

**Studies on the Nucleic Acid Interactions of *Xenopus* Transcription Factor IIIA**

by

NICHOLAS J. VELDHUEN

B. Sc., University of Victoria, 1989

A Dissertation Submitted in Partial Fulfillment of the  
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of  
Biochemistry and Microbiology

We accept this thesis as conforming  
to the required standard

---

Dr. Paul J. Romaniuk, Supervisor (Dept. of Biochemistry & Microbiology)

---

Dr. Juan Ausio, Departmental Member  
(Dept. of Biochemistry & Microbiology)

---

Dr. Robert W. Olafson, Departmental Member  
(Dept. of Biochemistry & Microbiology)

---

Dr. Peter C. Wan, Outside Member (Dept. of Chemistry)

---

Dr. Gordon W. Bushnell, Outside Member (Dept. of Chemistry)

---

Dr. Debbie Johnson, External Examiner (University of Southern California)

© NICHOLAS J. VELDHUEN, 1995  
University of Victoria

All rights reserved. This thesis may not be reproduced in whole or in part,  
by mimeograph or other means, without the permission of the author.

Supervisor: Dr. Paul J. Romaniuk

### Abstract

The expression of the 5S ribosomal RNA genes during the development of the South African clawed toad, *Xenopus laevis*, has provided a model system for the analysis of developmental control at the level of transcription. The primary step required for expression of the 5S rRNA genes in *Xenopus* is the binding of the positive transcription factor IIIA (TFIIIA) to the internal control region of the 5S RNA gene (Engelke et al, 1980). TFIIIA binding permits the subsequent ordered assembly of a transcription complex involving at least two other transcription factors (TFIIIB and TFIIIC) and RNA polymerase III (Shastry et al, 1982; Lassar et al, 1983). Once transcribed, 5S RNA is stored in the oocyte cytoplasm associated with TFIIIA as a 7S ribonucleoprotein particle (Picard & Wegnez, 1979). Thus, TFIIIA can interact specifically with both nucleic acids during 5S RNA biosynthesis.

In order to study the interaction of TFIIIA with the 5S RNA gene, a series of single substitution mutations within the box C and intermediate elements of the internal promoter were assayed for TFIIIA binding affinity using a nitrocellulose filter binding experiment. In addition, base pair sequence within the box C promoter element that contributes to high affinity TFIIIA binding was determined by a selected amplification and binding analysis of a synthetic 5S RNA gene promoter. Intermediate element sequences at positions +70 and +71 and box C element sequences from base pair position +80 to +92 contribute energetically to TFIIIA-DNA interaction. The contribution to the free energy of DNA binding is non-equivalent between base pairs within this contact region. Local DNA conformation from base pair position +79 to +94 also contributes to high affinity interaction of TFIIIA with the 5S RNA gene promoter.

The nucleic acid binding domain of TFIIIA consists of nine zinc finger motifs (Miller et al, 1985). In the present study, recombinant TFIIIA proteins containing a series of scanning sequence substitution mutations within the N-terminal first three zinc fingers were purified to homogeneity and assayed for binding to the 5S RNA gene using the nitrocellulose filter binding experiment. Amino acid substitutions within finger two reduced 5S RNA gene association four-fold, while substitutions within finger three resulted in a six-fold reduction in 5S RNA gene binding. These results support a role for the  $\alpha$ -helices within zinc fingers two and three in establishing direct contacts with specific base pairs in the major groove of the 5S RNA gene.

The central zinc fingers four through seven of TFIIIA contribute the majority of the free energy of 5S RNA binding (Clemens et al, 1993). The present study identifies the position of a purified polypeptide containing these zinc fingers bound to 5S RNA using a ribonuclease protection assay. Zinc fingers four through seven protect 5S RNA helices II, IV, and V, in addition to loops A, B, and E from nuclease attack. Full length TFIIIA provides additional protection to helix III and loops C and D. These regions of protection correspond to sequence substitution mutants within 5S RNA that reduce TFIIIA binding two-fold to twenty-five-fold (McBryant et al, 1995).

It appears that each zinc finger within TFIIIA provides different energetic contributions to DNA and RNA binding. Interaction with the 5S RNA gene promoter is established primarily by the N-terminal fingers, while the central zinc fingers are responsible for 5S RNA binding. Thus, different regions of the nucleic acid binding domain of TFIIIA have evolved and specialized in order to confer both gene activation and RNA storage activities to the *Xenopus* transcription factor.

Examiners:

---

Dr. Paul J. Romaniuk, Supervisor (Dept. of Biochemistry & Microbiology)

---

Dr. Juan Ausio, Departmental Member  
(Dept. of Biochemistry & Microbiology)

---

Dr. Robert W. Olafson, Departmental Member  
(Dept. of Biochemistry & Microbiology)

---

Dr. Peter C. Wan, Outside Member (Dept. of Chemistry)

---

Dr. Gordon W. Bushnell, Outside Member (Dept. of Chemistry)

---

Dr. Debbie Johnson, External Examiner (University of Southern California)

## Table of Contents

Abstract .....	ii
Table of Contents .....	v
List of Tables .....	viii
List of Figures .....	ix
List of Abbreviations .....	xii
Acknowledgments .....	xv
Dedication .....	xvi

### Chapter 1.0 Biosynthesis of *Xenopus* 5S ribosomal RNA

1.1 The 5S RNA genes of <i>Xenopus</i> .....	1
1.2 Transcription and transport of 5S RNA .....	6
1.3 The TFIIIA genes of <i>Xenopus</i> .....	13
1.4 <i>Xenopus</i> TFIIIA protein structure .....	17
1.5 Developmental regulation of 5S RNA expression .....	22
1.6 Biological Function of 5S RNA .....	26

### Chapter 2.0 Interaction of TFIIIA with the 5S RNA gene

2.1 Introduction .....	29
2.1.1 Structural properties of the 5S RNA gene .....	29
2.1.2 Interaction of TFIIIA with the 5S RNA gene .....	31
2.2 Materials and Methods .....	44
2.2.1 Bacterial strains and DNA vectors .....	44
2.2.2 Purification of recombinant TFIIIA .....	44
2.2.3 PCR-based labeling of the 5S RNA gene .....	46
2.2.4 Equilibrium binding of TFIIIA to mutant 5S RNA genes .....	49
2.2.5 Selected amplification and binding (SAAB) assay .....	50

2.3	Results .....	54
2.3.1	Effects of base pair mutations within the 5S RNA gene on TFIIIA binding .....	54
2.3.2	Identification of high affinity Box C elements within the 5S RNA gene .....	58
2.4	Discussion .....	59

### Chapter 3.0 Interaction of TFIIIA with 5S RNA

3.1	Introduction .....	71
3.1.1	General structure of 5S RNA .....	71
3.1.2	Structure of <i>Xenopus</i> 5S RNA .....	76
3.1.3	Interaction of 5S RNA with <i>Xenopus</i> TFIIIA .....	80
3.2	Methods and Materials .....	88
3.2.1	Bacterial strains and DNA vectors .....	88
3.2.2	Transcription of <i>Xenopus</i> oocyte-type 5S RNA .....	89
3.2.3	Labeling of 5S RNA .....	90
3.2.4	Protein purification .....	90
3.2.5	5S RNA footprinting analysis .....	91
3.3	Results .....	95
3.3.1	Ribonuclease footprinting of TFIIIA and <i>zf4-7</i> with <i>Xenopus</i> 5S RNA .....	95
3.4	Discussion .....	98

### Chapter 4.0 Characterization of the nucleic acid binding domain of TFIIIA

4.1	Introduction .....	106
4.1.1	The C <sub>2</sub> H <sub>2</sub> zinc finger domain .....	106
4.1.2	DNA sequence recognition by C <sub>2</sub> H <sub>2</sub> zinc finger domains .....	118

4.1.3	The TFIIIA nucleic acid binding domain .....	121
4.2	Methods and Materials .....	125
4.2.1	Bacterial strains and DNA vectors .....	125
4.2.2	Construction of mutant TFIIIA expression vectors .....	125
4.2.3	Expression and purification of recombinant wild type and mutant TFIIIA proteins .....	131
4.2.4	Synthesis and radiolabeling of the 5S RNA gene and 5S RNA .....	131
4.2.5	Equilibrium binding of the 5S RNA gene and 5S RNA to TFIIIA substitution mutants .....	133
4.3	Results .....	133
4.3.1	Substitution mutagenesis of TFIIIA .....	133
4.3.2	Effects of finger swap mutations of TFIIIA on nucleic acid binding activity .....	134
4.3.3	Effects of scanning substitution mutations of TFIIIA on nucleic acid binding activity .....	136
4.4	Discussion .....	141
5.0	Conclusions .....	152
6.0	Literature cited .....	154

**List of Tables**

Table 2.1	Effects on TFIIIA binding affinity of 5S RNA gene point mutants within the intermediate promoter element .....	56
Table 2.2	Effects on TFIIIA binding affinity of 5S RNA gene point mutants within the box C promoter element .....	57
Table 2.3	Comparison of the change in the free energy of TFIIIA association with clustered and point mutants of the 5S RNA gene .....	65
Table 3.1	Relative affinities for zf4-7 and TFIIIA binding to wild type and mutant <i>Xenopus</i> 5S RNAs .....	100
Table 4.1	The Effects of TFIIIA finger substitution mutations on binding activity to the 5S RNA gene and 5S RNA .....	135
Table 4.2	The Effects of TFIIIA scanning substitution mutations on binding activity to the 5S RNA gene and 5S RNA .....	140

### List of Figures

Figure 1.1	Organization of the <i>Xenopus</i> 5S RNA multi-gene families .....	2
Figure 1.2	Sequence alignment of the <i>Xenopus</i> 5S RNA genes .....	5
Figure 1.3	Simplified kinetic scheme for 5S RNA transcription complex formation and 5S RNA synthesis .....	8
Figure 1.4	Schematic model of 5S RNA transport during different stages of <i>Xenopus</i> oogenesis .....	12
Figure 1.5	Organization of the <i>Xenopus</i> TFIIIA gene .....	15
Figure 1.6	Schematic representation of the different upstream <i>cis</i> -elements within the <i>Xenopus</i> TFIIIA promoter active during oogenesis and early embryogenesis .....	16
Figure 1.7	The structure of <i>Xenopus</i> TFIIIA protein .....	19
Figure 1.8	Interaction of eukaryotic 5S RNA with other components of the ribosome .....	28
Figure 2.1	Sequence of the <i>Xenopus</i> 5S RNA genes .....	30
Figure 2.2	Structural polymorphism in the internal promoter of the 5S RNA gene .....	32
Figure 2.3	Proposed models for the interaction of a multi-zinc finger protein with the major groove of DNA .....	38
Figure 2.4	Models for the interaction of TFIIIA with the internal promoter of the 5S RNA gene .....	39
Figure 2.5	Location of point mutations and randomized base pairs within the <i>Xenopus</i> 5S RNA gene internal control region .....	47
Figure 2.6	SDS PAGE analysis of purified recombinant TFIIIA used in the present 5S RNA gene promoter analysis .....	48
Figure 2.7	The synthetic internal control region designed to identify box C promoter elements that bind to TFIIIA with high affinity .....	51

Figure 2.8	Sample nitrocellulose filter binding curves of 5S RNA gene mutants with recombinant TFIIIA indicating the average of three or more determinations .....	55
Figure 2.9	The frequency of base pair occurrence within box C element sequences selected by high affinity TFIIIA binding .....	60
Figure 2.10	Summary of the base pair interactions within the intermediate and box C promoter elements of the 5S RNA gene .....	66
Figure 3.1	The general eukaryotic consensus sequence for 5S RNA .....	73
Figure 3.2	Secondary structure of eukaryotic 5S RNA with somatic and oocyte substitutions indicated .....	77
Figure 3.3	Three-dimensional models of spinach chloroplast 5S RNA, <i>Xenopus</i> oocyte-type 5S RNA, and loop E of <i>Xenopus</i> 5S RNA .....	78
Figure 3.4	Secondary structure of <i>Xenopus</i> 5S RNA indicating the TFIIIA footprint area .....	83
Figure 3.5	SDS-PAGE analysis of TFIIIA and zf4-7 proteins .....	93
Figure 3.6	Ribonuclease footprinting of TFIIIA:5S RNA and zf4-7:5S RNA complexes using short and long electrophoretic runs .....	96
Figure 3.7	Summary of the footprinting results of TFIIIA-5S RNA and zf4-7-5S RNA complexes .....	97
Figure 3.8	Summary of the effects that mutations in 5S RNA have on the binding of TFIIIA and zf4-7 .....	101
Figure 3.9	Proposed secondary structure of the truncated 5S RNA molecules analyzed for zf4-7 binding affinity .....	105
Figure 4.1	Tertiary structure of the C <sub>2</sub> H <sub>2</sub> zinc finger .....	107
Figure 4.2	Sequence alignment of odd and even C <sub>2</sub> H <sub>2</sub> zinc fingers .....	109
Figure 4.3	Tertiary structure of SWI5 zinc fingers one and two .....	112

Figure 4.4	Schematic representation of the interfinger orientations of MBP-1 zinc fingers and Zif268 fingers one and two .....	117
Figure 4.5	DNA sequence recognition by zinc finger domains .....	120
Figure 4.6	Schematic representation of the recombinant TFIIIA expression plasmid, pTF4 .....	128
Figure 4.7	Schematic representation of the template-independent PCR mutagenesis procedure used to create the TFIIIA finger two substitution mutant, TX2 .....	129
Figure 4.8	Schematic representation of the PCR-mediated site-directed mutagenesis procedure used in construction of scanning substitution mutations within TFIIIA .....	130
Figure 4.9	SDS-PAGE analysis of TFIIIA mutants containing scanning substitution mutations within fingers two and three .....	132
Figure 4.10	Location of scanning substitution mutations within the first three zinc fingers of TFIIIA .....	137
Figure 4.11	Sample nitrocellulose filter binding curves of TFIIIA mutants with the oocyte-type 5S RNA gene indicating the average of three or more determinations .....	138
Figure 4.12	Sample nitrocellulose filter binding curves of TFIIIA mutants with oocyte-type 5S RNA indicating the average of three or more determinations .....	139
Figure 4.13	Structural representation of a C <sub>2</sub> H <sub>2</sub> zinc finger and helical wheel diagrams of TFIIIA zinc fingers two and three .....	147
Figure 4.14	Alignment of zinc finger sequences indicating the location of amino acid residues that contact DNA .....	148

**List of Abbreviations**

bp: base pair

BSA: bovine serum albumin

cDNA: complementary deoxyribonucleic acid

cpm: counts per minute

deoxynucleotide triphosphates:

dATP, deoxyadenosine triphosphate

dCTP, deoxycytidine triphosphate

dGTP, deoxyguanine triphosphate

dTTP, deoxythymidine triphosphate

DEPC: diethyl pyrocarbonate

DNA: deoxyribonucleic acid

DTT: dithiothreitol

DTE: dithioerythritol

*E. coli*: *Escherichia coli*

EDTA: ethylenediamine-tetraacetic acid

Hepes: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

ICR: internal control region

IE: intermediate element

IPTG: isopropyl- $\beta$ -D-thiogalactopyranoside

LB: Luria-Benton broth

mRNA: messenger ribonucleic acid

MW: molecular weight

NMR: nuclear magnetic resonance

nt: nucleotide

**nucleotide bases:**

A, adenine

C, cytosine

G, guanine

T, thymidine

U, uracil

N, either A, C, G, or T

**nucleotide triphosphates:**

ATP, adenosine triphosphate

CTP, cytidine triphosphate

GTP, guanine triphosphate

UTP, uridine triphosphate

PAGE: polyacrylamide gel electrophoresis

PAR: 4-(2-pyridylazo) resorcinol

pCp: cytidine 3',5'-bisphosphate

PEG: polyethylene glycol

PMPS: p-(hydroxymercuri)phenylsulfonate

PMSF: phenylmethylsulfonyl fluoride

PPO: 2,5-diphenyloxazole

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

RNase: ribonuclease

RNP: ribonucleoprotein

S: svedberg unit

SAAB: selected amplification and binding

SDS: sodium dodecyl sulphate

TBE: Tris base, borate, EDTA

TFIIIA: transcription factor IIIA

TFIIIB: transcription factor IIIB

TFIIIC: transcription factor IIIC

tRNA: transfer ribonucleic acid

Tris-HCl: tris-(hydroxymethyl)aminomethane hydrochloride

Xbo: *Xenopus borealis* oocyte

Xbs: *Xenopus borealis* somatic

Xlo: *Xenopus laevis* oocyte

Xls: *Xenopus laevis* somatic

Xlt: *Xenopus laevis* trace-oocyte

**Acknowledgments**

I am grateful to Dr. Paul Romaniuk for introducing me to the scientific process and overseeing my progress through the years. Valuable assistance was generously provided by all members of Dr. Romaniuk's laboratory, past and present. I am also appreciative of productive collaborations with the laboratories of Dr. Joel Gottesfeld at the Scripps Clinic and Research Institute and Dr. David Setzer at Case Western Reserve University. The support of my wife, Kathy Veldhoen, was invaluable in the completion of this work. I would also like to thank my father, Ben Veldhoen, for convincing me to "leave all doors open" in my travels through the various halls of learning.

**Dedication**

This body of work is dedicated to my mother, Kandy Veldhoen, who brought out the best in all with encouragement and patience.

## Chapter 1.0 Biosynthesis and Function of *Xenopus* 5S ribosomal RNA

### 1.1 The 5S RNA genes of *Xenopus*

The genomic DNA of *Xenopus* contains four classes of 5S RNA genes. Each class is organized into tandemly repeated units that are clustered at unique positions in the chromosomes. The major-oocyte 5S RNA genes and pseudogenes are the most abundant classes, with approximately 24,000 copies each per haploid genome (Fedoroff & Brown, 1978). In *Xenopus laevis*, these two gene classes are present within a single genetic unit 650 to 860 base pairs long that contains a 360 to 570 base pair spacer element (Fedoroff & Brown, 1977; Fedoroff & Brown, 1978; Miller et al., 1985) (Figure 1.1A). This spacer element also exhibits internal sequence repetition of similar, but not identical, AT-rich sequences (region A) (Fedoroff & Brown, 1978). DNA sequence that includes 73 base pairs upstream of the start site of transcription and the first 101 base pairs of the oocyte gene comprise a GC-rich region that is duplicated to produce the 3' end of the genetic unit containing the pseudogene (region B) (Miller et al., 1978) (Figure 1.1B).

