive sites to the appropriate mutated sites on the TFIIIA amino acid sequence. These results also demonstrate that the zinc finger mutations have resulted in local structural instability and not in global disruption of the tertiary structure of the TFIIIA molecule.

The mutation in the seventh zinc finger of TFIIIA, H210N, did not result in any new thermolysin sensitive site, even with increasing amounts of enzyme (Figure 20). Limited digestion with thermolysin was then carried out at
Figure 19. Proteolytic analysis of TFIIIA zinc finger mutants using thermolysin. Silver-stained SDS-polyacrylamide gel of TFIIIA and TFIIIA zinc finger mutants partially digested with thermolysin. The reactions were at 25°C for 30 minutes with an enzyme-to-substrate-ratio of 1:100. "+" and "-" indicate that thermolysin was added or not added, respectively. Molecular weight standards are indicated.
Figure 20. Proteolytic analysis of H210N and TFIIIA. Silver-stained SDS-polyacrylamide gel of TFIIIA and H210N digested with thermolysin. Lanes 1-6, H210N; lanes 7-11, TFIIIA; and lane 12, molecular weight standards. Reactions were at 25°C with enzyme-to-substrate-ratios of 0:1, lane 1; 1:100, lanes 2 and 7; 1:20, lanes 3 and 8; 1:10, lanes 4 and 9; 1:3.3, lanes 5 and 10; and 1:2, lanes 6 and 11.
Table IV. Thermolysin analysis of TFIIIA zinc finger mutants: expected versus observed polypeptides.

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<td>6-7, 25-29</td>
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<td>15-19, 20-21</td>
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<td>14-16, 21-24</td>
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<td>H210N 7</td>
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<td>H241N 8</td>
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<td>H272N 9</td>
<td>30.3-31.0 3.8-4.5</td>
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</table>

a Sizes calculated assuming (i) hydrolysis of the peptide bonds involving the conserved phenylalanine and leucine residues in the loop region of the zinc finger, ie., between the invariant cysteine and histidine residue; and (ii) a 115.6 daltons per amino acid (39750 daltons/344 amino acids).

b The 35 kdal polypeptide observed for TFIIIA was also expected for each of the TFIIIA zinc finger mutants.

c The 35 kdal polypeptide was observed for all the TFIIIA zinc finger mutants.

d No other bands detected besides the 35 kdal polypeptide.
37°C for H210N and the wild-type protein (data not shown). The increase in temperature to 37°C did not demonstrate any additional thermolysin sensitive site specific for the H210N mutant.

4. Scatchard Analyses

The equilibrium dissociation constants (K₀'s) for the various preparations of TFIIB zinc finger mutants was measured by binding to a DNA fragment containing the *Xenopus borealis* somatic-type 5S rRNA gene. This was accomplished using a gel retardation assay (35,37,87) to separate DNA fragments bound to TFIIB zinc finger mutants from those that are unbound and analyzing the resulting data according to Scatchard (100). Figures 22A-30A demonstrate that the nine TFIIB zinc finger mutants were all able to bind the somatic-type 5S rRNA gene, making it possible to perform Scatchard analysis on the gel-shift data as was done for the wild-type TFIIB (Figure 21A). The results of multiple Scatchard analyses for the TFIIB zinc finger mutant proteins are tabulated in Table V. These results demonstrate that for specific TFIIB zinc finger mutants, the K₀ values obtained from multiple experiments were within a factor of two. The K₀ and percent standard error of the regression coefficient values from Table V were used to calculate a weighted
average (109) for the $K_0$ of individual TFIIIA zinc finger mutants (Table VI). To do so, the $K_0$ from individual experiments receive a weight inversely proportional to the standard error (Figure 31). The weighted $K_0$ averages (Table VI) demonstrate that the mutations in TFIIIA zinc fingers 3 and 4 result in the greatest decrease in binding affinity, 27-fold and 12-fold respectively. The Scatchard curves in Figures 21B-30B were generated by fixing the line for a particular zinc finger mutant to the X-axis ([bound DNA]) and using a slope for the line equal to $-1/K_0$, where $K_0$ is the weighted $K_0$ average. The 95% and 99% confidence intervals for the weighted $K_0$ averages for the TFIIIA zinc finger mutants are listed in Table VI. The standard error used to calculate the confidence intervals was obtained by (i) scaling the data points from the Scatchard curves of a particular TFIIIA zinc finger mutant from Table V to pass through the origin; (ii) plotting the multiple scaled data points from the separate experiments onto one plot, and (iii) determining the error associated with a line passing through the origin with a slope equal to $-1/K_0$, where $K_0$ is the weighted $K_0$ average.

The change in free energy ($\Delta G$) for the TFIIIA-5S DNA interaction at 25°C was determined to be -12.8 kcal/mole (Table VII) in good agreement with a previously reported
Figure 21. Scatchard analysis: TFIIIA.

A. Autoradiograph of a gel-shift experiment. The concentration of TFIIIA was kept constant, 10.0 nM, while the concentration of the 5S DNA was varied, 1.53-7.60 nM. The positions of the TFIIIA-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the TFIIIA-5S DNA interaction. The slope of the plot is equal to -1/K_0 (where K_0 is the weighted average K_0 for TFIIIA, 0.42 nM) and scaled to pass through the point ([B]=3.5, B/F=0). The scaled data points are indicated by the open diamonds (⋄). This curve is used in Figures 22-30 as the WT control.
Table V. Scatchard analyses of TFIIIA zinc finger mutants

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<tr>
<th>Mutant:</th>
<th>WT</th>
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Table V. Scatchard analyses of TFIIIA zinc finger mutants

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<td>Kd (nM)</td>
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<tr>
<td>%STD error</td>
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<td>1.32</td>
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<td>7.7</td>
<td>2.9</td>
<td>3.3</td>
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Figure 22. Scatchard analysis: H33N.

A. Autoradiograph of a gel-shift experiment. The concentration of H33N was kept constant, 10.0 nM, while the concentration of the 5S DNA was varied, 1.75-8.00 nM. The positions of the H33N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H33N-5S DNA interaction. The slope of the plot is equal to -1/K₀ (where K₀ is the weighted average K₀ for H33N, 1.09 nM) and scaled to pass through the point ([B]=3.5, B/F=0). The scaled data points are indicated by the open circles (C). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 23. Scatchard analysis: H63N.

A. Autoradiograph of a gel-shift experiment. The concentration of H63N was kept constant, 17.7 nM, while the concentration of the 5S DNA was varied, 0.83–18.43 nM. The positions of the H63N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H63N-5S DNA interaction. The slope of the plot is equal to -1/K₀ (where K₀ is the weighted average K₀ for H63N, 2.9 nM) and scaled to pass through the point ([B]=8, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 24. Scatchard analysis: H93N.

A. Autoradiograph of a gel-shift experiment. The concentration of H93N was kept constant, 250 nM, while the concentration of the 5S DNA was varied, 10.0-40.5 nM. The positions of the H93N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H93N-5S DNA interaction. The slope of the plot is equal to $-1/K_0$ (where $K_0$ is the weighted average $K_0$ for H93N, 11.2 nM) and scaled to pass through the point ([B]=15, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 25. Scatchard analysis: H125N.

A. Autoradiograph of a gel-shift experiment. The concentration of H125N was kept constant, 200 nM, while the concentration of the 5S DNA was varied, 4.30-35.2 nM. The positions of the H125N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H125N-5S DNA interaction. The slope of the plot is equal to -1/Kₐ (where Kₐ is the weighted average Kₐ for H125N, 4.9 nM) and scaled to pass through the point ([B]=12, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 26. Scatchard analysis: H155N.

A. Autoradiograph of a gel-shift experiment. The concentration of H155N was kept constant, 70.0 nM, while the concentration of the 5S DNA was varied, 2.68-22.72 nM. The positions of the H155N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H155N-5S DNA interaction. The slope of the plot is equal to -1/K₀ (where K₀ is the weighted average K₀ for H155N, 3.6 nM) and scaled to pass through the point ([B]=9, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 27. Scatchard analysis: H183N.