Comparison of the repetitious sequences within the *Xenopus laevis* oocyte-pseudogene repeat suggests that this genetic unit is, in general, a fixed collection of old duplications/deletions (regions A<sub>3</sub>, B, A<sub>1</sub>) bounded by a variable number of recent duplications (region A<sub>2</sub>) that could function as a recombination 'hotspot' (Fedoroff & Brown, 1978) (Figure 1.1B). Thus, duplication of the oocyte-pseudogene unit that created the 24,000 gene copies appears to be a relatively recent event in *Xenopus laevis* evolution. The organization of the oocyte-pseudogene cluster is different in *Xenopus borealis* than for *Xenopus laevis*, with 9000 oocyte gene copies per haploid genome and variability in the number and relative positions of genes and

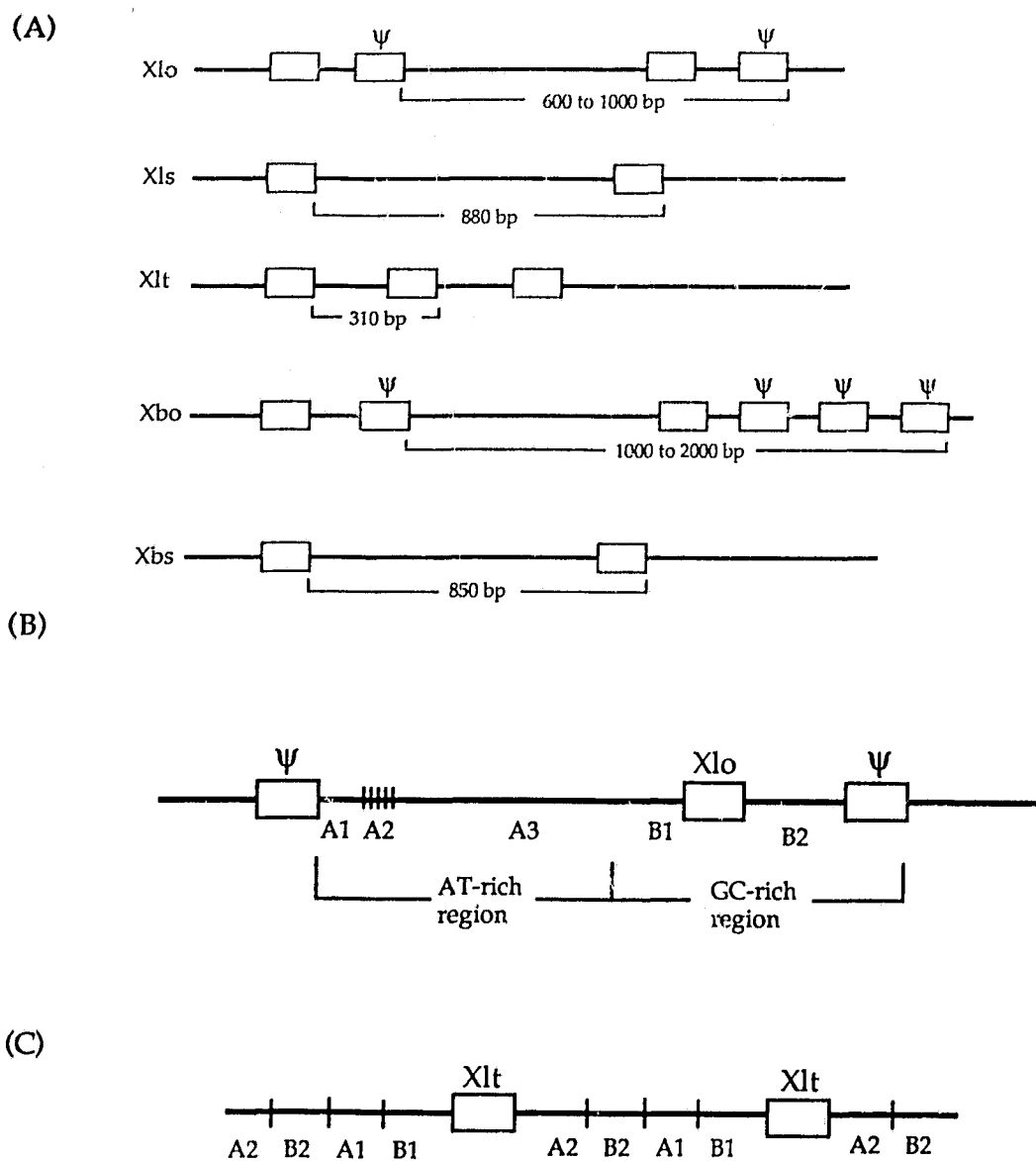


Figure 1.1 Organization of the *Xenopus* 5S RNA multi-gene families. (A) *Xenopus laevis* oocyte (Xlo), somatic (Xls), and trace-oocyte (Xlt) as well as *Xenopus borealis* oocyte (Xbo) and somatic (Xbs) gene repeats are shown, with the location of pseudogenes depicted by a 'Ψ' (Korn, 1982). *Xenopus laevis* oocyte (B) and trace-oocyte (C) gene repeats are shown in greater detail (Miller et al., 1978; Peterson et al., 1980).

pseudogenes between repeats (Korn & Brown, 1978; Korn, 1982).

The other two classes of 5S RNA genes are present in lower abundance and carry only one gene per repeat. In *Xenopus laevis*, the trace-oocyte class is found in gene repeats of 1300 copies per haploid genome with a short 190 base pair intergenic spacer element (Brown et al., 1977). This spacer contains AT-rich and GC-rich sequences that are duplicated twice between each gene copy (Figure 1.1C). Similarity between the GC-rich regions of oocyte and trace-oocyte indicate a common origin for these two gene classes (Peterson et al., 1980). Unlike the AT-rich spacer region of the oocyte-pseudogene genetic unit, the trace-oocyte spacer does not contain a repetitive simple sequence (Peterson et al., 1980). The *Xenopus laevis* somatic 5S RNA gene class occurs in gene repeats of 400 copies per haploid genome and contain a 760 base pair intergenic spacer element that is GC-rich (Peterson et al., 1980). The organization of the somatic 5S RNA genes in *Xenopus borealis* is similar to that of *Xenopus laevis*, with a repeat unit of 850 base pairs and 700 gene copies per haploid genome (Peterson et al., 1980; Korn, 1982). The somatic genes and adjacent spacer regions exhibit a greater conservation in length and sequence between the two *Xenopus* species than do the oocyte genes and their related spacer elements (Peterson et al., 1980). The dual somatic-oocyte 5S RNA gene system is not unique to *Xenopus* and predates the evolution of amphibians (Denis & Wegnez, 1977). This suggests that similarities and differences between the repeat organization of the 5S RNA gene families may reflect different functional constraints imposed during amphibian evolution.

Unlike RNA polymerase II-transcribed genes, the 5S RNA gene families are transcribed by RNA polymerase III from a promoter that is within the coding region of the genes (Bogenhagen et al., 1980; Sakonju et al., 1980) (Figure 1.2). This internal control region (ICR) is similar in sequence

between the different 5S RNA genes and is the site of transcription factor binding and assembly of a functional transcription preinitiation complex (Engelke et al., 1980; Shastry et al., 1982). DNA sequences flanking the internal promoter may also contribute to efficient 5S RNA transcription (Majowski et al., 1987; Keller et al., 1990; Wolffe & Morse, 1990). The upstream spacer sequence is not required for *in vivo* transcription of major-oocyte or somatic 5S RNA but may function in correctly identifying the precise start site of transcription (Korn & Brown, 1978; Sakonju et al., 1980). Conserved sequences AAAAG, AGAAG, and GAC are located approximately 15, 25, and 35 base pairs upstream from the transcription start site, respectively, in a number of RNA polymerase III-transcribed genes (Korn & Brown, 1978) (Figure 1.2). These sequences, positioned along the same face of the DNA helix, may represent interaction sites for accessory proteins that modulate transcription of the 5S RNA genes (Wormington et al., 1981). A two-component activity within *Xenopus* oocytes and mammalian HeLa cells has been identified that interacts with the conserved sequences at positions -15 and -25 and increases 5S RNA expression four-fold (Oei & Pieler, 1990). The first transcribed nucleotide is generally a purine residue flanked by pyrimidine bases (Korn, 1982). Transcription of the major-oocyte and somatic genes terminates primarily within the first of a number of T clusters on the non coding strand (Korn & Brown, 1978; Bogenhagen & Brown, 1981) (Figure 1.2). Differences in gene sequence between the three transcribed 5S RNA genes (major-oocyte, trace-oocyte, and somatic) may function in their differential regulation during development (Xing & Worcel, 1989a).

The oocyte-pseudo 5S RNA gene repeats are clustered in the telomeric region of the long arm of most or all of the 18 haploid *Xenopus* chromosomes (Pardue et al., 1973; Harper et al., 1983). Trace-oocyte 5S RNA genes are

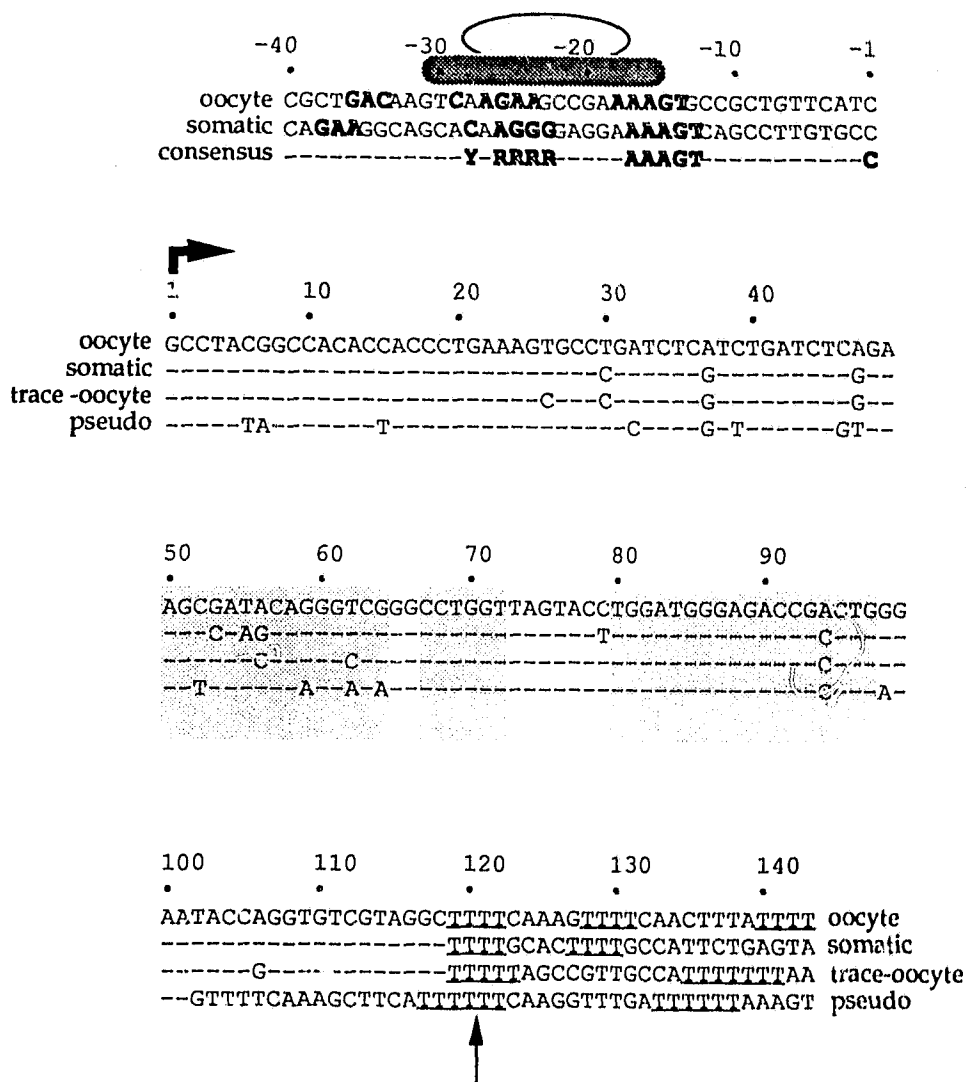


Figure 1.2 Sequence alignment of the *Xenopus* 5S RNA genes. A dash denotes identical base pair sequence at that position, while shaded sequences identify the internal promoter. Sequences upstream that influence transcription are shown outlined, with a proposed two-component activation complex positioned at  $-31$  to  $-15$  (Oei & Pieler, 1990). The bold arrow shows the start site of transcription and the major termination sites are indicated with a thin arrow (Korn & Brown, 1978). Termination signals in the downstream region of the genes are underlined (Korn & Brown, 1978).

present at the distal end of the long arm of chromosome 13, while somatic gene clusters are located in the telomeric region of the long arm of chromosome nine (Harper et al., 1983). The somatic and trace-oocyte 5S RNA genes may also be present at a number of minor sites within the *Xenopus* genome (Peterson et al., 1980).

## 1.2 Transcription and transport of 5S RNA

Investigation into the process of eukaryotic RNA transcription has benefited from the early development of *in vitro* 5S RNA transcription systems (Parker & Roeder, 1977; Birkenmeier et al., 1978; Ng et al., 1979; Weil et al., 1979; Wormington et al., 1981) and from micro injection of oocyte nuclei with purified 5S RNA genes (Brown & Gurdon, 1978). Accurate initiation of 5S RNA transcription requires a functional transcription complex assembled within the coding region of the gene and is sensitive to sequence context at the initiation site (Sakonju et al., 1980; Segall et al., 1980; Cozzarelli et al., 1983). The *Xenopus* protein factors involved in 5S RNA gene transcription have been isolated either to homogeneity, in the case of TFIIA (Engelke et al., 1980), or as a partially purified chromatographic fraction, as is the case for TFIIC and TFIIIB (Shastry et al., 1982; Keller et al., 1992). Similar 5S RNA gene transcription factors have been characterized in a variety of diverse organisms, including humans (Segall et al., 1980; Schneider et al., 1990) and yeast (Braun et al., 1989; Parsons & Weil, 1990).

The active *Xenopus* 5S RNA gene transcription complex contains the 5S-specific transcription factor IIA (TFIIIA) in addition to two (or more) general RNA polymerase III transcription factors (TFIIC and TFIIIB) that assemble onto the gene promoter in an ordered sequence (Segall et al., 1980; Shastry et al., 1982) (Figure 1.3). First, TFIIA binds to the ICR of the 5S RNA

gene forming a metastable complex (Engelke et al., 1980; Lassar et al., 1983; Setzer & Brown, 1985). TFIIC recognizes and binds to this metastable complex predominantly through protein-protein interactions with the C-terminal domain of TFIIIA (Lassar et al., 1983; Setzer & Brown, 1985; Hayes et al., 1989). However, regions of the 5S RNA gene that influence TFIIC association have also been identified (Majowski et al., 1987; Keller et al., 1990; Keller et al., 1992). The TFIIIA-TFIIC complex associated with the ICR is substantially more stable than the TFIIIA-ICR binary complex (Lassar et al., 1983; Setzer & Brown, 1985; Keller et al., 1992). TFIIB then binds at a considerably slower association rate to complete the assembly of the 5S RNA gene preinitiation complex (Lassar et al., 1983; Setzer & Brown, 1985; Bieker & Roeder, 1986) (Figure 1.3). Studies performed on the yeast 5S RNA gene system suggest that TFIIB is the primary transcription initiation factor of RNA polymerase III, while TFIIIA and TFIIC represent assembly factors (Kassavetis et al., 1990). One TFIIB component recently identified is the TATA-box binding protein (TBP) that is required in transcription of eukaryotic genes by RNA polymerase I, II, and III (Gottesfeld et al., 1994).

The *Xenopus* somatic 5S RNA gene interacts with the assembled transcription complex over an extended 180 base pair region (-24 to +159), with DNase I protected sequences located at the transcription initiation site, within the ICR, and at the termination signal (Wolffe & Morse, 1990). Such an extended protection region is also observed for the yeast 5S RNA gene transcription complex (Braun et al., 1989). Transcription complexes formed on trace-oocyte and major-oocyte 5S RNA genes are not as active as those assembled onto the somatic-type gene, as determined in both oocyte and somatic cell extracts (Wormington et al., 1981; Millstein et al., 1987; Keller et al., 1990). This differential transcription activity may be due, in part, to a

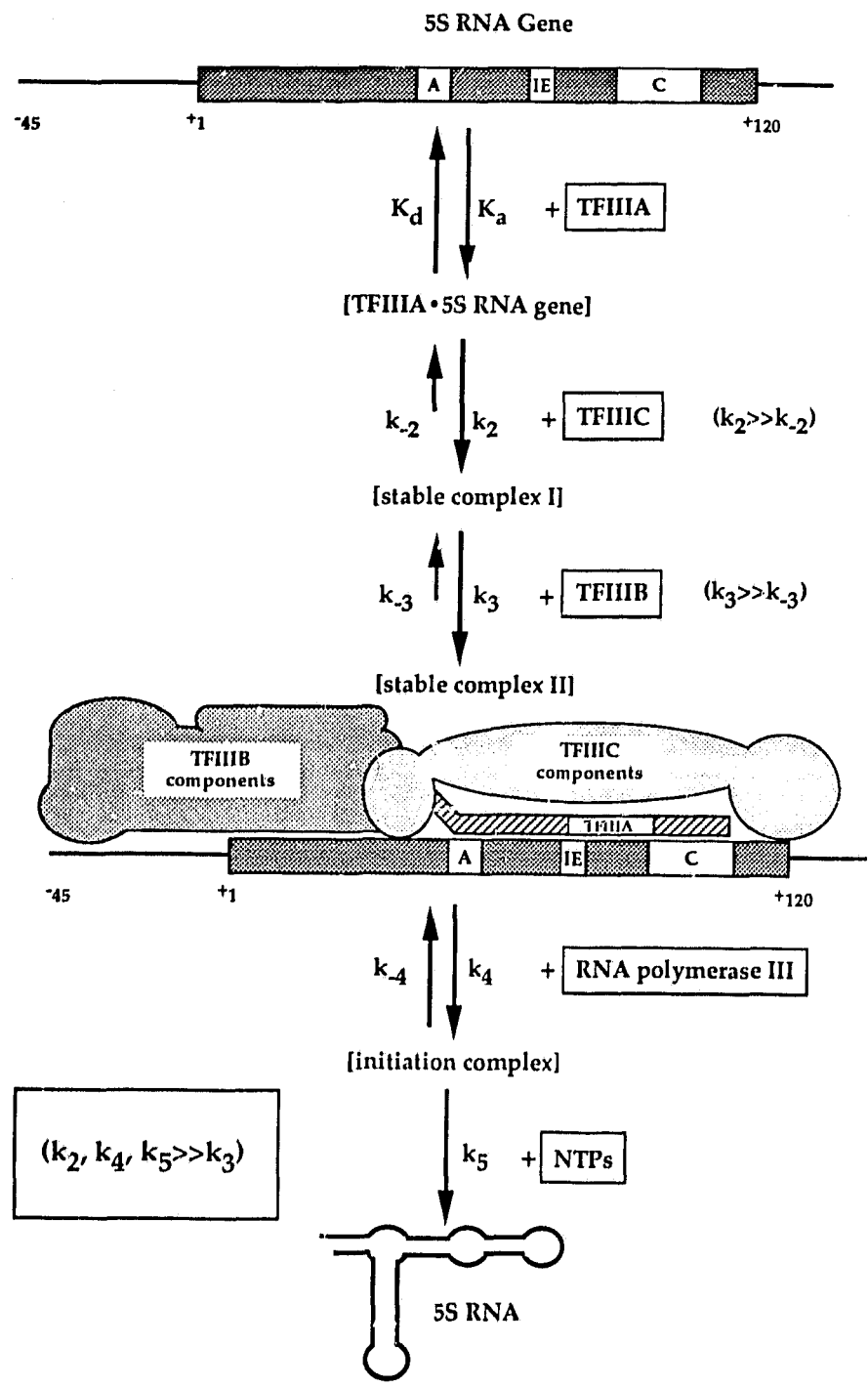


Figure 1.3 Simplified kinetic scheme for 5S RNA transcription complex formation and 5S RNA synthesis (Del Rio et al., 1993a).

greater affinity of TFIIC for metastable TFIIIA-DNA complexes assembled on the somatic 5S RNA gene rather than on the oocyte-type gene (Keller et al., 1990; Keller et al., 1992). Complex discrimination by TFIIC involves base pair sequences within the box A promoter element and sequences immediately upstream of the ICR (Xing & Worcel, 1989a; Keller et al., 1990). Such a discriminatory activity of TFIIC, coupled with differences in TFIIIA association, could contribute to selective repression of the oocyte 5S RNA genes during early *Xenopus* development (Wolffe, 1988; Xing & Worcel, 1989a; Keller et al., 1990; Keller et al., 1992).