A. Autoradiograph of a gel-shift experiment. The concentration of H183N was kept constant, 60.0 nM, while the concentration of the 5S DNA was varied, 2.65-13.76 nM. The positions of the H183N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H183N-5S DNA interaction. The slope of the plot is equal to -1/K₀ (where K₀ is the weighted average K₀ for H183N, 1.5 nM) and scaled to pass through the point ([B]=4.5, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 28. Scatchard analysis: H210N.

A. Autoradiograph of a gel-shift experiment. The concentration of H210N was kept constant, 40.0 nM, while the concentration of the 5S DNA was varied, 1.85-10.40 nM. The positions of the H210N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H210N-5S DNA interaction. The slope of the plot is equal to -1/K₀ (where K₀ is the weighted average K₀ for H210N, 2.6 nM) and scaled to pass through the point ([B]=5, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIA (Figure 21) is indicated by WT.
Figure 29. Scatchard analysis: H241N.

A. Autoradiograph of a gel-shift experiment. The concentration of H241N was kept constant, 40.0 nM, while the concentration of the 5S DNA was varied, 0.1.90-11.20 nM. The positions of the H241N-5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.

B. Scatchard plot for the H241N-5S DNA interaction. The slope of the plot is equal to -1/K₀ (where K₀ is the weighted average K₀ for H241N, 2.9 nM) and scaled to pass through the point ([B]=5, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFIIIA (Figure 21) is indicated by WT.
Figure 30. Scatchard analysis: H272N.
A. Autoradiograph of a gel-shift experiment. The concentration of H272N was kept constant, 50.0 nM, while the concentration of the 5S DNA was varied, 4.60–25.60 nM. The positions of the H272N–5S DNA complex and free 5S DNA are indicated, "<Bound" and "<Free", respectively.
B. Scatchard plot for the H272N–5S DNA interaction. The slope of the plot is equal to $-1/K_0$ (where $K_0$ is the weighted average $K_0$ for H272N, 1.13 nM) and scaled to pass through the point ([B]=3.5, B/F=0). The scaled data points are indicated by the open circles (O). The Scatchard plot for TFI11A (Figure 21) is indicated by WT.
### Table VI. Equilibrium binding constants for TFIIIA zinc finger mutants

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<th>n</th>
<th>Kd (nM)</th>
<th>Relative decrease</th>
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<td>(0.37 - 0.48)</td>
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<td>11.75</td>
<td>(4.2 - 6.0)</td>
<td>(4.0 - 6.5)</td>
</tr>
<tr>
<td>H155N</td>
<td>5</td>
<td>4</td>
<td>32</td>
<td>3.60</td>
<td>8.63</td>
<td>(3.1 - 4.4)</td>
<td>(3.0 - 4.8)</td>
</tr>
<tr>
<td>H183N</td>
<td>6</td>
<td>3</td>
<td>27</td>
<td>1.50</td>
<td>3.60</td>
<td>(1.3 - 1.7)</td>
<td>(1.3 - 1.9)</td>
</tr>
<tr>
<td>H210N</td>
<td>7</td>
<td>2</td>
<td>20</td>
<td>2.60</td>
<td>6.23</td>
<td>(2.2 - 3.0)</td>
<td>(2.2 - 3.1)</td>
</tr>
<tr>
<td>H241N</td>
<td>8</td>
<td>2</td>
<td>19</td>
<td>2.90</td>
<td>6.95</td>
<td>(2.4 - 3.7)</td>
<td>(2.3 - 4.0)</td>
</tr>
<tr>
<td>H272N</td>
<td>9</td>
<td>4</td>
<td>29</td>
<td>1.13</td>
<td>2.71</td>
<td>(0.99 - 1.34)</td>
<td>(0.94 - 1.43)</td>
</tr>
</tbody>
</table>

N: number of individual experiments averaged to obtain Kd  
n: total number of points from all experiments  
*: based on Student's t-test  
Kd: weighted average
value of -12.4 kcal/mole (94). The ΔG and the change in ΔG (ΔΔG) for the nine TFIIII A zinc finger mutants were also determined (Table VII).

Theoretically, the absolute value of the sum of the individual ΔΔG's for the mutant protein-5S DNA interaction should be greater than the absolute value of ΔG for the WT TFIIII A-5S DNA interaction, 12.8 kcal/mole. This prediction is based on the following assumptions:

(i) individual zinc fingers are functionally independent;
(ii) disruption of a single zinc finger results in complete loss of function of the disrupted finger; and
(iii) the energetic contributions made by individual zinc fingers in the DNA-protein complex must, in aggregate, compensate for the relatively large decrease in entropy which occurs when two free-moving molecules, TFIIII A and 5S DNA, come together to form one bimolecular complex.

However, the absolute value of the calculated sum of the mutant protein ΔΔG's totaled 10.3 kcal/mole, a value less than 12.8 kcal/mole (Table VII). Two possibilities may account for this discrepancy: (i) disrupted zinc fingers may still be able to interact with the 5S DNA, albeit with reduced affinity; and (ii) loss of function of a zinc finger may result in an increase in the affinity of the remaining fingers for the 5S DNA. The latter possibility could result from loss of steric hindrance between adjacent fingers or a reduction in "strain" or
Figure 31. Calculation of weighted $K_0$ average. The formula used to calculate the average $K_0$. $s_\downarrow$ refers to the standard error of the regression coefficient. (109)
\[
K_D = \frac{\sum_{i=1}^{m} \omega_i K_{Di}}{\sum_{i=1}^{m} \omega_i}
\]

where, \( \omega_i = \frac{s_i}{s_i} \)
"tension" between opposite ends of the molecule that exists when all fingers bind simultaneously. These hypotheses could be tested experimentally by analyzing mutant forms of TFIIIA containing two or more mutated zinc fingers.

The mutations in TFIIIA zinc fingers 3 and 4 accounted for 34% (-3.5 kcal/mole) of the relative binding energy contributed by the zinc fingers of TFIIIA, -10.3 kcal/mole (Table VII). The three C-terminal zinc fingers of TFIIIA, fingers 7, 8 and 9, contributed 28% (-2.9 kcal/mole) of the relative binding energy (Table VII).

5. Kinetics of Dissociation

The dissociation rate of the mutant protein-5S DNA complexes were measured at 25°C, in the presence of an excess of pC9Xbs201, a plasmid containing the 5S RNA gene. Dissociation rate constants (k_d) were determined by linear least squares analysis of ln(B/Bo) versus time (35), where B/Bo is equal to the fraction of TFIIIA-5S DNA complexes remaining. Assuming first-order dissociation kinetics, the k_d for TFIIIA was 0.152 ± 0.002 min⁻¹ (Figure 32; Table VIII), corresponding to a half-life of 4.6 minutes. This half-life is similar to that reported by Hanas et al. (47), 5-6 minutes, for the dissociation of the ovarian purified TFIIIA-5S RNA gene
Table VII. ΔG and ΔΔG values for TFIIIA zinc finger mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Finger repeat</th>
<th>$K_D$ (nM)</th>
<th>$ΔG^a$ (kcal/mol)</th>
<th>$ΔΔG$ (kcal/mol)</th>
<th>% of WT</th>
<th>% of mutant $^b$</th>
<th>ΔΔG</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>0.42</td>
<td>-12.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H33N</td>
<td>1</td>
<td>1.09</td>
<td>-12.2</td>
<td>-0.6</td>
<td>4.7%</td>
<td>5.8%</td>
<td></td>
</tr>
<tr>
<td>H63N</td>
<td>2</td>
<td>2.9</td>
<td>-11.6</td>
<td>-1.2</td>
<td>9.4%</td>
<td>11.7%</td>
<td></td>
</tr>
<tr>
<td>H93N</td>
<td>3</td>
<td>11.2</td>
<td>-10.8</td>
<td>-2.0</td>
<td>15.6%</td>
<td>19.4%</td>
<td></td>
</tr>
<tr>
<td>H125N</td>
<td>4</td>
<td>4.9</td>
<td>-11.3</td>
<td>-1.5</td>
<td>11.7%</td>
<td>14.6%</td>
<td></td>
</tr>
<tr>
<td>H155N</td>
<td>5</td>
<td>3.6</td>
<td>-11.5</td>
<td>-1.3</td>
<td>10.2%</td>
<td>12.6%</td>
<td></td>
</tr>
<tr>
<td>H183N</td>
<td>6</td>
<td>1.5</td>
<td>-12.0</td>
<td>-0.8</td>
<td>6.3%</td>
<td>7.8%</td>
<td></td>
</tr>
<tr>
<td>H210N</td>
<td>7</td>
<td>2.6</td>
<td>-11.7</td>
<td>-1.1</td>
<td>8.6%</td>
<td>10.7%</td>
<td></td>
</tr>
<tr>
<td>H241N</td>
<td>8</td>
<td>2.9</td>
<td>-11.6</td>
<td>-1.2</td>
<td>9.4%</td>
<td>11.7%</td>
<td></td>
</tr>
<tr>
<td>H272N</td>
<td>9</td>
<td>1.13</td>
<td>-12.2</td>
<td>-0.6</td>
<td>4.7%</td>
<td>5.8%</td>
<td></td>
</tr>
</tbody>
</table>

-10.3  80.5%  100.0%

$a$: where $ΔG = -RT \ln(K_A)$

$b$: relative binding energy
complex, under similar buffer conditions but using single stranded DNA as the competitor. The dissociation rate constants were determined for TFIIIA mutants H125N (1.6 ± 0.1 min⁻¹), H183N (0.99 ± 0.06 min⁻¹), H210N (0.62 ± 0.02 min⁻¹), H241N (1.3 ± 0.2 min⁻¹) and H272N (0.39 ± 0.04 min⁻¹), corresponding to half-lives of 26 seconds, 42 seconds, 68 seconds and 108 seconds, respectively (Figures 34-38; Table VIII). The solution half-life of the H93N-5S DNA complex proved too short to measure with any accuracy, with the half-life being less than 15 seconds (Figure 33; Table VIII). Figure 39 contains the dissociation rate curves for all the TFIIIA zinc finger mutants tested and the dissociation rate curve for TFIIIA. The relative decrease in half-lives compared to that of the wild-type protein are listed in Table VIII. Since a decrease in half-life is a direct result of the instability of the protein-DNA complex, the relative decrease in half-life of the protein-DNA complex is a measure of the relative decrease in affinity of the protein for the ICR. Comparison between the relative decreased affinities calculated from either the equilibrium binding constants (Table VI) or kinetic dissociation rates (Table VIII) for the various TFIIIA zinc finger mutants reveal that for the most part they are in good agreement (Table IX). This observation
Figure 32. Dissociation analysis of the TFIllA-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the TFIllA-5S DNA complex. Lane 1, 0 min.; lane 2, 0.25 min.; lane 3, 0.5 min.; lane 4, 0.75 min.; lane 5, 1.25 min.; lane 6, 1.75 min.; lane 7, 3.75 min.; lane 8, 7.75 min.; and lane 9, 15.75 min..

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 0.152 min⁻¹, corresponding to a half-life of 4.56 min.
Figure 33. Dissociation analysis of the H93N-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the H93N-5S DNA complex. Lane 1, 0 min.; and lane 2, 0.25 min..

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 2.870 min⁻¹, corresponding to a half-life of 14 sec. The dissociation plot the TFI11A-5S DNA complex from Figure 32 is included for a reference line.
A. 

B. 

B. 

$\ln(1/T_{1/2})$

Time (min)

H93N

WT
Figure 34. Dissociation analysis of the H125N-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the H125N-5S DNA complex. Lane 1, 0 min.; lane 2, 0.25 min.; lane 3, 0.5 min.; lane 4, 0.75 min.; and lane 5, 1.25 min.

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 1.573 min⁻¹, corresponding to a half-life of 26 sec.. The dissociation plot the TFIIB-5S DNA complex from Figure 32 is included for a reference line.
Figure 35. Dissociation analysis of the H183N-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the H183N-5S DNA complex. Lane 1, 0 min.; lane 2, 0.25 min.; lane 3, 0.5 min.; lane 4, 0.75 min.; lane 5, 1.25 min.; and lane 6, 1.75 min..

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 0.989 min⁻¹, corresponding to a half-life of 42 sec. The dissociation plot the TFIIB-5S DNA complex from Figure 32 is included for a reference line.
Figure 36. Dissociation analysis of the H210N-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the H210N-5S DNA complex. Lane 1, 0 min.; lane 2, 0.25 min.; lane 3, 0.5 min.; lane 4, 0.75 min.; lane 5, 1.25 min.; lane 6, 1.75 min.; and lane 7, 3.75 min.

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 0.615 min⁻¹, corresponding to a half-life of 68 sec. The dissociation plot the TFIllA-5S DNA complex from Figure 32 is included for a reference line.
Figure 37. Dissociation analysis of the H241N-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the H241N-5S DNA complex. Lane 1, 0 min.; lane 2, 0.25 min.; lane 3, 0.5 min.; lane 4, 0.75 min.; lane 5, 1.25 min.; and lane 6, 1.75 min..

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 1.256 min⁻¹, corresponding to a half-life of 33 sec. The dissociation plot the TFIIIA-5S DNA complex from Figure 32 is included for a reference line.
Figure 38. Dissociation analysis of the H272N-5S DNA complex.

A. Autoradiograph of a dissociation experiment of the H272N-5S DNA complex. Lane 1, 0 min.; lane 2, 0.25 min.; lane 3, 0.5 min.; lane 4, 0.75 min.; lane 5, 1.25 min.; lane 6, 1.75 min.; lane 7, 3.75 min.; and lane 8, 7.75 min..

B. Ln plot of the dissociation data from A. The calculated dissociation rate constant is 0.386 min⁻¹, corresponding to a half-life of 108 sec. The dissociation plot the TFIIIA-5S DNA complex from Figure 32 is included for a reference line.
Figure 39. Compilation of the dissociation curves for TFIIIA and the TFIIIA zinc finger mutants. Curves are replotted from Figures 32B-38B. The corresponding half-lives for TFIIIA, H93N, H125N, H183N, H210N, H241N and H272N are 273 sec., 14 sec., 26 sec., 42 sec., 68 sec., 33 sec. and 108 sec., respectively.
Table VIII. Dissociation rate constants for TFIIBA zinc finger mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Finger repeat</th>
<th>kd (min⁻¹)</th>
<th>t(1/2) (min)</th>
<th>t(1/2) (sec)</th>
<th>n</th>
<th>% error</th>
<th>Relative decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>0.152</td>
<td>4.56</td>
<td>273</td>
<td>9</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>H33N</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H63N</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H93N</td>
<td>3</td>
<td>2.870</td>
<td>0.24</td>
<td>14</td>
<td>2</td>
<td>*</td>
<td>19</td>
</tr>
<tr>
<td>H125N</td>
<td>4</td>
<td>1.573</td>
<td>0.44</td>
<td>26</td>
<td>5</td>
<td>6.5</td>
<td>10</td>
</tr>
<tr>
<td>H155N</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H183N</td>
<td>6</td>
<td>0.989</td>
<td>0.70</td>
<td>42</td>
<td>6</td>
<td>6.2</td>
<td>7</td>
</tr>
<tr>
<td>H210N</td>
<td>7</td>
<td>0.615</td>
<td>1.13</td>
<td>68</td>
<td>7</td>
<td>3.4</td>
<td>4</td>
</tr>
<tr>
<td>H241N</td>
<td>8</td>
<td>1.256</td>
<td>0.55</td>
<td>33</td>
<td>6</td>
<td>13.3</td>
<td>8</td>
</tr>
<tr>
<td>H272N</td>
<td>9</td>
<td>0.386</td>
<td>1.80</td>
<td>108</td>
<td>8</td>
<td>11.6</td>
<td>3</td>
</tr>
</tbody>
</table>

kd: dissociation rate  
n: number of data points  
ND: not determined  
*: can not be calculated with only two data points  
% error: % standard error of the regression coefficient
Table IX. Comparison between Kd and t(1/2) for TFIIIA zinc finger mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Finger repeat</th>
<th>Dissociation analysis</th>
<th>Scatchard analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>t(1/2) (sec)</td>
<td>Kd (nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relative decrease</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>273</td>
<td>0.42</td>
</tr>
<tr>
<td>H93N</td>
<td>3</td>
<td>14</td>
<td>11.2</td>
</tr>
<tr>
<td>H125N</td>
<td>4</td>
<td>26</td>
<td>4.9</td>
</tr>
<tr>
<td>H183N</td>
<td>6</td>
<td>42</td>
<td>1.5</td>
</tr>
<tr>
<td>H210N</td>
<td>7</td>
<td>68</td>
<td>2.6</td>
</tr>
<tr>
<td>H241N</td>
<td>8</td>
<td>33</td>
<td>2.9</td>
</tr>
<tr>
<td>H272N</td>
<td>9</td>
<td>108</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Kd: equilibrium dissociation constant