The preinitiation complex is highly stable and persists on 5S RNA genes *in vivo* for weeks, even in the absence of active 5S RNA transcription or significant concentrations of free factors (Darby et al., 1988). In addition, it has been suggested that the 5S RNA gene transcription complex remains bound to the internal promoter through multiple rounds of 5S RNA transcription (Bogenhagen et al., 1982; Setzer & Brown, 1985; Wolffe et al., 1986). This may be accomplished either by transient association with the non coding DNA strand (Sakonju & Brown, 1982) or by sequential dissociation and reassociation of individual protein domains with the DNA (Miller et al., 1985). Thus, the assembled transcription complex plays a role in establishing a transcriptionally 'committed' state for the 5S RNA gene classes. In contrast, TFIIIA complexed with the 5S RNA gene promoter is readily removed by a transcribing RNA polymerase *in vitro* (Campbell & Setzer, 1991).

Transcription of the major-oocyte and somatic genes terminates primarily within the first of a number of T clusters on the non coding strand (Korn & Brown, 1978; Bogenhagen & Brown, 1981) (Figure 1.2). The activity of this AT-rich termination region is enhanced by GC-rich sequence immediately flanking the T cluster (Korn & Brown, 1978; Bogenhagen &

Brown, 1981). Transcription termination appears to be a multi-step process that includes cessation of elongation, termination signal recognition, RNA strand displacement, and polymerase release (Bogenhagen & Brown, 1981; Gottlieb & Steitz, 1989a; Campbell & Setzer, 1992). The simple 5S RNA gene termination signals are recognized by RNA polymerase III in the absence of auxiliary factors (Cozzarelli et al., 1983). The polymerase then pauses at these T clusters (Campbell & Setzer, 1992). However, the 50 kD La protein, which copurifies with TFIIC activity, may be required for the polymerase to complete transcription through the termination signal and release 5S RNA (Gottlieb & Steitz, 1989b). Once the termination signal has been identified, polymerase release may also be dependent on RNA strand displacement during the elongation process (Campbell & Setzer, 1992).

Although the majority of *Xenopus* 5S RNA is transcribed in a mature form, inefficient termination and readthrough to a downstream termination site can occur, particularly with the oocyte 5S RNA gene (Bogenhagen & Brown, 1981). These RNA molecules are processed by a 3' RNA exonuclease to the mature form (Xing & Worcel, 1989b). It is interesting to note that the pseudogene is efficiently transcribed *in vitro*, but a transcript is not observed *in vivo* (Ford & Southern, 1973; Wormington et al., 1981; Xing & Worcel, 1989b). The same ribonuclease activity that processes 142 nt and 130 nt readthrough products of the oocyte gene to the mature 5S RNA form is also suggested to rapidly degrade the pseudogene transcript *in vivo* (Xing & Worcel, 1989b). Differential stability of the oocyte and somatic 5S RNA compared with the pseudogene transcript may be attributed to formation of a stable helical structure by the 5' and 3' ends of the RNA molecule (Xing & Worcel, 1989b). All mature 5S RNA transcripts are subsequently associated transiently through their 3' uridylyate tails with the La protein (Gottlieb &

Steitz, 1989a; Guddat et al., 1990; Allison et al., 1991). This nuclear phosphoprotein appears to be a RNA polymerase III-specific termination factor required for transcription of the termination signal and transcript release (Gottlieb & Steitz, 1989b).

TFIIIA associates with nascent 5S RNA molecules within the nucleus and the La protein may then be recycled into the transcription complex assembled on the active 5S RNA gene (Gottlieb & Steitz, 1989b) (Figure 1.4). In immature oocytes (stages I to III), 5S RNA is transported to the cytoplasm and stored as a 7S ribonucleoprotein (RNP) particle in association with TFIIIA and as a 42S RNP in association with protein p43, tRNA, and protein p50 (Picard & Wegnez, 1979; Picard et al., 1980) (Figure 1.4). Independent studies have also shown association of nascent 5S RNA with ribosomal protein L5 and transport of the 5S RNP particle to the cytoplasm (Guddat et al., 1990). Migration of 7S RNP from the nucleus, during oogenesis, may result in a limited concentration of nuclear TFIIIA and a reduction in the transcriptional competence of the 5S RNA genes (Guddat et al., 1990). As the oocyte matures (around stages III and IV) TFIIIA and 42S RNP levels begin to decrease and maximal synthesis of ribosomal protein L5 is observed (Picard et al., 1980; Wormington, 1989). The stored 7S RNP then undergoes an exchange reaction and a 5S RNP containing L5 protein and 5S RNA accumulates (Allison et al., 1991) (Figure 1.4). The binding sites of TFIIIA and L5 protein on 5S RNA may overlap which suggests that a direct competition for complex formation occurs (Nazar & Wildeman, 1983; Romaniuk et al., 1987a). The 5S RNP particle is subsequently transported back into the nucleus to the nucleolus for ribosome assembly (Allison et al., 1991). In somatic cells, 5S RNA may associate with La protein in the nucleoplasm, exchange into a 5S RNP with L5

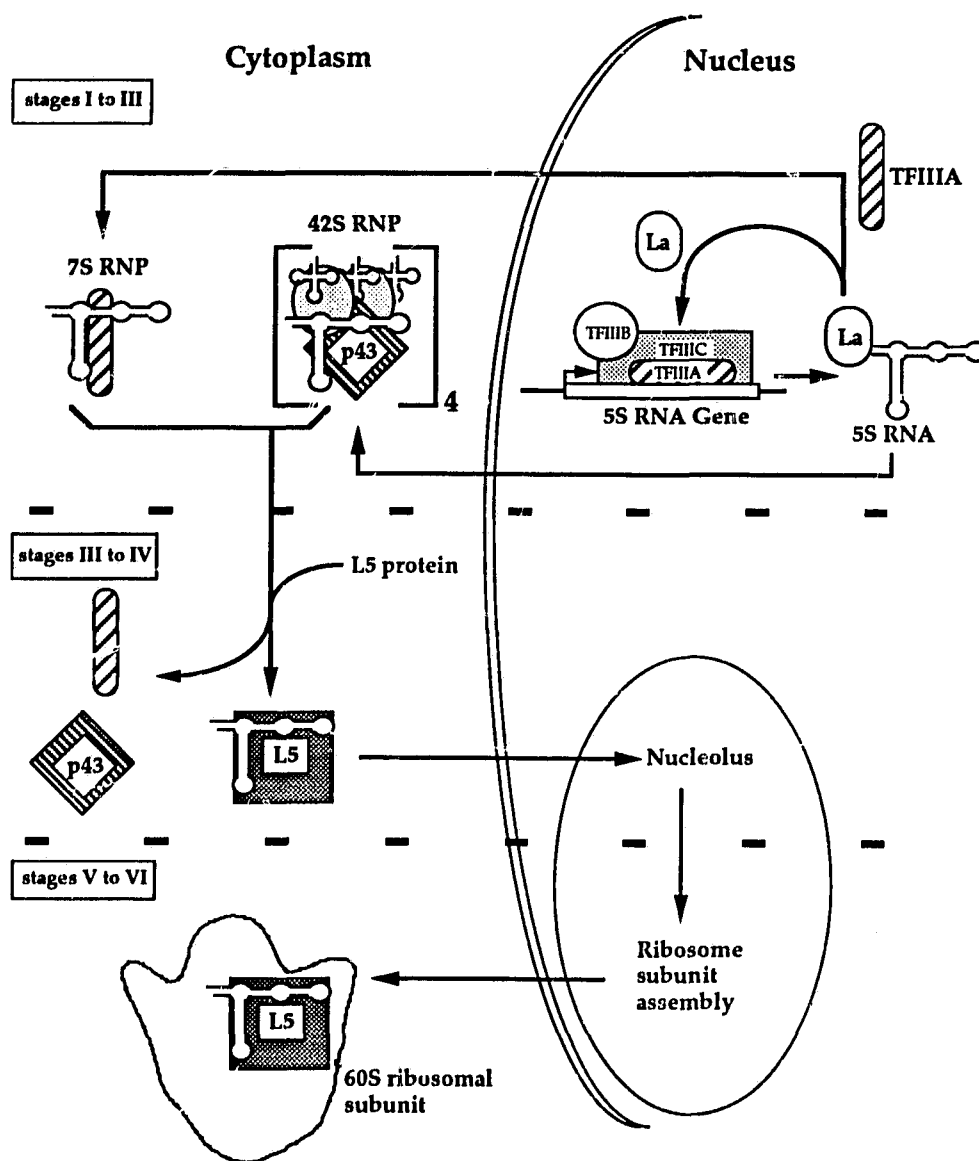


Figure 1.4 Schematic model of 5S RNA transport during different stages of *Xenopus* oogenesis (Wormington, 1989; Gottlieb & Steitz, 1989b; Allison et al., 1991).

protein, and migrate directly to the nucleoli without the need for cytoplasmic targeting (Steitz et al., 1988).

### 1.3 The TFIIIA genes of *Xenopus*

The TFIIIA gene is present as a single or low copy gene within the *Xenopus* genome and is polymorphic, with at least two allelic forms (Taylor et al., 1986; Tso et al., 1986; Scotto et al., 1989). Multiple gene copies and sequence polymorphism in such a functionally important gene may have arisen by a chromosome duplication event occurring relatively early in *Xenopus* evolution (30 million years ago) that generated an essentially tetraploid *Xenopus laevis* genome (Bisbee et al., 1977). Polymorphism is also observed for the genes encoding the *Xenopus* L5 protein (Wormington, 1989). The TFIIIA gene is approximately 11 kilobases in length and contains nine coding regions separated by eight introns (Tso et al., 1986) (Figure 1.5). The location of exon-intron boundaries with respect to encoded structural domains within the protein suggests that TFIIIA evolved from a primordial genetic unit encoding the zinc finger DNA binding domain (Tso et al., 1986).

The expression of TFIIIA is developmentally regulated, exhibiting a strong correlation with the level of 5S RNA synthesis (Ginsberg et al., 1984; Shastry et al., 1984; Taylor et al., 1986). The region adjacent to the transcription start site of the TFIIIA gene is similar to many other genes transcribed by RNA polymerase II (Figure 1.6). A consensus TATA box sequence is found at position  $-32$  and a CAAT box sequence is centered at base pair  $-96$  (Tso et al., 1986). The sequence further upstream of the TFIIIA coding region contains four negative ( $-425$  to  $-350$ ,  $-306$  to  $-289$ ,  $-235$  to  $-175$ , and  $-200$  to  $-159$ ) and seven positive ( $-671$  to  $-629$ ,  $-289$  to  $-253$ ,  $-250$  to  $-173$ ,  $-167$  to  $-122$ ,  $-159$  to  $-110$ ,  $-144$  to  $-101$ ,  $-110$  to  $-58$ ) *cis*-acting elements (Matsumoto & Korn, 1988;

Hall & Taylor, 1989; Scotto et al., 1989; Pfaff et al., 1991; Pfaff & Taylor, 1992). These *cis*-elements function in unique combinatorial patterns during the immature oocyte, mature oocyte, and embryonic stages of *Xenopus* development (Pfaff et al., 1991; Pfaff & Taylor, 1992) (Figure 1.6).

Both constitutively expressed and developmentally regulated transcription factors interact with the TFIIA promoter (Pfaff et al., 1991). When incubated with a mature oocyte or somatic cell extract two protein-DNA complexes are observed to form within this upstream region; complex B1 at  $-271$  to  $-253$  and complex B2 at  $-253$  to  $-221$  (Scotto et al., 1989; Pfaff et al., 1991). Protein associated with the former complex (B1) has been identified as a *Xenopus* homolog to the adenovirus major late transcription factor (MLTF) and is a member of the helix-loop-helix class of trans-acting factors (Sawadogo & Roeder, 1985; Hall & Taylor, 1989; Scotto et al., 1989; Kaulen et al., 1991). This *Xenopus* protein, termed the TFIIA distal element factor (TDEF), recognizes the core sequence  $-269$ CACGTG $-264$  and contributes to a three-fold enhancement of TFIIA expression in oocytes, although it is also present in somatic cells (Hall & Taylor, 1989; Scotto et al., 1989; Kaulen et al., 1991). The oocytic and embryonic TDEF-DNA complexes may contain different post-translationally modified forms of the transcription factor indicating an additional level of TFIIA gene regulation (Kaulen et al., 1991). A third complex formed in immature oocytes, termed B3, is located on a large inverted repeat sequence at  $-670$  to  $-635$  and increases TFIIA expression two-fold (Pfaff & Taylor, 1992). The protein factor associated with the B3 complex is developmentally regulated and is expressed in oocytes but not in somatic cells. TFIIA itself may also interact with a region upstream from the TFIIA gene (Hanas & Smith, 1990). This interaction is suggested to occur within the negative element from  $-306$  to  $-289$  and could interfere with TDEF binding

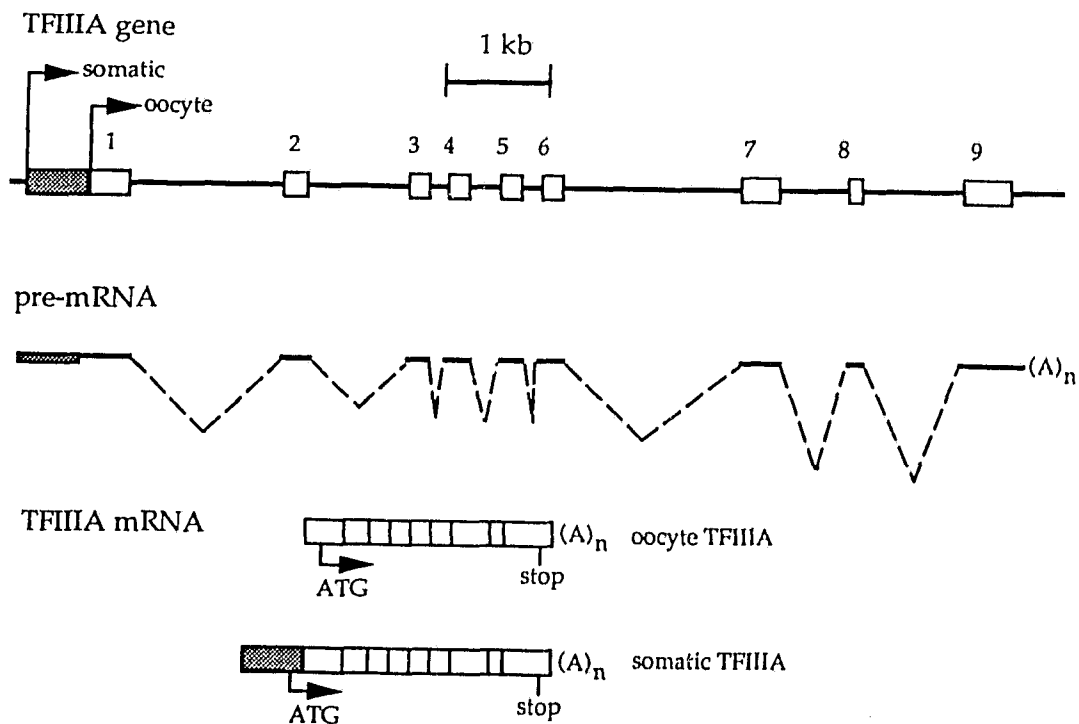


Figure 1.5 Organization of the *Xenopus* TFIIIA gene. The nine coding exons are represented by boxes (Tso et al., 1986). Transcription start sites for oocyte and somatic forms of TFIIIA are shown with additional somatic exon 1 sequence shaded. The pre-mRNA and mRNA for both TFIIIA forms are similar with additional RNA sequences at the 5' end of the somatic message shaded and a translation initiation signal indicated further upstream (Ginsberg et al., 1984; Kim et al., 1990).

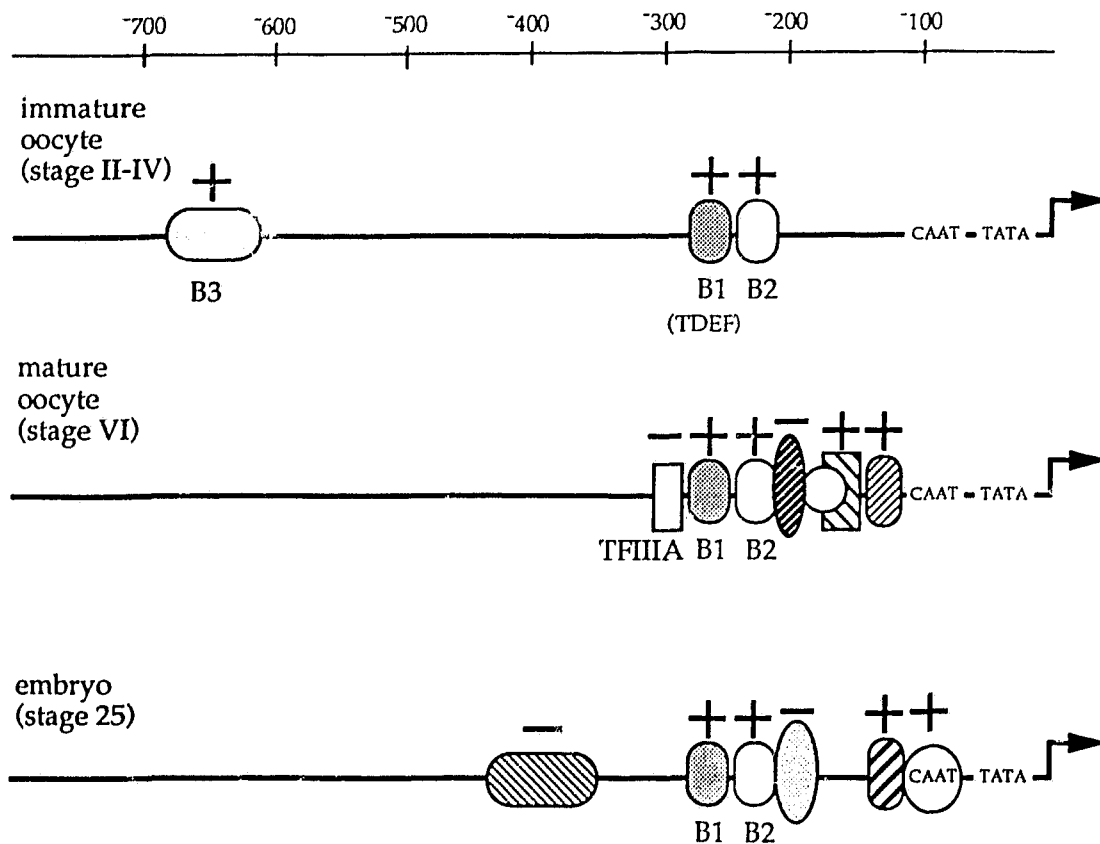


Figure 1.6 Schematic representation of the different upstream *cis*-elements within the *Xenopus* TFIIIA promoter active during oogenesis and early embryogenesis. Activator and repressor activities are denoted by '+' and '-', respectively. Stage-specific complexes B1, B2, B3 and a putative binding site for TFIIIA are shown (Matsumoto & Korn, 1988; Hall & Taylor, 1989; Scotto et al., 1989; Hanas & Smith, 1990; Pfaff et al., 1991; Pfaff & Taylor, 1992).

at the adjacent positive control element (-271 to -253) (Hanas & Smith, 1990) (Figure 1.6).

Oocyte and somatic forms of TFIIIA have been identified (Pelham et al., 1981; Shastry et al., 1984; Kim et al., 1990). The somatic-form TFIIIA, which contains 22 additional amino acid residues at the N-terminus, is not expressed until the embryonic stage of *Xenopus* development and is functionally indistinguishable from oocyte-form TFIIIA (Shastry et al., 1984; Kim et al., 1990). Adult kidney cells are found to express 800 molecules of oocyte-form TFIIIA and 2000 molecules of somatic-form TFIIIA protein (Shastry et al., 1984). It is proposed that these different forms of TFIIIA are expressed from two overlapping promoters, which are differentially active in oocyte and somatic cells and utilize different start sites of transcription (Kim et al., 1990; Martinez et al., 1994) (Figure 1.5). RNA polymerase III has been shown to initiate transcription 70 base pairs upstream of the oocyte promoter in somatic cell nuclear extracts (Martinez et al., 1994). It is suggested that this activity may represent an RNA polymerase III-mediated down regulation of the oocyte-specific TFIIIA gene promoter in somatic cells. Termination of the 1.4 kilobase TFIIIA mRNA transcript occurs 255 residues downstream of the translational stop codon and the message is subsequently polyadenylated (Tso et al., 1986). Sequences within the 3' end of TFIIIA mRNA have been found to significantly reduce the half life of a chimaeric mRNA construct and may contain an endonuclease recognition site that functions in rapid turnover of TFIIIA transcripts (Harland & Misher, 1988).

#### **1.4 *Xenopus* TFIIIA protein structure**

TFIIIA is involved in the developmental regulation of 5S RNA gene transcription by RNA polymerase III (Engelke et al., 1980; Sakonju et al., 1980).

In *Xenopus* oocytes TFIIIA not only acts as a positive transcription factor but also interacts with 5S RNA to form a 7S ribonucleoprotein particle (RNP) (Pelham & Brown, 1980). *Xenopus* TFIIIA is 344 amino acids in length, 38.5 kD in size, and contains nine C<sub>2</sub>H<sub>2</sub> zinc finger motifs tandemly repeated through the N-terminal two thirds of the protein (Brown et al., 1985; Miller et al., 1985) (Figure 1.7). This 30 kD N-terminal region comprises the nucleic acid binding domain of TFIIIA, while a 10 kD C-terminal region functions in transcription activation (Smith et al., 1984). The DNA-binding domain of TFIIIA may have evolved through multiple gene duplicating events of an ancient finger sequence followed by sequence divergence (Ginsberg et al., 1984). This is supported by exon-intron boundaries observed in the TFIIIA gene (Tso et al., 1986). The first six zinc fingers of TFIIIA and the C-terminal transactivation domain are all encoded by separate exons. The conformation of the TFIIIA nucleic acid binding domain does not appear to change upon 5S RNA binding (Hanas et al., 1989). In contrast, interaction with the 5S RNA gene results in a structural change in the zinc finger region of TFIIIA concomitant with enhanced protease sensitivity (Hanas et al., 1989). A conformational change in TFIIIA structure may be necessary for correct positioning along the DNA promoter sequence and/or for efficient transactivation of transcription.

The requirement of zinc for the folding of individual finger structures and the contributions made by each zinc finger of TFIIIA to nucleic acid binding would suggest that all nine fingers of TFIIIA associate with zinc in the fully functional protein. However, conflicting evidence exists for the number of bound zinc ions in active TFIIIA. The apparent discrepancies may result from differences in the experimental techniques (Miller et al., 1985; Han et al., 1990) and/or in the relative affinities of individual fingers for

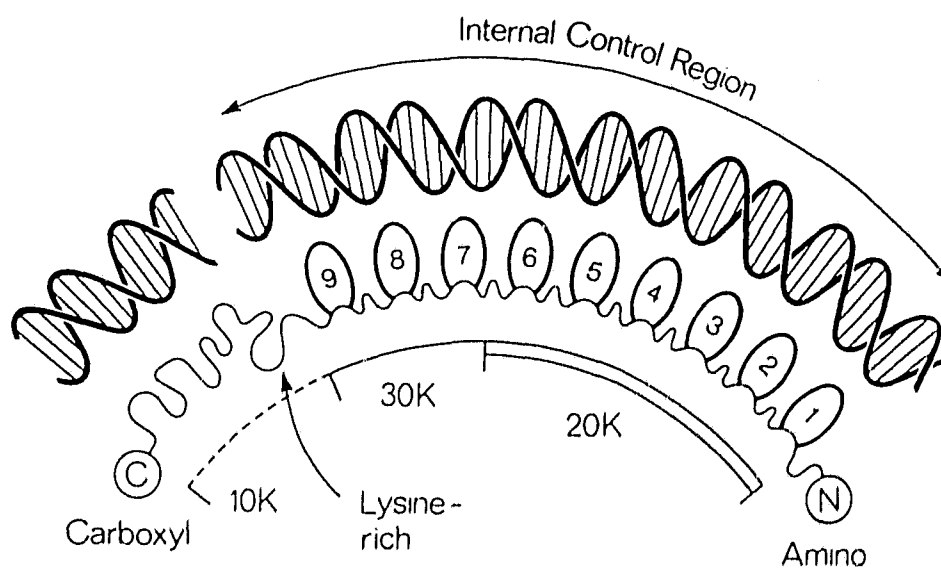
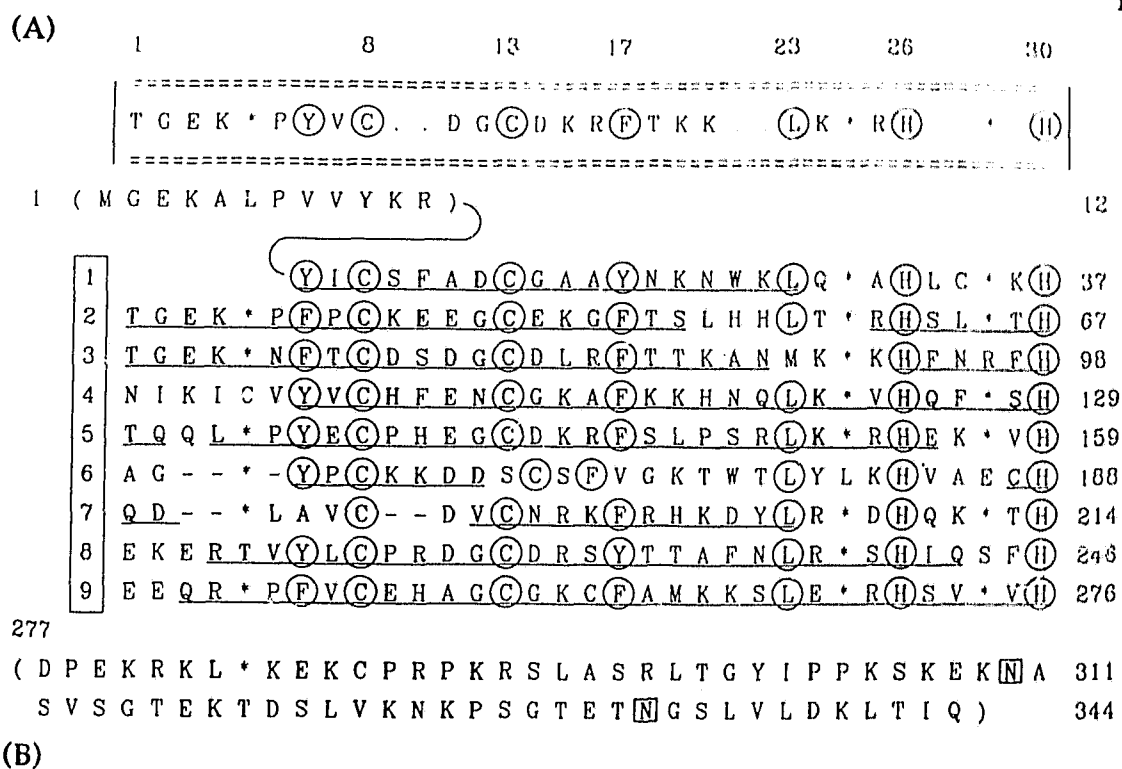


Figure 1.7 The structure of *Xenopus* TFIIIA protein. (A) Sequence alignment of TFIIIA indicating the repeated C<sub>2</sub>H<sub>2</sub> zinc finger motif (Miller et al., 1985). (B) Schematic depicting the extended TFIIIA protein containing nine zinc fingers and a C-terminal transactivation domain (Miller et al., 1985).

zinc(II) (Han et al., 1990). Independent atomic absorption analyses of EDTA-treated 7S RNP particles have shown two mole equivalents (Hanas et al., 1983a) and seven to eleven moles (Miller et al., 1985) of zinc associated with TFIIA. Experiments using a sulfhydryl modifier, PMPS, and the metallochromophore PAR have identified nine mole equivalents of zinc per TFIIA molecule (Han et al., 1990). Treatment with PAR alone released five moles of zinc per mole TFIIA. This suggests that the nine fingers of TFIIA bind zinc with varying affinity. In contrast, an independent chemical modification study using PMPS (modifies cysteines) and DEPC (modifies histidines) showed two mole equivalents of zinc associated with the TFIIA molecule (Shang et al., 1989). Therefore, it appears that only two intrinsic zinc ions within TFIIA are required for the nucleic acid binding activity (Hanas et al., 1989; Shang et al., 1989).

Other structural features that may contribute to transcription factor DNA-binding and transactivation activities include post-translational modification and electrostatic charge distribution. Glycosylation has been shown to function in transactivation by Sp1 (Jackson & Tjian, 1988). Unlike many RNA polymerase II transcription factors, TFIIA does not appear to be O-glycosylated (Jackson & Tjian, 1988). In contrast, sequence analysis has identified two putative N-linked glycosylation sites within the C-terminal region of TFIIA (Miller et al., 1985) (Figure 1.7). Charge clusters are found associated with the DNA-binding zinc fingers of Sp1, Krox20, and GLI, while TFIIA and ADR1 display a more evenly distributed charge over the whole protein (Miller et al., 1985; Brendel & Karlin, 1989). Thus, the presence and possible function of electrostatic charge and post-translational modifications within TFIIA remains unclear at present. It is important to note, however, that bacterially produced TFIIA maintains nucleic acid binding and

transactivation activity similar to TFIIIA isolated from *Xenopus* (Del Rio and Setzer, 1991). If post-translational modification of TFIIIA does occur in *Xenopus*, it may play a role in protein transport and/or turnover.

The transactivation domain of TFIIIA has been localized by two groups from amino acid positions 294 to 313 (Vrana et al., 1988) and positions 284 to 297 (Mao & Darby, 1993) within the C-terminal region. This region exhibits position-dependent activity and may interact directly with other RNA polymerase III factors and stabilize the functional transcription preinitiation complex (Braun et al., 1989; Mao & Darby, 1993). It is interesting to note a minor lysine cluster that overlaps the transactivation region of TFIIIA (Miller et al., 1985) (Figure 1.7). This hydrophilic region could serve as a flexible intradomain linker or function directly in transactivation (Smith et al., 1984; Miller et al., 1985). The precise structure of the TFIIIA transactivation domain remains unknown.

Oocyte and somatic forms of TFIIIA may exist that are expressed from two different promoters within the single-copy TFIIIA gene (Pelham et al., 1981; Shastry et al., 1984; Kim et al., 1990; Martinez et al., 1994). The somatic TFIIIA contains 22 additional residues at the N-terminus but appears to be immunologically and functionally indistinguishable from the oocyte form of the protein (Pelham et al., 1981; Kim et al., 1990). However, independent studies suggest that, while both proteins exhibit similar DNA binding affinities, the somatic form of TFIIIA is unable to support oocyte-type 5S RNA gene transcription (Blanco et al., 1989). This suggests that somatic TFIIIA could function as a repressor of oocyte 5S RNA genes and as an activator of somatic-type 5S RNA genes in somatic tissue (Blanco et al., 1989). The oocyte form of TFIIIA is present at  $10^{12}$  copies per cell in immature oocytes and declines in abundance by as much as 20-fold during oogenesis. In contrast,

somatic TFIIIA represents only two percent of the total TFIIIA concentration in immature oocytes and increases significantly during oogenesis. Thus, both the effective cellular concentrations of both forms of TFIIIA and a switch in transactivation activity may contribute to the regulation of 5S RNA biosynthesis during *Xenopus* development.

### 1.5 Developmental regulation of 5S RNA expression

The *Xenopus* oocyte develops through six stages that are characterized by gross changes in morphology and gene expression patterns. These developing amphibian oocytes accumulate massive numbers of ribosomes in preparation for embryogenesis and thus require the synthesis of large amounts of ribosomal RNA. To support this high rate of synthesis, the 450 genes for 18S, 5.8S, and 28S rRNA are amplified specifically in oocyte nuclei by disproportionate replication (Brown & Dawid, 1968; Gall, 1968). An alternative scheme is used for 5S RNA synthesis, with the activation of a large 5S RNA gene family early in *Xenopus* oogenesis. The major-oocyte, trace-oocyte, and somatic genes are all expressed in immature oocytes (stages I to III). Transcription of 5S RNA then ceases during late oogenesis (stages V and VI) and early embryogenesis. Somatic 5S RNA gene expression is subsequently renewed in the blastula stage of development during gastrulation (about 12 to 15 cell divisions after egg fertilization) (Ford & Southern, 1973; Shastry et al., 1984). Thus, the different classes of 5S RNA genes exhibit different expression patterns during *Xenopus* development.

Since TFIIIA has been identified as a 5S RNA gene-specific transcription factor, investigations into the mechanisms underlying the developmental regulation of 5S RNA synthesis have focused on TFIIIA expression patterns and function in the assembled transcription complex.

Changes in the expression pattern of TFIIA during *Xenopus* development and the differential stability of the fully assembled transcription complex on the 5S RNA gene families play a significant role in the timing of 5S RNA gene expression. Steady state levels of TFIIA mRNA range from  $2 \times 10^7$  to  $5 \times 10^6$  in immature oocytes (stages I to III) and from six to one or less TFIIA mRNA per somatic cell (Ginsberg et al., 1984; Taylor et al., 1986). This suggests that a  $10^6$ -fold reduction occurs in TFIIA expression during *Xenopus* development from oocyte to embryo. This massive reduction in mRNA levels is reflected by TFIIA protein levels, which drop from  $8 \times 10^{11}$  to  $1 \times 10^{12}$  molecules in an immature oocyte (stages I to III) to about  $4 \times 10^5$  molecules per somatic cell in a tailbud stage embryo (Shastri et al., 1984; Andrews & Brown, 1987). Developmental regulation of TFIIA expression is mediated through stage-specific transcription complexes positioned within the oocyte and somatic promoters of the TFIIA gene (Pfaff et al., 1991). A reduction in TFIIA levels may be required for reprogramming the expression of the 5S RNA gene families during embryogenesis.

Subtle differences in the interaction of TFIIA with the somatic and oocyte 5S RNA genes may indirectly contribute to a 40-fold or greater enhancement of somatic 5S RNA transcription over the oocyte-type gene *in vitro* (Wormington et al., 1981; Millstein et al., 1987; McConkey & Bogenhagen, 1988; Xing & Worcel, 1989a). TFIIC recognizes these TFIIA-DNA complexes with different affinities resulting in a more stable ternary complex on the somatic 5S RNA gene (Wolffe, 1988; Keller et al., 1992). The assembly of transcription complexes *in vivo* with different stabilities and, perhaps, different association kinetics may be part of an important regulatory mechanism within the somatic cell that includes differences in the timing of 5S RNA gene replication. Somatic 5S RNA genes are replicated earlier in the

S-phase of the cell cycle than the oocyte-type genes (Gilbert, 1986). During embryogenesis, the newly replicated somatic 5S RNA genes could sequester a limiting pool of TFIIA (and/or TFIIC) before replication of the oocyte 5S RNA gene class and assemble stable transcription complexes (Gottesfeld & Bloomer, 1982; Wormington & Brown, 1983; Wolffe & Brown, 1986). It has been suggested that late replication of the oocyte-type genes removes pre-existing transcription complexes leaving this gene class susceptible to global mechanisms of gene repression (Brown, 1984; Wolffe & Brown, 1988). This repression model is supported by the early replication and expression of oocyte-type 5S RNA genes in a *Xenopus* somatic cell line containing a chromosome translocation (Guinta et al., 1986).

Oocyte 5S RNA genes that lack an associated transcription complex are assembled into chromatin containing histone H1 and actively repressed (Bogenhagen et al., 1982; Schlissel & Brown, 1984; Gilbert, 1986; Tremethick et al., 1990; Chipev & Wolffe, 1992; Bouvet et al., 1994). TFIIA has been observed to associate with the somatic 5S RNA gene promoter in partially assembled or loosely organized chromatin (Rhodes, 1985; Tremethick et al., 1990; Hayes & Wolffe, 1992). This metastable TFIIA-DNA complex can be subsequently displaced *in vitro* by completion of nucleosome assembly or H1-mediated chromatin compaction (Tremethick et al., 1990; Chipev & Wolffe, 1992; Hansen & Wolffe, 1992; Hayes & Wolffe, 1992). In contrast, the fully assembled 5S RNA gene transcription complex is refractory to chromatin assembly and H1-dependent repression (Tremethick et al., 1990; Chipev & Wolffe, 1992). In the somatic cell, transcriptionally competent somatic 5S RNA gene repeats are found to contain few positioned nucleosomes within the promoter region, while repressed oocyte-type gene repeats are incorporated into a loosely positioned array of nucleosomes that provide a

substrate for active H1-mediated repression (Chipev & Wolffe, 1992; Bouvet et al., 1994). Single nucleosomes that are located on active 5S RNA genes apparently do not impede RNA polymerase passage and completion of RNA transcription but can interfere with the initial interaction of the polymerase with the gene promoter (Lorch et al., 1987; Losa & Brown, 1987; Wolffe & Drew, 1989; Clark & Felsenfeld, 1992). It is important to note that both *in vitro* initiation and elongation by RNA polymerase III can be inhibited by nucleosomal arrays in the absence of histone H1 (Hansen & Wolffe, 1992). This transcriptional repression is dependent on the degree of internucleosome contacts and chromatin compaction, which could be mediated through histone H1 *in vivo*.

Transient repression of RNA polymerase III-mediated transcription is found to occur in cells entering mitosis and can be reproduced *in vitro* (Gottesfeld et al., 1994). This regulation involves the reversible phosphorylation of protein factors associated with the TATA-box binding protein (TBP) component of TFIIB by mitotic protein kinases (Gottesfeld et al., 1994). During interphase this factor is dephosphorylated and RNA polymerase III transcription is reactivated. Thus, somatic 5S RNA gene expression appears to be transiently repressed in a cell cycle-dependent manner, while oocyte 5S RNA genes are permanently repressed by a more global repression mechanism within the somatic cell.