$t(1/2)$: half-life of dissociation
suggests that the major factor responsible for the
decrease in affinity of the TFIIIA zinc finger mutants to
the 5S DNA is an increase in the dissociation rates of
the mutant proteins and not a decrease in the association
rates.

6. DNaseI Protection

Initial attempts at "footprinting" the N-terminal
TFIIDA zinc finger mutants on the non-coding strand of
5S DNA as described under "DNaseI Protection" in the
Materials and Methods section of Chapter II yielded poor
quality footprints, especially for the weak binders, ie,
H93N and H125N (data not shown). The footprints appeared
to consist of a combination of a zinc finger mutant-DNA
protection pattern superimposed on a naked-DNA cleavage
pattern. The lower the affinity for 5S DNA for a
particular mutant, the more the actual footprint was
obscured by the naked-DNA cleavage pattern. It seemed
likely that one of the reasons for the combined footprint
patterns was a failure to saturate binding sites on the
end-labeled DNA fragment. To overcome this problem, we
devised a modified DNaseI protection method to analyze
the TFIIDA zinc finger mutant-5S DNA binary complexes
(see Materials and Methods section in this chapter). Even
with these conditions the measured half-lives for the
TFIIIA zinc finger mutant-5S DNA binary complexes suggested that we were analyzing a population of DNA molecules in which individual DNA molecules were cycling between bound and unbound states during the period of DNaseI treatment. This phenomena now probably contributes to a low-level contamination of the mutant footprints with the naked-DNA cleavage pattern.

After the protein-DNA complexes were treated with DNaseI, the reactions were directly applied to a running non-denaturing gel (Figures 40 and 41). The mobility of the various protein-5S DNA complexes varied slightly from each other. The TFIIIA sixth zinc finger mutant-5S DNA complex (H183N-5S DNA complex) was observed to have the slowest mobility. The 5S DNA complexed with the various wild-type and mutant forms of TFIIIA were eluted and then resolved on sequencing gels.

Upon visual inspection of the DNaseI protection patterns illustrated in Figures 42 and 43, non-coding strand and coding strand, respectively, it becomes evident that globally the N-terminal zinc fingers of TFIIIA interact with the 3' end of the control region on both the non-coding and coding strands whereas the C-terminal fingers of TFIIIA interact with the 5' end of the control region on both the non-coding and coding strands. Details of the footprints for the individual
TFIIIA zinc finger mutants are described below. In the descriptions below, "95-98" refers to changes between residues 95 through 98 and "96/97" refers to the 3' phosphate ester between residues 96 and 97.

a. **Mutation in finger 1, mutant H33N.** On both the non-coding and coding strands, the 5' ends of the footprint are identical to that of the wild-type protein. The only differences are located at the 3' end of the ICR, between residues 93-97 (NC strand) and 91-95 (C strand). The major change in the C strand footprint is loss of protection at position 94/95, whereas the NC strand footprint reveals changes at positions 96/97 and 95/96 (loss of protection) and 93/94 (loss of a hypersensitive site).

b. **Mutation in finger 2, mutant H63N.** The changes on the NC strand are located between 84-94. The region between 95-97 which was accessible to DNaseI in the first zinc finger mutant, H33N, is protected in the second zinc finger mutant. These data suggest that the first zinc finger domain in the H63N mutant is functional and able to interact with the 3' end of the ICR in the presence of a mutated second finger. The hypersensitive site at 93/94 has been reduced but not eliminated and the site at 86/87 is no longer protected from DNaseI cleavage. On the C strand, the major changes extend from 86-91 with a
Figure 40. Non-coding strand footprint analysis of TFIIB zinc finger mutants: gel-shift assay. Autoradiograph of gel-shift assay to separate bound protein-5S DNA complexes (B) from dissociated free (F) 5S DNA after digestion with DNaseI. The protein-5S DNA complexes were eluted from the gel slices and resolved over a sequencing gel (Figure 42).
Figure 41. Coding strand footprint analysis of TFIIBA zinc finger mutants: gel-shift assay. Autoradiograph of gel-shift assay to separate bound protein-5S DNA complexes (B) from dissociated free (F) 5S DNA after digestion with DNaseI. The protein-5S DNA complexes were eluted from the gel slices and resolved over a sequencing gel (Figure 43).
Figure 42. Non-coding strand footprint analysis of TFIIIA zinc finger mutants: sequencing gel. The eluted 5S DNA fragments (Figure 40) were analyzed on a sequencing gel. Lanes 1 and 15, guanine and adenine chemical cleavage reactions (denoted G/A); lanes 2 and 14, DnaseI digestion pattern of protein-free DNA; lanes 3 and 13, TFIIIA protection pattern; lanes 4-12, protection patterns for TFIIIA zinc finger mutants H33N, H63N, H93N, H125N, H155N, H183N, H210N, H241N AND H272N, respectively. The location and orientation of the TFIIIA footprint is indicated with the vertical bar; nucleotide positions within the 120 bp gene are also indicated.
Figure 43. Coding strand footprint analysis of TFIIIA zinc finger mutants: sequencing gel. The eluted 5S DNA fragments (Figure 41) were analyzed on a sequencing gel. Lanes 1 and 15, guanine and adenine chemical cleavage reactions (denoted G/A); lanes 2 and 14, DnaseI digestion pattern of protein-free DNA; lanes 3 and 13, TFIIIA protection pattern; lanes 4-12, protection patterns for TFIIIA zinc finger mutants H33N, H63N, H93N, H125N, H155N, H183N, H210N, H241N AND H272N, respectively. The location and orientation of the TFIIIA footprint is indicated with the vertical bar; nucleotide positions within the 120 bp gene are also indicated.
complete loss of protection occurring at 91/92 and a partial loss of protection at 86/87.

c. **Mutation in finger 3, mutant H93N.** No localized changes were apparent on the C strand footprint of the third zinc finger mutant, H93N. The pattern obtained contains the appropriate hypersensitive sites at 38/39 and 40/41 indicating that the H93N mutant is at least interacting with the 5' end of the ICR, while partial loss of protection throughout the ICR suggests that the H93N mutant is interacting with the entire length of the ICR including the 3' end. On the NC strand, the H93N mutant reveals good protection of the 5' end of the ICR, 46-60, with the expected hypersensitive sites at 41/42 and 40/41. The hypersensitive sites present in the wild-type footprint on the NC strand are all present in the footprint pattern of H93N; in addition, there is increased hypersensitivity between sites 76-78 which are specific to the H93N mutant (See Figure 44 for details).