It is clear from the above studies that a number of gene-specific and global regulatory mechanisms within *Xenopus* oocyte and somatic cells function to program the differentiated state of the 5S RNA gene families. Control mechanisms that contribute to the overall regulation of 5S RNA synthesis during *Xenopus* development include: modulation of TFIIB and histone H1 nuclear concentrations, post-translational modification of RNA

polymerase III factors, assembly of different chromatin organizations on the somatic and oocyte gene clusters, and cell cycle-dependent access restrictions to the different 5S RNA gene classes.

### 1.6 Biological Function of 5S RNA

Eukaryotic 5S RNA interacts predominantly with the ribosomal L5 protein in vertebrate ribosomes and with the L5 homolog, YL3, in yeast (Nazar & Wildeman, 1983; Chan et al., 1987; Wormington, 1989; Tang & Nazar, 1991). There is a significant lag between L5 protein accumulation and the synthesis and cytoplasmic storage of 5S RNA in immature oocytes (stages I to III) (Allison et al., 1991). Expression of *Xenopus* L5 protein and other ribosomal proteins during oogenesis coincides with transcription of 18S and 28S ribosomal RNAs from the nucleoli and ribosome assembly (stages III and IV) (Wormington, 1989). During embryogenesis ribosomal protein mRNAs are synthesized during gastrulation (stage 11) but are not maximally translated until the tailbud stage of development (stages 24 through 28) (Pierandrei-Amaldi & Campioni, 1982; Wormington, 1989). However, sufficient L5 mRNA translation does occur before this stage and coincides with renewed somatic 5S RNA gene expression (Pierandrei-Amaldi & Campioni, 1982; Shastry et al., 1984; Wormington, 1989). The L5 protein-5S RNA complexes accumulate in a ribosome-free pool that accounts for up to fifty percent of cellular 5S RNA (Steitz et al., 1988). Thus, L5 protein translation appears to be uncoupled from the synthesis of other ribosomal proteins during embryogenesis but coincides with ribosomal protein and RNA synthesis in the growing *Xenopus* oocyte.

Ribosomal 5S RNA is an essential constituent of the large ribosomal subunits of prokaryotes and eukaryotes. Although several biological

functions have been ascribed to 5S RNA, its precise role in ribosome assembly and translational fidelity remain unclear. Eukaryotic 5S RNA is found to associate with L5 protein and two acidic phosphoproteins, P2 and P3, in the 60S subunit of rat liver at a ratio of 2:1:1 (P2/P3:L5:5S RNA) (Nendza et al., 1987). A number of bases within ribosome-bound 5S RNA are solvent accessible and located within the interface between the two ribosomal subunits (Nygård & Nilsson, 1987; Holmberg et al., 1992). In eukaryotic ribosomes, 5S RNA is found to contribute to the elongation factor 2 (eEF-2) binding site (Nygård & Nilsson, 1987) and may be involved in activating eEF-2-dependent GTP hydrolysis (Figure 1.8). Elongation factor 2 catalyzes the translocation of peptidyl-tRNA from the ribosomal A-site to the P-site. In the post-translocation state of the ribosome (mimicked by treatment of the ribosome with ricin), 5S RNA regions within loops E and D and helix IV become available for eEF-2 binding (Holmberg et al., 1992) (Figure 1.8). Both helix IV and loop D sequences are protected from chemical or nuclease attack upon eEF-2 binding. Thus, 5S RNA may be involved in translocation of the growing polypeptide during translation elongation. It has also been suggested that base pair interaction between 5S RNA in the 60S subunit and 18S RNA in the 40S subunit may contribute to ribosome assembly (Sarge et al., 1991). Thus, 5S RNA appears to play a crucial role in the assembly and function of the eukaryotic ribosome.

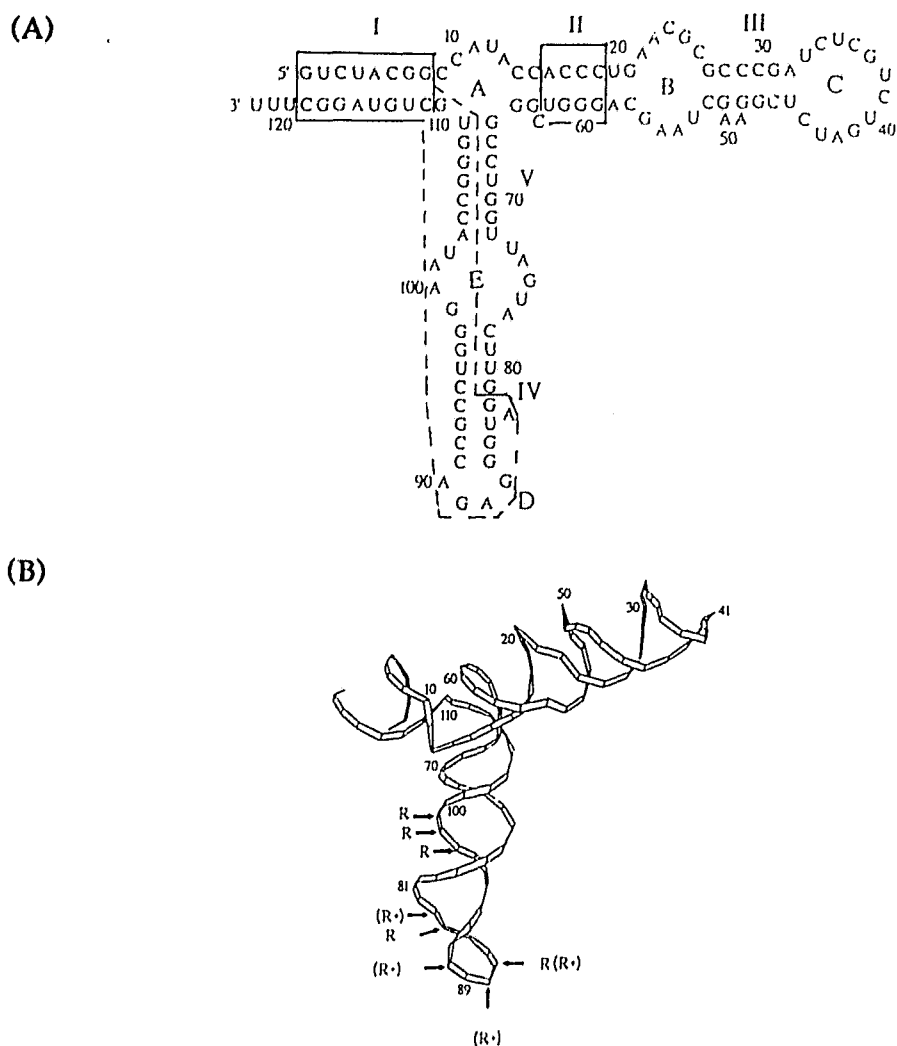


Figure 1.8 Interaction of eukaryotic 5S RNA with other components of the ribosome. (A) The binding site of yeast YL3 protein on rat 5S RNA. Boxed regions indicate sites of protection from chemical modification, while the region enclosed with dashes identifies sequences further protected on the yeast 5S RNA (Nazar & Wildeman, 1983). (B) Region of 5S RNA involved in ribosome translocation. Sequences with increased accessibility in ricin-treated (post-translocative) ribosomes are denoted with an 'R', while sequences protected by eEF-2 binding in ricin-treated ribosomes are shown by an 'R+' (Holmberg et al., 1992).

## Chapter 2.0 Interaction of TFIIA with the 5S RNA gene

### 2.1 Introduction

#### 2.1.1 Structural properties of the 5S RNA gene

There are four types of 5S RNA gene within the *Xenopus* genome. These include the somatic, oocyte, trace-oocyte, and pseudo 5S RNA genes (Figure 2.1). Studies using somatic 5S RNA gene deletion mutants initially defined the minimal sequence of the internal promoter required for transcription initiation as base pairs +50 to +83 (where +1 denotes the site of transcription initiation) (Bogenhagen et al., 1980; Sakonju et al., 1980). Similar studies using a series of point mutants extended this internal control region (ICR) from base pairs +45 to +97 (Pieler et al., 1985a; McConkey & Bogenhagen, 1987). The ICR is tripartite in structure, consisting of box A (+50 to +64) and box C (+80 to +97) elements flanking a small intermediate element (+67 to +72) (Pieler et al., 1985b; Pieler et al., 1987; You et al., 1991). Spacer regions between these promoter elements are quite tolerant to changes in sequence (Pieler et al., 1985b). The box A sequence is homologous to the box A or D-control region of tRNA genes (Ciliberto et al., 1983), while the intermediate and box C elements are specific to the 5S RNA gene. Similarity between promoter elements of 5S RNA and tRNA genes is not surprising, as they share common transcription factors (Shastry et al., 1982; Lassar et al., 1983).

TFIIA recognizes both RNA and DNA helical conformations, therefore, considerable interest exists as to the structure of the internal control region of the 5S RNA gene. Similarity to A-form DNA may indicate a common mechanism of TFIIA recognition and binding to both nucleic acids. In contrast, TFIIA interaction with RNA and DNA may be fundamentally

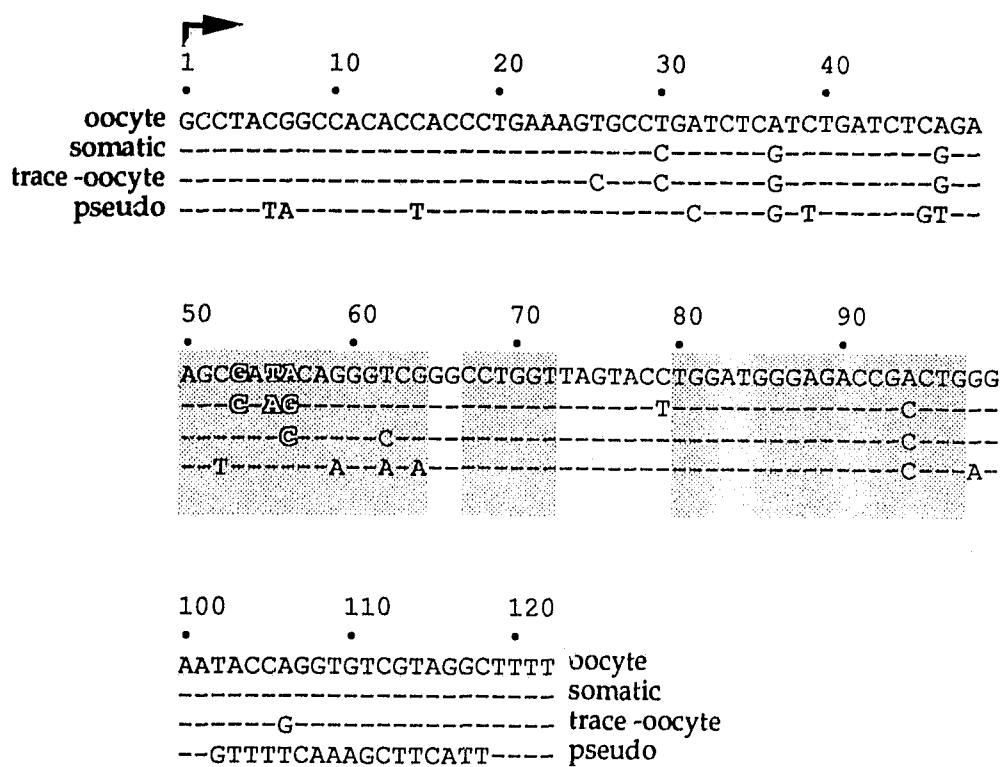


Figure 2.1 Sequence of the *Xenopus* 5S RNA genes. The non coding DNA strand is shown. A dash indicates identical base sequence at that position. The tripartite internal control region is shaded and base pairs that influence transcription complex activity are outlined. The arrow denotes the start site of transcription and RNA synthesis terminates within the thymidine tract centered at position +120.

different if the ICR adopts a B-DNA structure. Contradictory results have been obtained from a number of independent studies. X-ray crystallography of a DNA fragment containing the box C element and enzymatic nuclease digestion of the intact gene have given support to an A-DNA structure within the internal promoter of the 5S RNA gene (McCall et al., 1986; Rhodes & Klug, 1986). However, circular dichroism and NMR analyses indicate that the 5S RNA gene promoter adopts a B-DNA conformation (Gottesfeld et al., 1987; Aboul-ela et al., 1988; Huber et al., 1991a) or a conformation intermediate between A-form and B-form DNA (Fairall et al., 1989). An analysis of a variety of zinc finger protein-DNA complexes has identified a characteristic DNA structure with an enlarged major groove (Nekludova & Pabo, 1994) (Figure 2.2A). This DNA structure is different from classical A-DNA and B-DNA conformations and may be an independent form of DNA often present within protein binding sites. Spectroscopic and chemical probing of the 5S RNA gene suggest that such a structural polymorphism exists within the TFIID binding site (Fairall et al., 1989; Huber et al., 1991b) (Figure 2.2B). Sequence-dependent major groove widening centered at positions +46, +53, +59, +64, +70, +81, +87, +92, and +96 could allow zinc finger entry and base recognition within the promoter. Thus the zinc fingers of TFIID can interact with B-DNA, that may contain local structural polymorphism, and A-type RNA.

### **2.1.2 Interaction of TFIID with the 5S RNA gene**

TFIID interacts with the internal promoter of the 5S RNA gene in a one to one ratio (Bieker & Roeder, 1984; Romaniuk, 1990). This interaction may be cooperative as suggested by gel mobility shift (Daly & Wu, 1989), and DNase I protection (Hanas et al., 1983b) analyses. TFIID binding affinity is

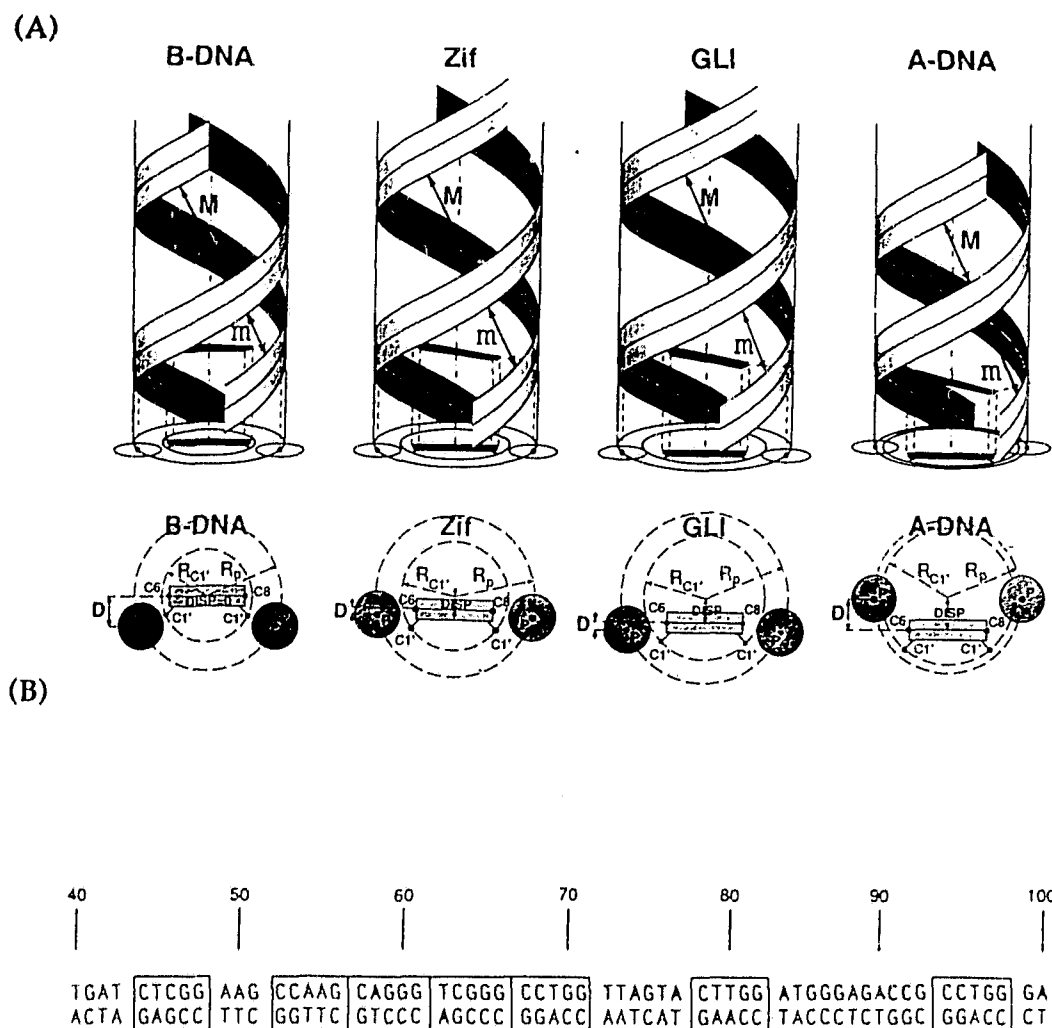


Figure 2.2 Structural polymorphism in the internal promoter of the 5S RNA gene. (A) Enlarged major groove structure in the DNA binding sites of regulatory proteins (Nekludova & Pabo, 1994). (B) Local regions of major groove widening within the *Xenopus* 5S RNA gene promoter. Boxed sequence denote regions of the DNA sequence exhibiting major groove widening (Huber et al., 1991b).

identical for both oocyte and somatic forms of the 5S RNA gene (Xing & Worcel, 1989a). The association constant for TFIIIA-oocyte 5S RNA gene complex formation at 22°C in a buffer containing 0.1 M salt at pH 7.5 is  $1.9 \times 10^9 \text{ M}^{-1}$  (Romaniuk, 1990). The binding interaction is largely enthalpy driven at temperatures above 19°C ( $\Delta G = -12.4 \text{ kcal/mol}$ ,  $\Delta H = -12.1 \text{ kcal/mol}$ , and  $\Delta S = +1.23 \text{ cal mol}^{-1} \text{ deg}^{-1}$  at 22°C) and entropy driven at lower temperatures ( $\Delta G = -11.6 \text{ kcal/mol}$ ,  $\Delta H = -0.1 \text{ kcal/mol}$ , and  $\Delta S = +42.3 \text{ cal mol}^{-1} \text{ deg}^{-1}$  at 0°C). The complex exhibits a half life of 15.6 minutes and has a broad pH optimum ranging from six to eight (Romaniuk, 1990). TFIIIA-5S RNA gene complex stability is maintained at a divalent cation ( $\text{Mg}^{2+}$ ) concentration of 5 mM but appears to be insensitive to the identity of the monovalent cation present in the binding buffer. In contrast, TFIIIA binding is strongly dependent on the identity of the anionic species (Romaniuk, 1990). This may indicate that there are a number of specific anion-binding sites on the TFIIIA surface and as many as eight lysine-phosphate type ionic bonds formed within the TFIIIA-5S RNA gene complex. Chemical modification studies have identified eight phosphates within the internal promoter that are essential for TFIIIA association (Sakonju & Brown, 1982).

Binding of TFIIIA to the 5S RNA gene alters the helical linking number of the 5S RNA gene by 0.2 to 0.4 helical turns (Reynolds & Gottesfeld, 1983; Sekiguchi et al., 1990). This effect could result from a local denaturation of DNA base pairing or from DNA wrapping about a protein core. The helical conformation of the 5S RNA gene does not appear to alter significantly upon TFIIIA binding (Gottesfeld et al., 1987; Huber et al., 1991a). However, there is evidence for TFIIIA-induced bending within the internal promoter. Electron spectroscopic imaging suggests that, in the presence of TFIIIA, approximately 60 base pairs of the 5S RNA gene is distorted into a hairpin-shaped structure

extending about 9.5 nm out from the flanking DNA and 5.2 nm along the helical axis (Bazett-Jones & Brown, 1989). In comparison, the extended ICR is greater than 17.5 nm (175Å) in length. Circular permutation analysis, which identifies protein-induced DNA bends, located the bend site to within the internal promoter of the oocyte and somatic forms of the 5S RNA gene (Schroth et al., 1989; Schroth et al., 1991). The degree of bending may be ionic strength-dependent, with a DNA bend of 25° to 30° under low ionic strength conditions (less than 20 mM) and about 60° to 65° in higher ionic strength buffers (45 to 90 mM) (Schroth et al., 1991). This apparent dependence on the ionic strength conditions may explain conflicting results on the existence of TFIIIA-induced DNA bending (Zwieb & Brown, 1990). Bending within the 5S RNA gene promoter region may be necessary for high affinity TFIIIA interaction due to the short linker sequences between zinc fingers five and six and between fingers six and seven (Miller et al., 1985; Berg, 1990). Alternatively, bending of the internal promoter may also be required for assembly of a functional transcription complex. Zinc(II) itself induces a significant local bend of about 55° in the 5S RNA gene centered at base pair +65 (Nickol & Rau, 1992). This effect is specific for zinc, requiring a metal ion concentration of only 5 µM, and may be caused by joint coordination of the metal ion to the N<sup>7</sup> atoms of stacked purine bases. The relationship between the metal and protein-induced bends within the ICR is unclear. However, it is possible that zinc induces a unique bend conformation within the ICR (requiring approximately 2 kcal/mole 5S RNA gene) that is further stabilized by TFIIIA association.

Studies using substitution mutants of the 5S RNA gene indicate that TFIIIA interacts with the internal promoter primarily at three contact regions that correspond to the three functional promoter elements identified by

transcription complementation studies (You et al., 1991) (Figure 2.1). The contribution to complex formation by these three protein-DNA contact points is not equivalent (Sakonju et al., 1981; You et al., 1991). Mutations within the box C region of the *Xenopus* 5S RNA gene severely affect TFIIIA binding and assembly of a functional transcription complex, while changes in the sequence of box A or the intermediate element result in moderate effects on TFIIIA association and 5S RNA transcription (Sakonju et al., 1981; Pieler et al., 1985a; McConkey & Bogenhagen, 1987; You et al., 1991). These latter two elements may function in correctly aligning the TFIIIA molecule along the ICR once a high affinity association with the box C promoter element has been established. It appears that different functional roles may exist for zinc fingers of TFIIIA and individual promoter elements within the ICR that help establish a biologically active TFIIIA-5S RNA gene complex.

Current experimental evidence is consistent with the existence of multiple energetically favorable contacts between TFIIIA and the 5S RNA gene. Experiments using a bacterially expressed polypeptide containing the first three fingers of TFIIIA showed that these N-terminal fingers interact with an affinity similar to that observed with intact TFIIIA (Christensen et al., 1991; Liao et al., 1992). These results supported earlier studies using truncated TFIIIA mutants produced in a cell-free translation system (Vrana et al., 1988). Recently, 'broken finger' mutants of TFIIIA, in which a coordinating histidine was replaced with asparagine, exhibited a more complex pattern of zinc finger contribution to the DNA binding energy (Del Rio et al., 1993a). Fingers three and four contribute a large part towards complex formation, while disruption of fingers one or two had moderate effects on the free energy of binding. It was proposed that disrupting a zinc finger in TFIIIA may be compensated for energetically by the remaining fingers (Del Rio et al., 1993a).

In this respect, the contribution made by each finger to DNA binding by TFIIIA may not be correctly determined by analysis of truncation mutants (Clemens et al., 1994). However, both experimental techniques point to a significant contribution by all zinc fingers, either positive or negative, to the free energy of DNA binding (Del Rio et al., 1993a; Clemens et al., 1994).

Two alternate models were originally proposed for the interaction of TFIIIA with the 5S RNA gene (Fairall et al., 1986) (Figure 2.3). In one model, successive zinc fingers follow the path of the major groove of DNA with each finger presenting the same contact surface to the base sequence. The second model proposed that TFIIIA bound to one side of the DNA helix, with successive fingers presenting different contact surfaces to the DNA base pairs. Electron spectroscopic imaging of TFIIIA-5S RNA gene complexes shows the protein and DNA components as being 'interspersed' (Bazett-Jones & Brown, 1989). This suggests that a more complex arrangement of components may exist than the above models predict.

A number of nuclease and chemical protection studies using full length and truncated forms of TFIIIA suggest that the TFIIIA-DNA complex may include characteristics from both of these early models. TFIIIA is proposed to interact with the internal promoter primarily along one face of the DNA helix. The new interaction models differ mainly in the organization of the middle three fingers of TFIIIA along the central region of the ICR (Figure 2.4). All recent models place the first three fingers of TFIIIA within the major groove of the box C promoter element (Vrana et al., 1988; Christensen et al., 1991; Clemens et al., 1992; Fairall & Rhodes, 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992; Liao et al., 1992; Hansen et al., 1993; Del Rio et al., 1993a; Clemens et al., 1994). The extended protein then crosses the minor groove at base pair positions +78 to +79 (all positions unless otherwise

stated refer to the non coding strand of the 5S RNA gene) (Clemens et al., 1992; Fairall & Rhodes, 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992). This may be accomplished either by the linker sequence between fingers three and four (Churchill et al., 1990; Kochoyan et al., 1991d; Fairall & Rhodes, 1992) (Figure 2.4B) or by finger four itself (Clemens et al., 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992; Hansen et al., 1993; Del Rio et al., 1993a; Clemens et al., 1994) (Figure 2.4A). Finger five alone (Clemens et al., 1992; Hayes & Clemens, 1992; Del Rio et al., 1993a; Clemens et al., 1994) or in combination with finger four (Fairall & Rhodes, 1992) contacts base pairs within the major groove of the intermediate promoter element. A second minor groove crossover at base pair position +65 to +67 occurs, with finger six aligned parallel to the DNA helical axis (Clemens et al., 1992; Fairall & Rhodes, 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992; Clemens et al., 1994) (Figure 2.4A and B). The remaining C-terminal three fingers follow the path of the major groove within the box A promoter element (Vrana et al., 1988; Clemens et al., 1992; Fairall & Rhodes, 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992; Clemens et al., 1994). One recent model attempts to account for the observed TFIIA-induced DNA bending within the ICR by placing fingers one through five in the major groove from position +75 to +92 and fingers seven through nine from position +46 to +58 (Berg, 1990) (Figure 2.4C). The sixth finger is proposed to lie approximately parallel to the helical axis centered at position +67. Due to the short linkers between fingers five and six and between six and seven, TFIIA binding would necessitate bending the ICR around position +67. However, with the N-terminal zinc fingers wrapped around one and a half to two turns of DNA it is difficult to envision how TFIIA could partially dissociate and reassociate during DNA strand separation and RNA transcription through the ICR.

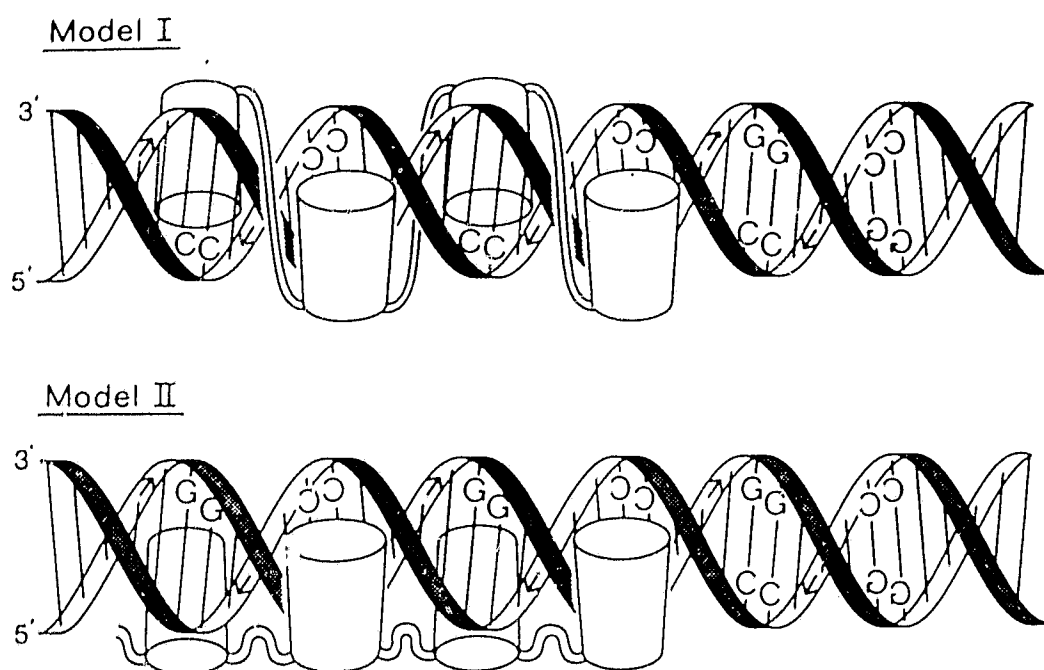


Figure 2.3 Proposed models for the interaction of a multi-zinc finger protein with the major groove of DNA (Fairall et al., 1986).

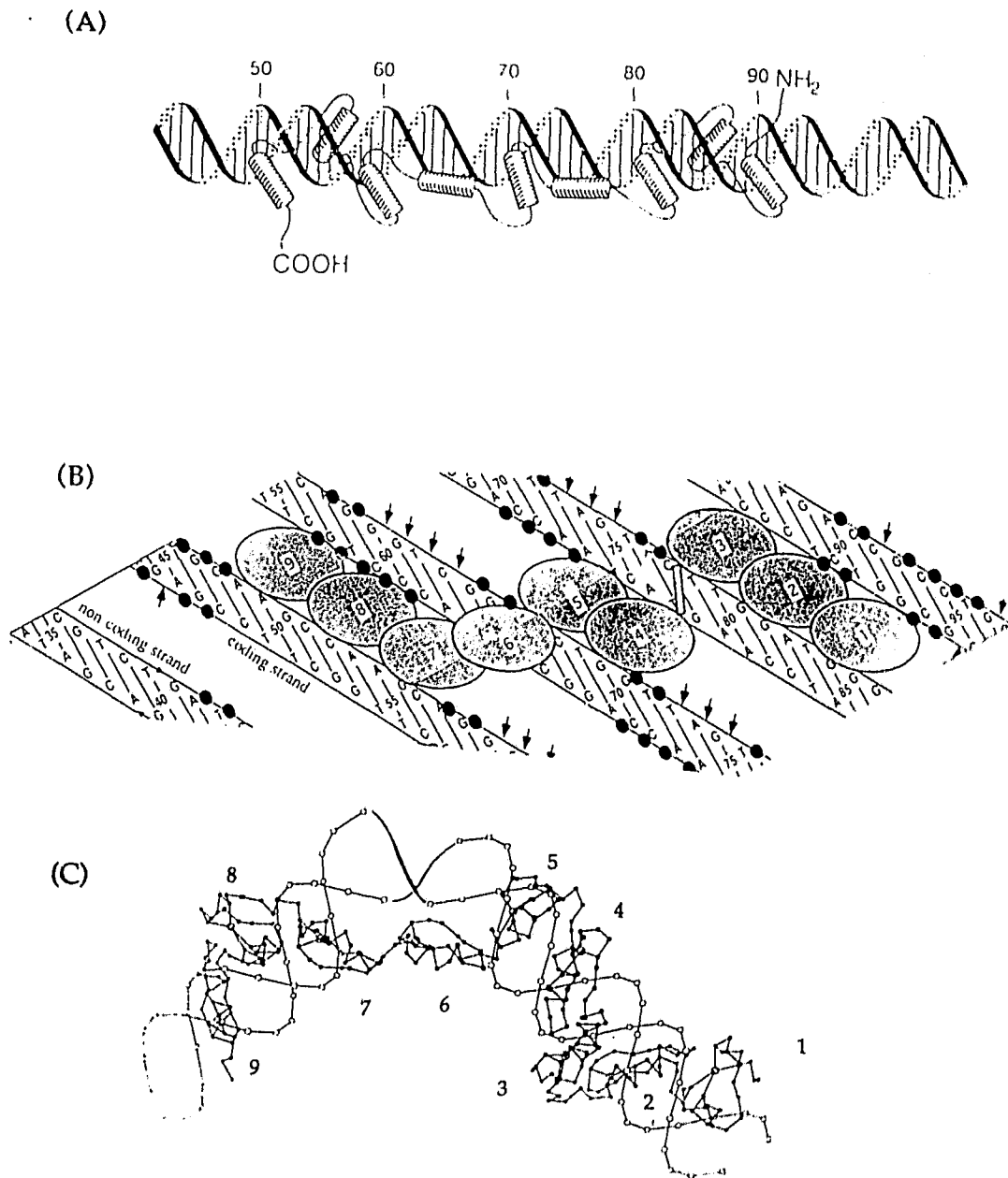


Figure 2.4 Models for the interaction of TFIID with the internal promoter of the 5S RNA gene. (A) Interaction model based on primer extension and oligonucleotide binding analysis (Clemens et al., 1992). (B) TFIID-DNA complex based on enzymatic nuclease analysis (Fairall & Rhodes, 1992). (C) Interaction model designed to account for possible promoter bending by TFIID (Berg, 1990).

Nuclease and chemical footprinting of TFIIIA on the ICR identify a region of protection extending from +44-+45 to +95-+97 and +40-+42 to +98, respectively (Churchill et al., 1990; Fairall & Rhodes, 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992). Differences in the footprints obtained with DNase I and hydroxyl radicals reflect the difference in size and reactivity of the probes. DNase I exhibits sequence specificity and can displace portions of DNA-associated protein that have weak affinity (Clemens et al., 1994). Hydroxyl radicals are not sequence specific but are sensitive to alterations in DNA minor groove structure and tend to identify regions of the DNA backbone protected from the solvent by protein (Churchill et al., 1990). Both probes cut across the minor groove and could be partially excluded by protein interactions in an adjacent major groove of DNA (Del Rio et al., 1993a). Thus, neither probe directly identifies base pairs contacted by TFIIIA but rather the region of the ICR where the TFIIIA protein is in close proximity to the DNA.

The base pair contact region appears to be smaller than the TFIIIA footprint, extending from +50 to +92 (Hayes & Tullius, 1992). There is a stronger interaction between TFIIIA and the non coding strand than with the coding strand of the 5S RNA gene (Sakonju & Brown, 1982; Churchill et al., 1990; Hayes & Tullius, 1992). The location of individual zinc finger interaction sites within this region of the ICR have been identified using truncated mutants of TFIIIA (Vrana et al., 1988; Christensen et al., 1991; Clemens et al., 1992; Hayes & Clemens, 1992; Liao et al., 1992; Hansen et al., 1993). Fingers one through three are situated within the box C promoter element from position +77-+79 to +95-+96 when probed with DNase I (Christensen et al., 1991; Clemens et al., 1992; Liao et al., 1992; Clemens et al., 1994) and from position +79 to +90 when probed with hydroxyl radicals (Vrana et al., 1988; Hayes & Clemens, 1992) (Figure 2.4A and B). Primer extension

and oligonucleotide binding studies suggest that the N-terminal three fingers directly contact base pairs +79 to +92 (Clemens et al., 1992; Liao et al., 1992). In addition, 'broken finger' mutations within TFIIA suggest that finger one interacts with positions +93 to +97, finger two with positions +86 to +87, and finger three with positions +76 to +78, as determined by DNase I protection (Del Rio et al., 1993a). Chemical crosslinking places finger two and the linker sequence between fingers two and three within the major groove at positions +84 and +88 (Lee et al., 1991b), while a missing nucleoside analysis identifies position +88 as a non-contact base pair between two contact regions (Hayes & Tullius, 1992). The latter study suggests that three contact regions are centered at positions +81, +84.5, and +90 on the coding strand (Hayes & Tullius, 1992). Recently, computer modeling of the N-terminal fingers of TFIIA was performed based on the X-ray crystallographic structure of a three-finger protein, Zif268 (Hansen et al., 1993). This model predicts base contacts between finger one and positions +88 through +89, finger two contacts with positions +83 through +85, and finger three contacts to positions +81 and +82. An earlier sequence analysis predicted fingers one and two interact with the box C element at positions +81 to +86, but also suggested that finger three does not make sequence specific contacts with the ICR (Klevit, 1991). The combination of these results indicates that the N-terminal three fingers of TFIIA interact with base sequence through one complete turn of the DNA major groove.

The C-terminal three fingers of TFIIA interact with the box A promoter element from positions +49 to +58 as determined by DNase I footprinting (Fairall & Rhodes, 1992) and positions +49-+50 to +57 as determined by hydroxyl radical footprinting (Clemens et al., 1992; Hayes & Clemens, 1992) (Figure 2.4A and B). Primer extension and missing nucleoside

analysis suggest that base pairs from positions +50 to +62 contribute to ICR interaction with zinc fingers seven through nine (Clemens et al., 1992; Hayes & Tullius, 1992). Additional interactions between the C-terminal domain of TFIIA and base pairs +44 to +48 have also been observed (Hayes & Clemens, 1992). Finger nine occupies a region of the ICR extending from position +49 to +51, while fingers seven and eight are located at positions +52 to +57 (Hayes & Clemens, 1992). Sequence analysis suggests that these three zinc fingers contact base pairs from position +51 to +59 (Klevit, 1991). Together, these results support an interaction of the C-terminal zinc fingers of TFIIA with base sequence through one complete turn of the DNA major groove. The interaction of the C-terminal fingers with different base sequences within the oocyte and somatic forms of the 5S RNA gene may be responsible for differences in TFIIA-DNA complex structure and transcription complex activity (Xing & Worcel, 1989a). Base pair identity at positions +53, +55, and +56 within the box A promoter element direct the formation of structurally distinct complexes with TFIIA that are differentially bound by TFIIC (Xing & Worcel, 1989a; Keller et al., 1990; Keller et al., 1992). The fully assembled transcription complex on the somatic 5S RNA gene appears to be 50 times more efficient than that assembled on the oocyte-type gene (Millstein et al., 1987; Wolffe & Brown, 1987; McConkey & Bogenhagen, 1988).

The central region of the ICR is protected from the solvent by TFIIA at positions +67 to +75, with the strongest protection centered at +74.5 on the coding and +69 on the non coding strand (Hayes & Tullius, 1992). This staggered pattern suggests protection of both strands across the major groove along one face of the DNA helix. Finger four contacts the DNA backbone just outside this region at position +80 and is influenced by base sequence from position +79 to +71 (Clemens et al., 1992; Hayes & Clemens, 1992). However,

finger four does not appear to interact directly with base pairs in the major groove of the ICR, as suggested by accessibility of guanines at +75 and +78 to methylation (Sakonju & Brown, 1982). In addition, finger four exhibits weak association with DNA when located as the C-terminal finger of a TFIID truncation mutant (Clemens et al., 1994). Finger five contacts base pairs in the DNA major groove at positions +68-+70 to +71 (Clemens et al., 1992; Hayes & Clemens, 1992; Del Rio et al., 1993a). Finger six is influenced by base pair sequence from positions +66 to +69 and also from positions +58 to +56 one helical turn away (Clemens et al., 1992; Hayes & Clemens, 1992). Similar to finger four, finger six participates in weak interactions with the DNA when present as the C-terminal finger (Clemens et al., 1994). Thus, the central region of the ICR is protected on one face of the DNA helix by three zinc fingers with a few base contacts made to the intermediate promoter element (Sakonju & Brown, 1982).