d. **Mutation in finger 4, mutant H125N.** Neither the NC nor the C strand footprints for the fourth zinc finger mutant, H125N, reveal any H125N specific hypersensitive sites or loss-of-protection sites. Both strands reveal a partial loss of protection throughout the ICR. All hypersensitive sites which are present in the wild-type footprint are also present with the same intensity on the
Figure 44. Non-coding strand footprint analysis of H93N. Detailed view of lanes 2, 3 and 6 from Figure 42. Lane 1, DnaseI digestion pattern of protein-free DNA; lane 2, protection pattern of H93N; and lane 3, TFIIIA protection pattern. The location and orientation of the TFIIIA footprint is indicated with the vertical bar; hypersensitive sites induced by TFIIIA binding are indicated (denoted by H). The hypersensitive site induced by H93N binding is indicated (*). Comparison of the DNaseI cleavage sites in lane 1 (marked a, b and c) with the corresponding sites in lane 2, indicate that sites a and c appear to be more protected than site b in lane 2 relative to the DNaseI pattern in lane 1.
H125N footprints.

e. **Mutation in finger 5, mutant H155N.** Changes on the NC strand footprint for the fifth zinc finger mutant, H155N, are located between 62-75. The major change was a hypersensitive site at 70/71, whereas the hypersensitive site present at 62/63 in the wild-type footprint was lost. Loss of protection occurred between nucleotides 73-75 and at the 65/66 site. On the C strand, the major change was a hypersensitive site at 72/73.

f. **Mutation in finger 6, mutant H183N.** The only changes present on the NC strand footprint of the sixth zinc finger mutant, H183N, were localized to 61-65. Compared to the wild-type protein footprint, a slight loss of hypersensitivity occurred at 62-65, where as site 61/62 showed increased hypersensitivity. On the C strand, a hypersensitive site was located at 60/61 with partial loss of protection at 50/51.

g. **Mutation in finger 7, mutant H210N.** The NC strand for the seventh zinc finger mutant, H210N, was similar to the NC strand footprint for the H183N mutant. Unlike the C strand footprint for H183N, the C strand footprint for H210N revealed a hypersensitive site at 50/51. On both the NC and C strand footprints for the H210N mutant, the 5' ends of the footprints were indistinguishable from the wild-type pattern of protection or hypersensitivity.
h. **Mutation in finger 8, mutant H241N.** Out of all the TFIIIA zinc finger mutants analyzed, the NC and C strand footprints of the eighth zinc finger mutant, H241N, revealed the most extensive changes extending from 60–64 to 38–40. The changes on the NC strand were as follows (i) loss of hypersensitivity between 61–64 and 41–40; (ii) loss of protection between 48–54; and (iii) new hypersensitive sites at 48/49 and 59/60. The changes on the C strand were as follows (i) new hypersensitive sites between 56–58 and 47–49; and (ii) loss of hypersensitivity between 38–41.

i. **Mutation in finger 9, mutant H272N.** The changes on the NC strand for the ninth zinc finger mutant, H272N, were mainly localized to the 5' end of the ICR with loss of hypersensitivity between 40–42 and increased hypersensitivity between 44–47. On the C strand, the footprint for the H272N mutant revealed (i) hypersensitive sites between 44–46; loss of hypersensitivity between 38–41; and partial loss of protection between 56–58 and 47–49.

We have mapped the various zinc fingers of TFIIIA onto the ICR (Figure 45) based on the footprint data. The figure includes box A, box C and intermediate regions (82–84), the G residues whose methylation interferes with the binding of TFIIIA (98), the phosphates that make
Figure 45. Schematic representation of footprint results. The TFIIIA zinc finger mutants have been mapped onto the 5S DNA based on alterations observed with DNaseI protection analyses, indicated by the numbers below the 5S DNA sequence and the boxed-in regions (See figure key). The phosphates which make contact with TFIIIA (98) are indicated by the open triangles (▼); the guanidine (G) residues that interfere with the binding of TFIIIA when methylated (98) are circled (○); the thymidine (T) residues which were crosslinked to TFIIIA are boxed (□) (61); and the A box, C box and I box (82-84) are indicated above the 5S DNA sequence.
contact with TFIIIA (98) and the T residues which were crosslinked to TFIIIA (61).

D. Discussion

We have analyzed a set of nine TFIIIA zinc finger mutants, each containing one mutant zinc finger and eight wild-type zinc fingers. In order to simplify the interpretation of the results, we have systematically mutated the same relative position in each of the nine zinc fingers of TFIIIA. Specifically, we have targeted the third conserved zinc ligand, that is, the first of the two conserved histidine residues. Limited proteolysis with thermolysin suggests that the histidine to asparagine substitutions result in local disruption of individual TFIIIA zinc fingers. This is direct evidence for two things: (i) the coordination of the zinc ion by the first conserved histidine is crucial for structural integrity of the zinc finger domain; and (ii) the fingers are largely structurally independent.

The calculated half-life for the wild-type TFIIIA-DNA complex (in 7 mM MgCl₂) of approximately 5 minutes is in good agreement with earlier reports using either single stranded DNA (in 7 mM MgCl₂) (47) or 5S RNA (in 5 mM MgCl₂) (94) as a competitor, but different from a recent study which reported a half-life of 100 minutes
(64) using double stranded DNA as the competitor (in 1 mM MgCl₂). This recent report (64) also reported the equilibrium binding constant of TFIIIA to 5S DNA in 1 mM MgCl₂ to be 2.2 nM, greater than a 5-fold decrease in the binding affinity of TFIIIA for the 5S DNA compared to our reported equilibrium binding constant of 0.4 nM in 7 mM MgCl₂. The apparent discrepancy in the half-life and binding constant determinations is most likely due to differences in MgCl₂ concentrations. This possibility can be tested by determining the half-life of dissociation for the TFIIIA-5S DNA complex at variable MgCl₂ concentrations.

While every mutant exhibits a lower binding affinity than observed for wild-type TFIIIA, indicating that every finger contributes to the binding energy, not all fingers contribute equally, with individual mutants exhibiting reductions in binding affinity from 2 to 25 fold. Fingers 3 and 4 make the largest contributions to the binding energy, with surprisingly small contributions made by zinc fingers 1 and 9. Analysis of the ΔG's and ΔΔG's of the zinc finger mutants suggests that binding of a zinc finger may effect the binding affinity of neighboring fingers or fingers at opposite ends of TFIIIA to 5S DNA.

Since changes in the pattern of cleavage by DNaseI can be due to direct protection by TFIIIA, local
distortion of the 5S DNA by TFIIIA, or even global distortions, such as bending induced by the protein, we cannot be quite certain of the origin of every change in the protection pattern obtained for the various TFIIIA zinc fingers. Nevertheless, the protection patterns considered as a whole indicate very clearly how the TFIIIA protein interacts with the 5S DNA ICR.

First, the fingers appear to be aligned in order along the length of the ICR, with the N-terminal fingers binding the 3' end of the control region and the C-terminal fingers binding to the 5' end of the control region. An exception to this co-linearity is finger 8, disruption of which leads to footprint alterations not only where expected, but also at some distance downstream. Second, the footprint alterations are localized, suggesting a rather high degree of functional independence between different fingers. Third, every mutated finger protein except one exhibits an altered footprint on one or both strands of 5S DNA. The exception is finger 4, which is the second most important finger energetically, exhibiting a 12-15 fold reduction in binding affinity when mutated. There are multiple explanations for this surprising result, but one interesting possibility is that finger 4 contributes to the binding energy not by making specific contacts to the
DNA template, but by positioning adjacent fingers so as to permit energetically optimal contacts by these fingers. Fourth, the spacing of interaction sites for the N-terminal fingers of TFIIIA appears to be too great to accommodate a Zif268-like mode of binding; the binding sites for fingers 1, 2, and 3 are separated by about 10 base pairs, while the sites for fingers 3 and 5 are separated by about 5 base pairs. In contrast, the 3 C-terminal fingers do appear to interact in a Zif268-like mode; each finger disruption results in a loss of protection of 2-3 nucleotides, spanning a total length of 8 nucleotides.