In a collaborative study with Dr. David Setzer's laboratory at Case Western Reserve University, the contribution of individual base pairs within the box C and intermediate promoter elements to the overall interaction of *Xenopus* TFIID with the *Xenopus borealis* somatic-type 5S RNA gene was investigated using a nitrocellulose filter binding assay (Figure 2.5). In addition, a selected amplification and binding (SAAB) assay (Blackwell & Weintraub, 1990) was performed to identify box C sequences, from positions +78 to +95, that bind with high affinity to TFIID (Figure 2.5). Dr. Setzer's laboratory provided the single base pair substitution mutants of the box C and intermediate promoter elements, while Dr. Romaniuk's laboratory provided the synthetic ICR for SAAB analysis. Single base pair substitutions at position +70 within the intermediate element and at positions +78 through +87 within the box C element were assayed for their effects on TFIID binding by Qimin

You. The SAAB analysis and the effects of the remaining 5S RNA gene substitution mutants on TFIIIA binding were performed by myself.

## 2.2 Materials and Methods

### 2.2.1 Bacterial strains and DNA vectors

Plasmid pT7-TF, which contains the TFIIIA cDNA from pUC 3a1.b (Ginsberg et al., 1984) cloned into the *Nde*I/*Bam*HI sites of pT7-7 was provided by J. Tso. The *Nde*I/*Bam*HI fragment of pT7-TF was cloned into the same site of pET-11b (Studier et al., 1990) to yield pTF4.

The phagemid pST5RD contains the *Xenopus borealis* somatic-type 5S RNA gene from pXbs201 cloned into the *Hinc* II site of pGP76. Construction of this clone and oligonucleotide-directed mutagenesis used to generate the series of 5S RNA gene point mutants used in this study were performed in the laboratory of Dr. David Setzer at Case Western Reserve University by Roberta Hmiel and Shaoyi Liao.

DNA vectors were maintained in *E. coli* K-12 strain NM522. TFIIIA expression from pTF4 was carried out using the *E. coli* B strain BL21(DE3) (F'ompTr<sup>-</sup>Bm<sup>-</sup>B).

### 2.2.2 Purification of recombinant TFIIIA

Expression and purification of recombinant *Xenopus* TFIIIA was performed as described by Del Rio and Setzer (1991). A 100 ml volume of LB media containing 50 µg/ml ampicillin was inoculated with 1 ml of an overnight culture of *E. coli* BL21(DE3) containing the pTF4 expression plasmid. The culture was grown at 37°C with shaking at 300 rpm to an O.D.<sub>600</sub> of between 0.4 and 0.6. TFIIIA protein synthesis was induced with the addition of ZnSO<sub>4</sub> to 50 µM and IPTG to 1 mM final concentration. Cells

were harvested following a further four hour incubation by centrifugation at 3800 x g for ten minutes in a Beckman JA-14 rotor and washed once with 5 ml of buffer A (20 mM Na<sup>+</sup>Hepes pH7.4, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 50 μM ZnSO<sub>4</sub>, 10% glycerol) containing 250 mM NaCl. The induced cells are pelleted once more at 3800 x g for ten minutes in a Beckman JA-20 rotor and resuspended in 4 ml of buffer A containing 250 mM NaCl and 1 mM PMSF. Sonication was performed using a microtip sonicator (Heat Systems-Ultrasonics W-385) at setting four and 50% duty cycle for 6 x 20 second intervals with one minute cooling between each pulse. The cell sample was immersed in an ethanol-ice bath during the sonication procedure. All subsequent handling of the cell and protein samples was carried out at 4°C unless otherwise stated. The sonicate was centrifuged for ten minutes at 12000 x g in a Beckman JA-20 rotor. The pellet was resuspended in 1 ml buffer A containing 250 mM NaCl, 1 mM PMSF, and 5 M urea and mixed by inversion overnight (16 hours).

The extract was centrifuged in a microcentrifuge at 14000 rpm for 20 minutes and the supernatant (approximately 1 ml) saved. Buffer A containing 250 mM NaCl, 1 mM PMSF, 5 M urea, and saturated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 4°C was added to bring the sample to 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (v/v) which was then mixed by inversion for one hour. Precipitated proteins were pelleted by centrifugation for 20 minutes at 12000 x g in a Beckman JA-20 and the pellet discarded. Additional (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-saturated buffer A was added to the 40% supernatant to achieve 80% saturation (v/v) and the sample mixed by inversion for one hour. Proteins were pelleted by centrifugation for 20 minutes at 12000 x g and the supernatant discarded. The protein pellet was redissolved in 10 ml buffer A containing 250 mM NaCl and 5 M urea. This protein preparation was applied to an 800 μl BioRex 70 (BioRad) column pre-equilibrated in buffer A containing 250 mM NaCl and 5 M urea. The loaded

column was washed with 1 ml of loading buffer followed by 700  $\mu$ l of buffer A containing 400 mM NaCl and 5 M urea. TFIIIA was eluted from the column with 800  $\mu$ l buffer A containing 600 mM NaCl and 5 M urea and stored at 4°C. The concentration of TFIIIA purified by this method ranged from five to 15  $\mu$ M. The nucleic acid binding activity of TFIIIA stored at 4°C gradually decreases over one week and is optimal within four days of purification. Alternatively, the purified TFIIIA fraction was aliquoted and stored at -70°C, where its nucleic acid binding activity was found to remain stable for up to two years. Protein concentration and purity were determined by the method of Bradford (1976), with bovine serum albumin as a standard, and by 15% SDS-polyacrylamide gel analysis, respectively (Figure 2.6).

### 2.2.3 PCR-based labeling of the 5S RNA gene

The 5S rRNA gene was internally labeled in a 10  $\mu$ l PCR reaction containing 10 mM Tris-HCl pH 8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 50  $\mu$ g/ml gelatin, 1ng template DNA, 150 pmol each of dTTP, dGTP, and dCTP, 5 pmol dATP, 6.6 pmol (20  $\mu$ Ci) [ $\alpha$ <sup>32</sup>P]-dATP (Dupont NEN), 0.5 pmol primers (550 and 632), and 0.25 units Taq DNA polymerase. Twenty-five rounds of thermal cycling were carried out using a denaturation temperature of 94°C (1.5 minutes), an annealing temperature of 45°C (1.5 minutes), and an extension temperature of 72°C (two minutes). The coding template strand of Xbs 5S DNA was primed by oligomer 550 (5'CCCCCAGAAGGCAGCACAAG<sup>3</sup>); its 5' end corresponds to position -45 upstream of the transcription start site. Oligomer 632 (5'AAGCCTACGACACCTGGT<sup>3</sup>) anneals to the non coding template strand of 5S DNA; its 5' end corresponds to position +120. Labeled DNA products were purified by electrophoresis on a 9% non-denaturing polyacrylamide gel (29:1, acrylamide:bis; 16 x 16 cm x 0.75 mm). Full length

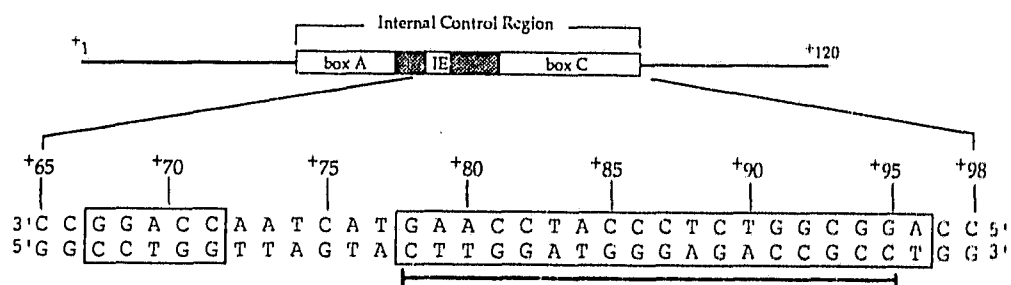


Figure 2.5 Location of point mutations and randomized base pairs within the *Xenopus* 5S RNA gene internal control region. A series of point mutations within the intermediate and box C promoter elements analyzed for TFIID binding affinity are shown as boxed sequence. The sequence randomized within a synthetic ICR for SAAB analysis is underlined. Position +1 denotes the start site of transcription.

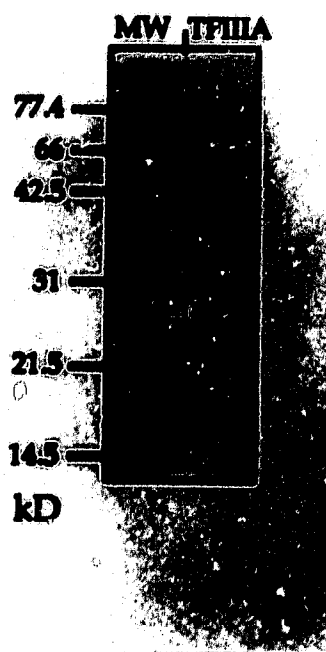


Figure 2.6 SDS PAGE analysis of purified recombinant TFIIIA used in the present 5S RNA gene promoter analysis.

PCR products were eluted from an isolated gel slice, ethanol precipitated, and resuspended in 40  $\mu$ l of water. The efficiency of radiolabel incorporation was measured using a LKB 1214 Rackbeta Scintillation counter and toluene with 0.4% (w/v) PPO (Sigma) added as scintillant.

#### 2.2.4 Equilibrium binding of TFIIIA to mutant 5S RNA genes

The equilibrium constants for the binding of radioactively labeled mutant 5S RNA genes to TFIIIA were determined by using a nitrocellulose filter binding assay (Romaniuk, 1985; Romaniuk, 1990). The TMK binding reaction buffer contained 20 mM Tris-HCl pH 7.5 at 20°C, 5 mM MgCl<sub>2</sub>, 100 mM KCl, 100  $\mu$ g/ml BSA, 1 mM DTT, and 10  $\mu$ M ZnCl<sub>2</sub>. TFIIIA was serially diluted in 180  $\mu$ l of TMK buffer to give final concentrations ranging from 63 nM to 0.18 nM and equilibrated at ambient temperature for 10 minutes. The assay was initiated with the addition of 10,000 cpm (approximately 0.005 pmol) labeled 5S RNA gene and 1  $\mu$ g poly dI-dC (Pharmacia) as a non-specific competitor DNA in 20  $\mu$ l TMK buffer. The binding reactions were equilibrated for 20 minutes at ambient temperature and a 180  $\mu$ l aliquot was vacuum filtered through pre-soaked 0.45  $\mu$ m nitrocellulose filters (Millipore). The filters were dried and counted in toluene-based scintillant.

Non-specific retention of labeled DNA on the filters (or background) was typically below 15% of input and this value was used to normalize measurements of complex formation using the equation: proportion of bound DNA=[retained cpm-background cpm]/[total input cpm-background cpm]. A minimum of three independent determinations were carried out for each mutant DNA, using the wild type *Xenopus borealis* somatic gene as a control in each experiment. The values of bound DNA were plotted against TFIIIA concentration and fit to a single component pseudo first-order reaction

using Kaleidagraph software (Synergy Software, Reading, PA) for an Apple Macintosh computer and the equation:  $\text{fraction bound} = \frac{[\text{protein}]}{[\text{protein}] + K_d}$ . The apparent dissociation constant ( $K_d$ ) for each DNA assayed was determined from the generated curve. The relative association constant ( $K_a$ ) for each mutant 5S RNA gene was determined by the equation:  $\text{apparent wild type } K_d / \text{apparent mutant } K_d$ . The experimental range or error between each independent determination was measured using the equation:  $(\text{highest value for the relative } K_a - \text{lowest value determined for the relative } K_a) / 2$ . The free energy of TFIIA-5S RNA gene complex formation was determined from the apparent association constant using the standard Gibbs free energy equation:  $\Delta G^\circ = -RT \ln(K_a)$ , where  $T = 295.15^\circ\text{K}$ .

### 2.2.5 Selected amplification and binding (SAAB) assay

The SAAB assay was carried out as described by Blackwell & Weintraub (1990) with several minor modifications. The 91 nucleotide long template consisted of the non coding strand of the 5S RNA gene from base pair +55 to +99, with the sequence of base pairs +78 to +95 randomized (Figure 2.7). Unique restriction sites (underlined) were incorporated immediately upstream and downstream of the *Xenopus* sequence. The 5' end of the template incorporated the sequence of the M13 universal forward sequencing primer, while the 3' end of the template consisted of the sequence complementary to the M13 universal reverse sequencing primer.

This template oligonucleotide was converted into double stranded DNA using the M13 universal primers. The 40  $\mu\text{l}$  fill-in reaction contained NTB buffer (50 mM Tris-HCl pH 7.2, 10 mM  $\text{MgCl}_2$ , 1 mM DTT, 50  $\mu\text{g/ml}$  BSA), 50 pmol R.U.P., and 11.5 pmol SAAB template. Primer annealing was accomplished by incubation at  $65^\circ\text{C}$  for five minutes followed by cooling to

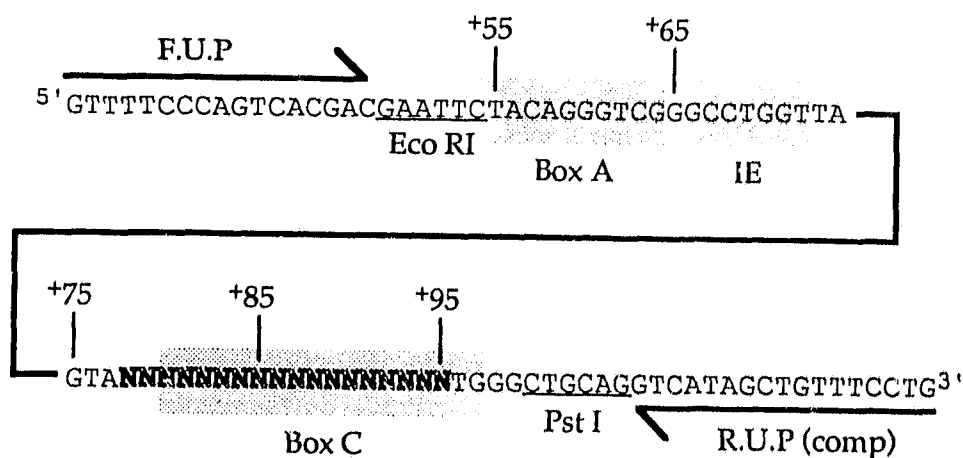


Figure 2.7 The synthetic internal control region designed to identify box C promoter elements that bind to TFIID with high affinity. Forward and reverse universal primer sites are shown flanking a portion of the ICR from position +55 to +95. Promoter elements are shaded and the random sequence region is outlined.

ambient temperature. The reaction was initiated with the addition of 8 nmol each dATP, dCTP, dGTP, and dTTP and 10 units of Klenow (New England Biolabs). After 45 minutes at ambient temperature, the enzyme was inactivated by incubation at 65°C for five minutes.

The first five selection rounds were performed using the nitrocellulose filter binding assay described in section 2.2.4. The TFIIIA concentration was maintained at 100 nM and protein-bound DNA was recovered by phenol:CHCl<sub>3</sub> extraction of the filter and ethanol precipitation with 20 µg glycogen (Boehringer) as a carrier. The selected DNA was resuspended in 20 µl water. Standard PCR amplification of each selected DNA was carried out in a 100 µl reaction containing 10 mM Tris-HCl pH 8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 50 µg/ml gelatin, 10 µl selected template DNA, 20 nmol each of dTTP, dGTP, dCTP, and dATP, 50 pmol F.U.P. and R.U.P. primers, and 2.5 units Taq DNA polymerase. Twenty-five rounds of thermal cycling were carried out using a denaturation temperature of 94°C (1.5 minutes), an annealing temperature of 55°C (1.5 minutes), and an extension temperature of 72°C (1.5 minutes). The amplified DNA product was phenol:CHCl<sub>3</sub> extracted, ethanol precipitated with 20 µg glycogen, and resuspended in 20 µl water. A 10 µl aliquot was used for further rounds of selection.

The DNA template selected from the final filter binding selection was radiolabeled in a 40 µl fill-in reaction containing NTB buffer, 40 pmol each of F.U.P. and R.U.P., 10 µl amplified DNA template, 8 nmol each dCTP, dGTP, and dTTP, 40 µCi [ $\alpha$ -<sup>32</sup>P]-dATP, and 10 units Klenow. The labeling reaction was incubated at ambient temperature for 45 minutes and chased with 8 nmol dATP for five minutes. The enzyme was inactivated by incubation at 65°C for five minutes and the reaction then loaded onto a 9% non-denaturing polyacrylamide gel (29:1, acrylamide:bis; 16 x 16 cm x 0.75 mm) and separated

from unincorporated nucleotide at 250V. Labeled DNA was identified by autoradiography of the wet gel and eluted from a gel slice overnight (Sambrook et al., 1989). The purified DNA was ethanol precipitated without carrier and resuspended in 15  $\mu$ l water.

The next three rounds of selection were performed using a gel retention assay. The TFIIIA concentration was sequentially reduced to 25 nM, 3 nM, and 1.5 nM. The 30  $\mu$ l binding reactions contained 20 mM Tris-HCl pH 7.5 at 20°C, 70 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 0.1% NP-40, 10  $\mu$ M ZnCl<sub>2</sub>, 2.5 mM DTT, 100  $\mu$ g/ml BSA, 6% glycerol, 1  $\mu$ g poly dI-dC (Pharmacia), 50,000 to 100,000 cpm (approximately 1.5 pmol) of radiolabeled DNA, and TFIIIA at the above concentrations. Complex formation was allowed to proceed at 22°C for 20 minutes and the reaction was loaded onto a 5% non-denaturing polyacrylamide gel (29:1, acrylamide:bis; 16 cm x 16 cm x 0.75 mm) at 4°C and electrophoresed at 300V in a buffer containing 27 mM Tris-borate pH 7.5, and 0.6 mM EDTA until the bromophenol blue dye marker was 7 cm from the bottom. (The gel was initially pre-electrophoresed at 4°C for 30 minutes prior to loading.) Protein-bound DNA was identified by autoradiography of the wet gel with an intensifying screen at ambient temperature for 90 minutes and eluted from a gel slice overnight (Sambrook et al., 1989). The purified DNA was ethanol precipitated with 20  $\mu$ g glycogen carrier and resuspended in 20  $\mu$ l water. The selected DNA was amplified in a standard PCR reaction and labeled by fill-in reaction as described above in preparation for further selection. DNA was isolated from the final selection pool in a gel shift assay using 5 nM TFIIIA and cloned into the *Eco* RI and *Pst* I sites of pUC19. Fifty independent isolates were chosen for DNA sequencing.

## 2.3 Results

### 2.3.1 Effects of base pair mutations within the 5S RNA gene on TFIIIA binding

The nitrocellulose filter binding assay was used to determine the TFIIIA binding affinities for mutant 5S RNA genes. This method identifies base pair positions that contribute to the energy of the 5S RNA gene-TFIIIA association. Each position on the DNA may influence TFIIIA binding by providing an optimal stereo-specific conformation or direct base contact. The severity on TFIIIA binding affinity of all substitutions at some base pair positions is consistent with the loss of direct base contacts necessary for protein binding. However, mutations at other base pair positions lead to more moderate reductions in TFIIIA association suggesting either weaker forms of direct contact or subtle DNA conformational effects on binary complex formation. An example of the effects of 5S RNA gene mutation on TFIIIA binding affinity as determined by nitrocellulose filter binding is shown in Figure 2.8.