The data support a model for the interaction which is complex and different between the two ends of the protein. They suggest that the C-terminal fingers interact in a Zif268-like mode, while the N-terminal fingers are separated from each other by single helical turns. Finger 4 may not make direct contacts with the DNA at all, and finger 5 appears to interact in isolation on the opposite side of the helix with the so-called intermediate box of the 5s ICR. Several different finger disruptions affect the pattern of hypersensitivity observed on the non-coding strand with the wild-type protein, and the adjacent loss of protection observed in the finger 8 mutant might be related to a major
distortion of the DNA helix in this region that is induced by binding of wild-type TFIIIA.
CHAPTER IV

TRANSCRIPTIONAL ANALYSIS OF ZINC FINGER MUTANTS OF

_Xenopus laevis_ TRANSCRIPTION FACTOR IIIA

A. Introduction

An important question concerning sequence-specific DNA-binding transcription factors is whether transcriptional activity of the DNA-binding protein is correlated with its ability to support transcription. In the case of TFIIIA, we wanted to not only test the relationship between DNA binding affinity and transcriptional activation, but also whether individual mutated zinc fingers had additional altered transcriptional activation phenotypes.

From analyses of mutant TFIIIA binding to the 5S rRNA gene (see Chapter III), we knew that all the zinc finger mutants were able to form a complex with 5S DNA, even though the relative stability of each mutant:DNA binary complex was quantitatively different, as demonstrated by our determination of equilibrium binding constants and dissociation rate constants. The availability of a collection of mutants displaying different affinities for 5S DNA made it possible to test whether transcriptional activation is correlated with DNA binding affinity.

For transcription to initiate on the 5S RNA gene, the TFIIIA-5S DNA binary complex has to be stabilized by
interacting with TFIIIC and TFIIIB (3). Although our zinc finger mutants could form a binary protein:DNA complex, it was possible that the mutants could have altered interactions with other components of the transcriptional machinery, such as TFIIIC, thereby affecting formation of higher order complexes needed to support transcription.

Using a TFIIIA-dependent in vitro transcription assay, we analyzed the transcriptional activity of our zinc finger mutant proteins H33N, H63N, H93N, H125N, H155N, H183N, H210N, H241N and H272N corresponding to histidine-to-asparagine substitutions in fingers 1, 2, 3, 4, 5, 6, 7, 8 and 9, respectively. The activities of the mutants were compared to that of the wild-type protein, which was also expressed and purified from E. coli, and previously shown to be as active as TFIIIA purified from Xenopus ovaries, (See Chapter II).

B. Methods and Materials

Transcriptional analyses of TFIIIA mutants.
The ability of TFIIIA mutants to support transcription of a 5S rRNA was assessed using a TFIIIA-dependent in vitro transcription assay as described by Setzer and Brown (105) and as modified by Del Rio and Setzer (22). Briefly, template DNA (pC9Xbs201) (2.5 nM) was preincubated at 25°C for 60 minutes in a 20 µl reaction containing buffer B
(20 mM Tris-Cl, pH 7.5; 7 mM MgCl₂; 10 μM ZnCl₂; 1 mM DTT; 10% glycerol; 70 mM KCl), 100 μg/ml BSA, 5 μl of a Dignam nuclear extract (25) prepared from a *Xenopus laevis* kidney cell line, 20 units RNAsin (Promega), 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.5 mM PMSF, and variable concentrations of TFIIIA or mutant proteins as indicated in the figure legends. Concentrations of TFIIIA and mutant proteins correspond to active protein concentrations determined from the X-intercept from Scatchard plots. Following the preincubation period, 10 μCi [α-32P]UTP (800 Ci/m mole) was added along with ATP, GTP, and CTP to a final concentration of 500 μM and UTP to a final concentration of 50 μM. Transcription reactions were incubated for an additional 60 minutes, and stopped by the addition of 150 μl of SETS/glycogen (150 mM NaCl; 50 mM Tris-HCl; 5 mM EDTA; 0.5% SDS; 50 μg/ml glycogen; pH 8.0). Following extraction with phenol and precipitation in ethanol, transcription products were resolved on a 10% polyacrylamide/8.33 M urea gel. Wet gels were fixed in 40% methanol/10% acetic acid, transferred to Whatman 3MM paper and directly scanned on an Ambis Radiographic Imaging System (RIS). The amount of 5S RNA produced in each reaction was determined by analyzing and quantifying the scan using the Ambis Image analysis software, version 3.02.
C. Results

To determine the variability and sensitivity of our \textit{in vitro} assay, we analyzed the activity of wild-type TFIIIA (WT) multiple times (WT\#1, WT\#2 and WT\#3) on different days. Since the maximal level of transcriptional activity varied with different preparations of [\textalpha^{32}\text{P}]UTP, quantitative transcription data were scaled relative to the level of 5S RNA synthesis obtained when no exogenous TFIIIA was added, \textit{i.e.}, the endogenous level of transcriptional activity (Figure 46).

Multiple WT analyses (Figure 46) showed that the maximal level of 5S RNA synthesis obtained was similar for the three experiments, with an average value of 4.1 relative to the level of 5S RNA synthesis in the absence of added TFIIIA. All three reached at least 90\% of maximal level of 5S RNA synthesis with the addition of 0.5 nM TFIIIA. Below a TFIIIA concentration of 0.5 nM, the curves for the separate experiments show increased variability with WT\#1 reaching 88\% of maximal level by 0.2 nM, WT\#2 reaching 73\% by 0.2 nM and WT\#3 reaching 73\% by 0.25 nM TFIIIA. Since the same preparation of recTFIIIA was used for the three separate experiments, with an additional freeze/thaw cycle between each experiment, and the chronological order of the experiments being WT\#1 on day 1, WT\#2 on day 2 and WT\#3 on day 3, the slight reduction in
Figure 46. Transcriptional analyses of TFIIIA. A constant concentration of 5S DNA (2.5 nM) was incubated with variable concentrations of TFIIIA (0-2.5 nM) and Dignam nuclear extract (25) for 60 minutes prior to the addition of NTPs. WT#1 (▲), WT#2 (●) and WT#3 (★) were done at different times to test the sensitivity and variability of the transcription assay.
activity observed in the later experiments might be attributed to decreases in the percent of active protein.

Mutations in the six N-terminal fingers, H33N, H63N, H93N, H125N, H155N and H183N (see Figures 47 and 48), resulted in mutant proteins with transcriptional activities similar to that of the WT protein. The relative maximal level of 5S RNA synthesis was 4.3, 5.1, 5.1, 3.7, 4.2 and 3.7 for H33N, H63N, H93N, H125N, H155N and H183N, respectively.

Unlike the 6 N-terminal zinc finger mutants, mutations in the three C-terminal fingers, H210N, H241N and H272N, resulted in proteins with decreased transcriptional activity (see Figure 49). The relative maximal level of 5S RNA synthesis for mutant H210N was 3.0, reaching 90% of its maximal level by 0.5 nM. Mutant H272N showed only minimal transcriptional activation with a relative maximal level of 5S RNA synthesis of 1.5, only 50% above background. Mutant H241N was completely inactive and inhibitory, reducing the relative level of 5S RNA synthesis to 0.5, 50% below background.

To investigate the H241N mutant further, additional transcriptional experiments were conducted to determine whether H241N was forming stable inactive transcription complexes. In the first set of experiments (outlined in Figure 50A), (i) a constant amount (2.5 nM) of either
Figure 47. Transcriptional analyses of TFIIIA zinc finger mutants: H33N, H63N and H93N. A constant concentration of 5S DNA (2.5 nM) was incubated with variable concentrations of H33N (●), H63N (+), H93N (■) or TFIIIA (♦) (0–2.0 nM) and Dignam nuclear extract (25) for 60 minutes prior to the addition of NTPs. The WT#2 transcription curve from Figure 46 was used as the TFIIIA control.
Figure 48. Transcriptional analyses of TFIILIA zinc finger mutants: H125N, H155N and H183N. A constant concentration of 5S DNA (2.5 nM) was incubated with variable concentrations of H125N (x), H155N (o), H183N (v) or TFIILIA (♦) (0-2.0 nM) and Dignam nuclear extract (25) for 60 minutes prior to the addition of NTPs. The WT#2 transcription curve from Figure 46 was used as the TFIILIA control.
Figure 49. Transcriptional analyses of TFIIIA zinc finger mutants: H210N, H241N and H272N. A constant concentration of 5S DNA (2.5 nM) was incubated with variable concentrations of H210N (I), H241N (□), H272N (▼) or TFIIIA (♦) (0–2.0 nM) and Dignam nuclear extract (25) for 60 minutes prior to the addition of NTPs. The WT#2 transcription curve from Figure 46 was used as the TFIIIA control.
Relative 5S RNA Synthesis

[protein] (nM)

○ WT    △ H210N    □ H241N    ▼ H272N
wild-type or H241N protein was added to the DNA template (2.5 nM) along with Dignam nuclear extract and preincubated for 60 minutes to allow formation of transcription complexes; (ii) variable amounts of H241N or wild-type protein were then added and allowed to incubate an additional 60 minutes; (iii) NTPs were added and incubated for 60 minutes before the reactions were stopped and products analyzed as described above under the Methods section. In the second set of experiments (outlined in Figure 50B), either (i) a constant amount of wild-type protein (0.25 nM) was preincubated with increasing amounts of H241N, DNA template (2.5 nM) and Dignam nuclear extract for 60 minutes prior to the addition of the NTPs or (ii) a constant amount of H241N protein (0.25 nM) was preincubated with increasing amounts of wild-type protein, DNA template (2.5 nM) and Dignam nuclear extract for 60 minutes prior to the addition of the NTPs. After 60 minutes the reactions were stopped and products analyzed as described above under the Methods section.

Figure 51 shows the transcription activity curves for TFIIIA and H241N used as controls for the mixing experiments. Figure 52 shows the results obtained from the first set of mixing experiments. When the wild-type protein was preincubated with the template and other transcription factors to allow for the formation of transcription
complexes, the subsequent addition of increasing amounts of H241N had essentially no effect on the level of 5S RNA synthesis; only a 3% reduction in transcriptional activity was observed. When the template was preincubated with H241N and the other transcription factors to allow for the formation of transcription complexes, the addition of wild-type protein still stimulated transcription; however, the maximal level of 5S RNA synthesis was reduced by approximately 25% even at high wild-type protein concentrations.

Figure 53 shows the results for the second set of mixing experiments using H241N and wild-type protein. The activity curve for the experiment where increasing amounts of WT protein were preincubated with a constant concentration of H241N protein was similar to the activity curve of the wild-type control except for the slight indication that at concentrations of WT protein below 0.5 nM a greater amount of WT protein was required to attain the same relative level of activity in the presence of H241N than when H241N was omitted from the reaction. The activity curve for the experiment where increasing amounts of H241N protein were preincubated with a constant concentration of wild-type protein may be divided into two parts. The initial portion of the curve reveals a slight decrease in activity with increasing addition of H241N,
Figure 50. Experimental design for TFIIIA and H241N transcription mixing experiments. A. 1.) Scheme used for the analysis illustrated in Figure 52 corresponding to preWT/varH241N (I). 2.) Scheme used for the analysis illustrated in Figure 52 corresponding to preH241N/varWT (II).

B. Scheme used for the analyses illustrated in Figure 53 corresponding to conWT/varH241N (I) and conH241N/varWT (II).
A. 1.) 5S DNA
Dignam extract

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<thead>
<tr>
<th></th>
<th>WT</th>
<th>H241N</th>
<th>NTP's</th>
<th>Stop</th>
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<tr>
<td>0</td>
<td>60'</td>
<td>120'</td>
<td>180'</td>
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Time,(min.)

2.) 5S DNA
Dignam extract

<table>
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<tr>
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<th>H241N</th>
<th>WT</th>
<th>NTP's</th>
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<td>0</td>
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Time,(min.)

B. 5S DNA
Dignam extract

<table>
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<tr>
<th></th>
<th>H241N</th>
<th>WT</th>
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Time,(min.)
Figure 51. Transcriptional analyses of TFIIA zinc finger mutants: TFIIA and H241N mixing experiments, control curves.

A. A constant concentration of 5S DNA (2.5 nM) was incubated with variable concentrations of TFIIA (WT) or H241N and Dignam nuclear extract for 60 minutes prior to the addition of NTPs. Lane 1, 0 nM protein; lane 2, 0.025 nM protein; lane 3, 0.05 nM protein; lane 4, 0.125 nM protein; lane 5, 0.25 nM protein; lane 6, 0.50 nM protein; lane 7, 1.25 nM protein; lane 8, 2.50 nM protein; lane 9, 5.00 nM protein; and lane 10, 12.50 nM protein.

B. Graphic presentation of the results from A. TFIIA (WT; ♦) and H241N (⊗).
Figure 52. Transcriptional analyses of TFIIIA zinc finger mutants: TFIIIA and H241N mixing experiment, A.

A. A constant concentration of 5S DNA (2.5 nM) and Dignam nuclear extract was incubated with either (i) a constant concentration of TFIIIA (preWT/varH241N; 2.5 nM) for 60 minutes prior to the addition of variable concentrations of H241N (lane 1, 0 nM; lane 2, 0.25 nM; lane 3, 0.50 nM; lane 4, 1.25 nM; lane 5, 2.50 nM; lane 6, 5.00 nM; and lane 7, 12.50 nM) and an additional 60 minutes incubation prior to the addition of the NTPs; or (ii) a constant concentration of H241N (preH241N/varWT; 2.5 nM) for 60 minutes prior to the addition of variable concentrations of WT TFIIIA (lane 1, 0 nM; lane 2, 0.25 nM; lane 3, 0.50 nM; lane 4, 1.25 nM; lane 5, 2.50 nM; lane 6, 5.00 nM; and lane 7, 12.50 nM) and an additional 60 minutes incubation prior to the addition of the NTPs.

B. Graphic presentation of the results from A. preWT/varH241N (I) and preH241N/varWT (®). The TFIIIA control from Figure 51 (WT; ♦) is included for reference.
A. 

preH241N/varWT 

preWT/varH241N 

1 2 3 4 5 6 7 

B. 

Relative 5S RNA Synthesis 

[protein] (nM) 

♦ WT  x preWT/varH241N  □ preH241N/varWT
Figure 53. Transcriptional analyses of TFIIIA zinc finger mutants: TFIIIA and H241N mixing experiment, B.
A. A constant concentration of 5S DNA (2.5nM) and Dignam nuclear extract was incubated with either (i) a constant concentration of TFIIIA (0.25 nM) and variable concentrations of H241N (lane 1, 0 nM; lane 2, 0.025 nM; lane 3, 0.050 nM; lane 4, 0.125 nM; lane 5, 0.25 nM; lane 6, 0.50 nM; lane 7, 1.25 nM; and lane 8, 2.50 nM) (conWT/varH241N) for 60 minutes prior to the addition of the NTPs; or (ii) a constant concentration of H241N (0.25 nM) and variable concentrations of TFIIIA (lane 1, 0 nM; lane 2, 0.025 nM; lane 3, 0.050 nM; lane 4, 0.125 nM; lane 5, 0.25 nM; lane 6, 0.50 nM; lane 7, 1.25 nM; and lane 8, 2.50 nM) (conH241N/varWT) for 60 minutes prior to the addition of the NTPs.
B. Graphic presentation of the results from A. conWT/varH241N (I) and conH241N/varWT (■). The TFIIIA control from Figure 51 (WT; ♦) is included for reference.
while the second portion of the curve plateaus demonstrating no further reduction in activity with increasing addition of H241N.

D. Discussion

Kinetic analysis has revealed that after TFIIIA binds to the ICR, TFIIIC binds to form a more stable complex, followed by TFIIIB and finally RNA polymerase III (3). The analysis of transcription complexes assembled on immobilized DNA templates supports this chronology, and showed that TFIIIB interaction is the kinetically limiting step in the formation of transcriptionally active complexes (14). Therefore, of the various interactions leading to transcription of a 5S RNA gene, only those which affect the formation and stability of transcription complexes would be detected in equilibrium experiments as having a major effect on transcription. In the TFIIIA-dependent in vitro assay, transcription was initiated by addition of NTPs after the reaction had been allowed to reach equilibrium and the level of transcriptional activity was limited by the concentration of active complexes. Therefore, our assay was only able to detect alterations in the equilibrium concentration of active transcription complexes or the efficiency at which transcription complexes were utilized by RNA polymerase III.
The level of 5S RNA synthesis for mutants H33N, H63N, H93N, H125N, H155N and H183N (corresponding to mutations in the 6 N-terminal fingers) was only dependent on the concentration of mutant protein. Our earlier results showed a reduced binding affinity for the 5S RNA gene for these N-terminal zinc finger mutants. Nonetheless, their transcriptional activity was not altered. These results suggest that the 6 N-terminal mutants are defective only in their initial interaction with the 5S DNA to form the binary complex; once the binary complex was formed, it was able to interact with the rest of the transcriptional machinery and be incorporated into active transcriptional complexes displaying wild-type activity.

In contrast, the three C-terminal zinc finger mutants, H210N, H241N and H272N, showed decreased or inhibitory transcriptional activity. H210N and H272N demonstrated transcriptional activity, 200% and 50% above background, respectively, suggesting that these mutants were able to form active transcription complexes. However, since the concentration of complexes was not known, we could not determine if the decrease in transcriptional activity as compared to that of the WT TFIIIA was due to (i) a decrease in the concentration of complexes or (ii) a decrease in the efficiency with which the complexes were utilized by the RNA polymerase.
Zinc finger mutant H241N, containing a disrupted eighth finger, did not stimulate transcription; instead, H241N displayed inhibitory activity, reducing background level of 5S RNA synthesis by 50%. To test the possibility that H241N was forming inactive but stable transcription complexes, thereby reducing the available pool of templates which could form active transcription complexes with endogenous TFIIIA, mixing experiments were carried out with the wild-type protein. The first set of experiments was designed to test whether the H241N mutant could form stable inactive complexes which would be resistant to addition of wild-type protein. The results suggest that the inactive transcription complexes formed with H241N are not sufficiently stable to prevent the formation of active transcription complexes in the presence of wild-type protein. However, since even high concentrations of wild-type protein could not completely suppress the inhibitory activity of the H241N protein, it is likely that inactive transcription complexes were still present and were decreasing the pool of DNA templates available to interact with wild-type protein to form active transcription complexes. Together, these results suggest that the H241N mutant protein is able to form inactive transcription complexes. However, the inactive complexes are not as stable as the active complexes formed by wild-type TFIIIA,
and during the subsequent 60 minute incubation with the wild-type protein, as the H241N-containing complexes dissociate, the DNA templates are liberated from inactive complexes and become available to interact with wild-type TFIIIA in active transcription complexes. This reasoning predicts that as the subsequent incubation period with wild-type protein is increased, the maximal level of 5S RNA synthesis attained should also increase. A longer incubation period would increase the time available for inactive H241N transcription complexes to dissociate, allowing the formation of more active transcription complexes with wild-type protein. With shorter incubation times, the level of 5S RNA synthesis should decrease. Fewer inactive H241N transcription complexes would dissociate, thereby reducing the pool of templates available for formation of active transcription complexes containing wild-type TFIIIA. These predictions can be readily tested in future experiments.

In the second set of experiments, H241N showed minimal effects on the ability of WT TFIIIA to activate transcription. With no additional experimental data, one may incorrectly conclude that loss of finger 8 function results in a protein which is unable to enter the pathway leading to formation of transcription complexes. However, we have shown that H241N may form inactive but unstable
complexes. H241N also has a seven-fold reduction compared to WT TFIIIA in its affinity for the 5S DNA. Together, both of these would minimize the effects of H241N in our assays, yielding results similar to those we observed.

In summary, mutations in any one of the 6 N-terminal zinc fingers of TFIIIA have no effect on the activity of the protein in our TFIIIA-dependent in vitro transcription assay even though they result in a decreased binding affinity for the 5S rRNA gene. Unlike the 6 N-terminal zinc fingers, the 3 C-terminal fingers are important for transcriptional activity. Since the C-terminal fingers are juxtaposed to the C-terminal domain of TFIIIA, previously shown to be important in transcriptional activation (108), the decrease in transcriptional activity observed in the finger 7-9 mutants could result from two different mechanisms. One possibility is that protein:protein interactions between the three C-terminal zinc fingers and other transcription factors would be important in stabilization of higher order transcription complexes or in the efficient use of transcription complexes by RNA polymerase III. A second possibility is that proper positioning of the C-terminal domain of TFIIIA would be dependent on the correct DNA contacts by the three C-terminal zinc fingers, and this positioning would be important for interactions with other transcription factors.
or RNA polymerase III. Whereas the first mechanism postulates direct protein:protein interactions involving zinc fingers, the second mechanism would account for loss of transcription activity in the finger 7-9 mutants as the indirect effect of disrupting zinc finger:DNA interactions adjacent to the transcriptional activation domain of TFIIIA.
CHAPTER 5
SUMMARY

Though TFIIIA is the prototype protein for the emerging class of zinc finger nucleic acid binding proteins, a systematic and rigorous analysis of the nine zinc fingers of TFIIIA had not been reported. As a first step toward such an analysis, much time and effort was expended on developing an expression and purification scheme for recombinant TFIIIA and TFIIIA mutants. The initial investment of our time and resources was key to the qualitative and quantitative analyses of TFIIIA zinc finger mutants presented in this thesis.

By analyzing TFIIIA mutants with one disrupted finger and eight wild-type zinc fingers, we were able to assign specific functions, 5S DNA interactions and kinetic and equilibrium parameters to the individual zinc fingers of TFIIIA. The ability to analyze TFIIIA zinc finger mutants with intact C-terminal domains made it possible to determine which TFIIIA zinc fingers were important for transcriptional activation.

The thermolysin analyses showed that the first of the two histidines of each zinc finger of TFIIIA is crucial in mediating zinc finger folding and that the fingers are largely structurally independent.
The TFIIIA zinc fingers most important for binding the somatic-type 5S rRNA gene are fingers 3 and 4. Although the first six N-terminal fingers of TFIIIA contribute greater than 70% of the relative binding energy to the 5S DNA, mutations in any one of these six N-terminal fingers did not affect the transcriptional activity of TFIIIA.

Quantitative measurements between TFIIIA and 5S DNA mutants (132) indicated that the specific subregions within the ICR reported to be target sites for TFIIIA binding did indeed contain specific nucleotide sequences which contributed directly to the free energy of binary complex formation. In particular, nucleotides 57-62 of box A and nucleotides 67-70 (the intermediate box) made roughly equivalent contributions to TFIIIA binding as indicated by the observation that substitution of these sequences reduced the binding constant by a factor of 3-4. In addition, the data demonstrated that nucleotides 78-86 of box C represented the major TFIIIA recognition feature since substitution of these nucleotides reduced TFIIIA binding affinity by 2 orders of magnitude. Thus, there is excellent agreement between the independent binding studies conducted with TFIIIA zinc fingers mutants and 5S DNA mutants of the relative contributions of protein and DNA regions to the overall free energy of the TFIIIA-5S DNA interaction.
The three C-terminal fingers of TFIIIA, fingers 7-9, though contributing less than 30% of the relative binding energy to the 5S DNA, are essential for transcriptional activity of TFIIIA. These three C-terminal fingers interact with the 5' end of the ICR. Future transcriptional analyses will concentrate on these three C-terminal fingers.

The results on the three C-terminal fingers complement the work by McConkey and Bogenhagen (67) of 5S DNA mutants. By analyzing transitions in the 5' end of the ICR, they were able to identify mutations which led to significant decreases in transcription efficiency, but resulted in barely detectable changes in TFIIIA binding. Together, our work and McConkey et al.'s (67) results suggest that the three C-terminal fingers of TFIIIA must make appropriate contacts within the 5' domain of the ICR to permit efficient transcription of the 5S RNA gene.
CHAPTER 6

LITERATURE CITED