Base pair substitutions from positions +67 to +71 within the intermediate element were assayed for TFIIIA binding affinity (Table 2.1). All substitutions at GC70 significantly reduced TFIIIA binding affinity, suggesting that this position might provide a direct contact site for the protein within the central region of the promoter. Changing the base pair identity at GC71 had a modest influence on the TFIIIA-5S RNA gene interaction. Substitution mutations at positions CG67 to TA69 did not affect TFIIIA binding affinity.

An extensive region within box C is important for high affinity binding of TFIIIA to the 5S RNA gene (Table 2.2). Substitution mutations from position +80 to +91 reduced TFIIIA binding affinity to varying degrees. Specific sequences within box C that flank this contact region (+78 to +79 and

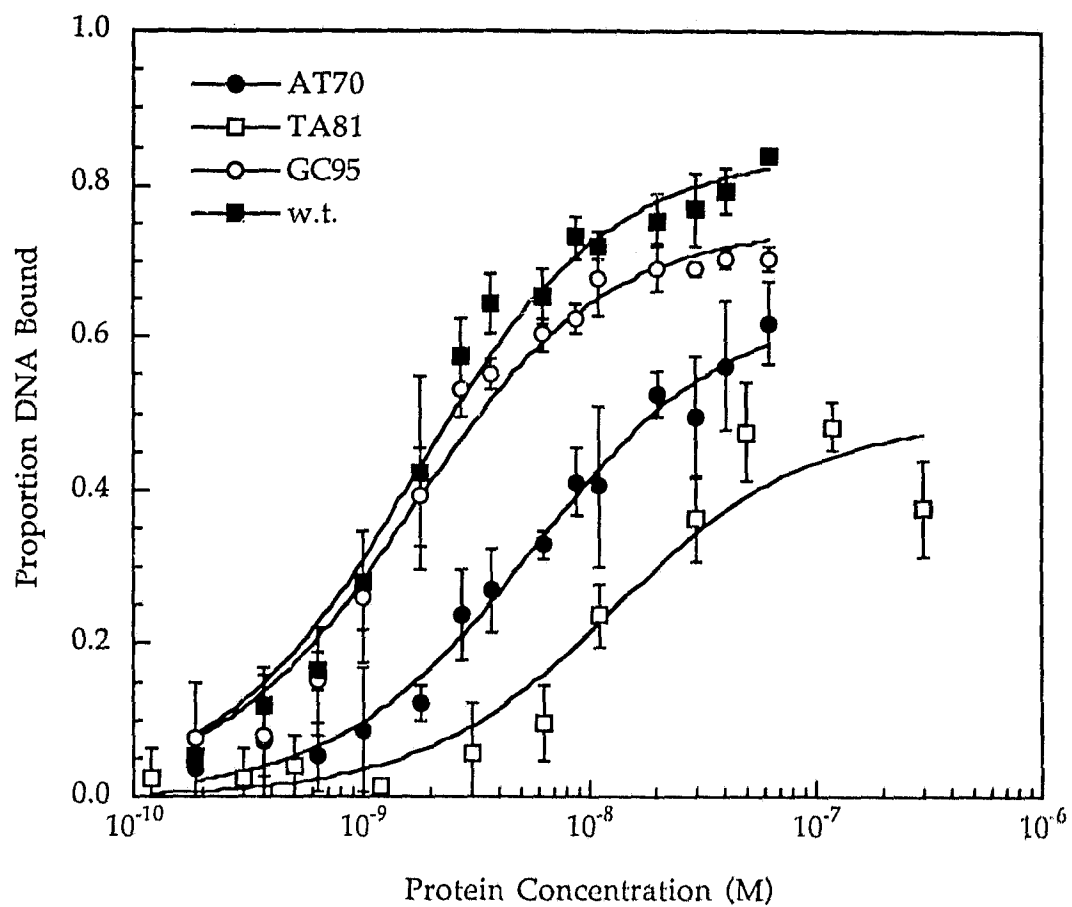


Figure 2.8 Sample nitrocellulose filter binding curves of 5S RNA gene mutants with recombinant TFIIA indicating the average of three or more determinations.

Table 2.1 Effects on TFIIA binding affinity<sup>a</sup> of 5S RNA gene point mutations within the intermediate promoter element .

Base Position	Base Pair Substitution			
	A-T	C-G	G-C	T-A
CG67	0.89 ± 0.07	•	1.08 ± 0.20	1.09 ± 0.18
CG68	1.04 ± 0.06	•	1.05 ± 0.14	1.07 ± 0.03
TA69	0.92 ± 0.03	0.95 ± 0.15	0.98 ± 0.04	•
GC70 <sup>b</sup>	0.31 ± 0.09	0.25 ± 0.04	•	0.67 ± 0.04
GC71	0.64 ± 0.11	0.74 ± 0.09	•	0.82 ± 0.11

<sup>a</sup>Determined as the ratio of apparent association constant for the mutant nucleic acid to the apparent association constant for the wild type nucleic acid. Average of three or more independent determinations. <sup>b</sup>This 5S RNA gene mutant was assayed by Qimin You.

Table 2.2 Effects on TFIIIA binding affinity<sup>a</sup> of 5S RNA gene point mutations within the box C promoter element.

Base Position	Base Pair Substitution			
	A-T	C-G	G-C	T-A
CG78 <sup>b</sup>	1.11 ± 0.25	•	1.22 ± 0.10	1.00 ± 0.20
TA79 <sup>b</sup>	1.12 ± 0.13	1.12 ± 0.13	1.09 ± 0.21	•
TA80 <sup>b</sup>	0.44 ± 0.17	0.98 ± 0.02	0.84 ± 0.04	•
GC81 <sup>b</sup>	0.30 ± 0.06	0.13 ± 0.03	•	0.22 ± 0.05
GC82 <sup>b</sup>	0.40 ± 0.01	0.54 ± 0.08	•	0.90 ± 0.10
AT83 <sup>b</sup>	•	0.70 ± 0.07	0.58 ± 0.04	0.20 ± 0.09
TA84 <sup>b</sup>	0.57 ± 0.13	0.39 ± 0.10	0.82 ± 0.14	•
GC85 <sup>b</sup>	0.23 ± 0.07	0.23 ± 0.15	•	0.19 ± 0.07
GC86 <sup>b</sup>	0.75 ± 0.18	0.35 ± 0.14	•	0.43 ± 0.22
GC87 <sup>b</sup>	0.49 ± 0.08	0.61 ± 0.19	•	0.56 ± 0.07
AT88	•	ND	0.70 ± 0.06	0.39 ± 0.07
GC89	ND	0.19 ± 0.06	•	0.25 ± 0.08
AT90	•	0.90 ± 0.10	0.40 ± 0.02	0.50 ± 0.06
CG91	0.48 ± 0.06	•	0.24 ± 0.05	0.39 ± 0.13
CG92	0.99 ± 0.22	•	1.27 ± 0.19	1.07 ± 0.04
GC93	1.34 ± 0.14	1.14 ± 0.16	•	1.06 ± 0.10
CG94	1.02 ± 0.19	•	1.13 ± 0.09	1.20 ± 0.25
CG95	1.14 ± 0.04	•	1.06 ± 0.05	1.07 ± 0.05
TA96	0.90 ± 0.14	0.92 ± 0.08	0.94 ± 0.10	•

<sup>a</sup>Determined as the ratio of apparent association constant for the mutant nucleic acid to the apparent association constant for the wild type nucleic acid. Average of three or more independent determinations. ND (not determined). <sup>b</sup>These 5S RNA gene mutants were assayed by Qimin You.

+92 to +96) are not required for high affinity interactions with TFIIIA. Different base pair substitutions at each position within the central region of box C had different effects on TFIIIA binding affinity, which may result from sequence-dependent changes in a DNA conformation optimal for binding or the loss of contact sites critical for complex formation. The diversity of responses to base pair substitution is perhaps best illustrated by comparing effects at positions with the same wild type base pair. For example, mutation of TA80 or TA84 to a GC base pair result in similar effects on TFIIIA interaction, while substitution of a CG base pair at position +84 leads to a 70 percent reduction in binding affinity, whereas the same change at position +80 results in no significant alteration in the binding constant (Table 2.2). The differential effects of substitution mutations can also be seen for AT base pairs at positions +83, +88, and +90. Substitution to GC or TA base pairs at these three sites affects TFIIIA binding to varying degrees. The clearest example of non equivalent contribution to TFIIIA association can be seen for GC base pairs within the box C contact region. The severity of the effects of all substitution mutations at positions GC81, GC85, GC89, and CG91 strongly support the existence of a role for these base pairs in providing direct base contacts to TFIIIA (Table 2.2). In contrast, the effects on TFIIIA binding affinity of different substitutions at GC82, GC86, and GC87 vary up to three-fold and are not the same for each base pair position. Perhaps these three GC base pairs influence TFIIIA binding affinity by contributing to an optimal DNA conformation within box C.

### **2.3.2 Identification of high affinity Box C elements within the 5S RNA gene**

Sequence specificity of TFIIIA binding was also analyzed using a SAAB assay. An ICR template pool, containing randomized sequence within the

box C element from positions +78 to +95, was subjected to repeated rounds of TFIIA selection and amplification. As a group, the DNAs selected for high affinity binding to TFIIA have a consensus sequence from +80 to +92 identical to that of the *Xenopus* wild type 5S gene sequence (Figure 2.9). The selected box C consensus sequence also shows strong similarity to the eukaryotic 5S DNA consensus sequence identified by Erdmann et al. (1985). The appearance of wild type base pairs in the box C selected sequences is more frequent at the 5' end (+80 to +87) than at the 3' end (+88 to +92). This pattern may result from differences in DNA binding affinity of the first three zinc fingers of TFIIA (Del Rio et al., 1993a). Base pairs flanking this recognition sequence (from +78 to +79 and from +93 to +95) remain relatively unselected in the ICR template pool (Figure 2.9). These positions, however, do exhibit a minor degree of wild type base pair identity within the selected DNA population and may influence promoter recognition by TFIIA.

Results of the SAAB assay may also reveal a significant contribution of DNA conformation to TFIIA binding. This is suggested by the apparent intolerance to many base pair substitutions within the box C element (positions +79, +80, +83, +84, +92, +94, and +95) that result in, at most, minor reductions in TFIIA binding affinity in the nitrocellulose filter binding assay. In this respect, the SAAB assay appears to identify the "sequence context" necessary for high affinity TFIIA binding in addition to possible individual base pair contacts.

## 2.4 Discussion

Assembly of an active RNA polymerase III transcription complex on the 5S gene requires recognition of the internal control region by TFIIA. Three promoter elements are required for the production of a functional

		Sequence Position																	
		78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
Base Occurrence	A	17	9	1	0	1	50	0	0	5	6	27	12	29	3	6	12	12	9
	C	6	17	13	0	0	0	1	0	1	2	2	4	4	39	23	12	21	14
	G	12	5	4	50	48	0	6	49	43	33	11	28	6	2	10	15	9	4
	T	14	19	32	0	1	0	41	1	1	7	10	6	11	6	11	11	8	17
SAAB consensus <sup>a</sup>		N	Y	T	G	G	A	T	G	G	G	R	R	A	C	Y	N	C	Y
Eukaryotic consensus <sup>b</sup>		S	Y	N	N	G	R	T	G	G	G	N	G	A	C	Y	N	Y	N

<sup>a</sup>Single letter codes are based on IUB nomenclature; R=A or G, Y=C or T, S=G or C.

<sup>b</sup>Eukaryotic 5S RNA gene consensus sequence from Erdmann, *et al.*, (1985).

Figure 2.9 The frequency of base pair occurrence within box C element sequences selected by high affinity TF.IIA binding. Shown are the bases selected on the non coding strand of the 5S RNA gene promoter, identified from sequencing fifty clones from the final selection round. The SAAB and eukaryotic consensus non coding strand sequences are shown.

transcription complex: the box A; intermediate; and box C elements (Bogenhagen et al., 1980; Sakonju et al., 1980; Pieler et al., 1987). TFIIA makes sequence-specific contacts within this internal control region (ICR) at three locations: box A (+57 to +62); intermediate (+67 to +70); and box C (+78 to +86) elements (You et al., 1991). Progressive 5' deletions that remove the box A promoter element result in a gradual loss of transcription efficiency and corresponding TFIIA binding ability (Sakonju et al., 1980). In contrast, the reduction in transcriptional competency and TFIIA binding is more severe for 3' deletions that enter the box C element (Bogenhagen et al., 1980). Clustered substitution mutations within the 5S RNA gene confirm the importance of the box C element in TFIIA binding (You et al., 1991). The simultaneous mutation of base pairs +78 to +86 results in a 100-fold reduction in transcription factor association. The present study further defines TFIIA contacts within the latter two promoter regions by using an extensive set of point mutants of the 5S RNA gene and a quantitative nitrocellulose filter binding assay for measuring TFIIA binding affinity. In addition, box C sequences recognized by TFIIA were isolated and characterized using a SAAB analysis.

The effects of point mutations in the 5S RNA gene on TFIIA binding affinity do not differentiate between contacts made to bases located on the coding and noncoding DNA strands. A reduction in complex stability due to a single base pair substitution identifies that position but not the individual base as being important for TFIIA association. This experimental limitation must be kept in mind when interpreting the binding data. However, evidence for the involvement of bases on the noncoding strand in TFIIA binding to the internal promoter has been elucidated using a variety of methods (Sakonju & Brown, 1982; Lee et al., 1991b). It is possible that TFIIA

association with the 5S RNA gene relies predominantly on an extensive set of hydrogen bond contacts to bases on this DNA strand.

TFIIIA interacts with two base pair positions (GC70 and GC71) within the intermediate promoter element (Table 2.1). Although these contacts have previously been identified (Sakonju & Brown, 1982; Fairall et al., 1986), the binding data indicate that their contribution to TFIIIA-5S gene complex stability is not equivalent. Mutation of GC70 is more deleterious to TFIIIA binding affinity than is substitution of the adjacent GC71 base pair. Recent studies suggest that zinc finger 5 contacts this base pair doublet in the center of the ICR (Clemens et al., 1992; Hayes & Clemens, 1992).

The apparent discrepancy between results obtained using chemical modification and substitution mutagenesis of the intermediate element may arise from inherent differences between these experimental procedures. Methylation of guanine bases within the ICR could negatively influence interactions between TFIIIA and base contacts adjacent to the modified guanine. The magnitude of such a "neighboring" effect would depend on the energetic contribution provided by the adjacent base contact to the overall free energy of binding and the degree of steric disruption with introduction of a methyl group in the major groove of DNA. Thus, studies based on chemical modification of DNA may not allow for an accurate measure of the energetic contribution of individual guanine base contacts within a GC-rich binding site such as the ICR of the 5S RNA gene. Single base pair substitution analysis could provide an advantage in that DNA structure is not likely to be significantly perturbed. In this respect, GC71 is shown to provide a small contribution to TFIIIA interaction with the intermediate element. Methylation of the guanine base at position +71 may abrogate TFIIIA binding

to GC70 as well as to GC71 resulting in a greater instability in the TFIIA-5S RNA gene complex.

DNase I protection by TFIIA encompasses residues +46 to +95 of the 5S RNA gene (Engelke et al., 1980; Sakonju & Brown, 1982). Functional studies using 5S DNA point mutants identified the ICR as including base pairs +50 to +97 (Pieler et al., 1985a; Pieler et al., 1985b). However, an investigation of the effects of point mutants centered around base pair +90 suggested the box C promoter element of the ICR extends only to CG91 (McConkey & Bogenhagen, 1987). TFIIA contacts within box C have also been defined by methylation and ethylation interference. These data showed guanine N<sup>7</sup> and backbone phosphate contacts extend from TA80 to CG91 (Sakonju & Brown, 1982). This identification of the box C element boundaries is supported by the present point mutant analysis. Base pairs that influence the thermodynamic stability of complex formation, either by direct contact or conformational restrictions, extend from position +80 to +91. Base pairs within this region are also responsible for the high affinity interaction of a truncated polypeptide containing only the first three zinc fingers of TFIIA (Liao et al., 1992). SAAB analysis indicates that base identity at position +79 and from positions +92 to +94 may influence promoter recognition by TFIIA, while the mutational data indicate these base pairs do not make a significant contribution to the free energy of protein binding. Weaker interactions within the box A and intermediate promoter elements may serve to correctly align the bound transcription factor on the internal promoter, producing a biologically functional complex. Oocyte and somatic forms of the 5S RNA gene do exhibit base differences at positions +53, +55, and +56 which lead to significant differences in TFIIA-5S DNA complex structure and developmental

regulation of transcription (Wormington et al., 1981; Sakonju & Brown, 1982; Xing & Worcel, 1989a).

TFIIIA interacts with the 5S RNA gene with an apparent association constant ( $K_a$ ) of  $1.9 \times 10^9 M^{-1}$  and a Gibbs free energy ( $\Delta G_c$ ) of -12.53 Kcal/mol (Romaniuk, 1990). In contrast, non specific DNA binding by TFIIIA has a  $K_a$  of less than  $3.3 \times 10^7 M^{-1}$  and a  $\Delta G_c$  of greater than -10.15 Kcal/mol. The difference in the free energy of binding ( $\Delta\Delta G_c$ ) of TFIIIA with the internal control region compared to non specific DNA is at least -2.38 Kcal/mol. A major contribution to this specific DNA binding energy can be attributed to interactions between TFIIIA and base sequence from positions +81 to +91 within the box C promoter element (Table 2.3). A comparison of the change in free energy of TFIIIA association with clustered and point substitution mutants of the 5S RNA gene was performed to determine the contribution of base sequence and DNA conformation to complex formation (Table 2.3). Changes in  $\Delta G_c$  correlate well between the two types of mutants in the intermediate element suggesting that individual base sequence positions contribute independently to the binding energy. However, there are moderate differences in  $\Delta\Delta G_c$  values for clustered mutants and point mutants within the box C element indicating that individual base contributions to the free energy of binding are dependent, to some extent, on the sequence context presented to TFIIIA. A recognition process that includes contributions from DNA context could be important in discrimination by a multi-zinc finger protein of the correct promoter sequence from non specific DNA sites that may contain a few correctly positioned contact bases.

Chemical modification, photocrosslinking, and hydroxyl radical footprinting studies have provided strong evidence for the close association

**Table 2.3** Comparison of the change in the free energy of TFIIIA association<sup>a</sup> with clustered and point mutants of the 5S RNA gene.

Clustered Mutants <sup>b</sup>		Point Mutants		Clustered Mutants <sup>b</sup>		Point Mutants	
Base Position	$\Delta\Delta G_c$ (Kcal/mol)	Base Position	$\Delta\Delta G_c$ (Kcal/mol)	Base Position	$\Delta\Delta G_c$ (Kcal/mol)	Base Position	$\Delta\Delta G_c$ (Kcal/mol)
Intermediate Element				Box C Element			
		67	-0.04±0.0003			78	-0.12±0.0004
		68	-0.03±0.0002			79	-0.05±0.0004
		69	+0.05±0.0001			80	NS
		70	+0.81±0.007			81	+1.2±0.015
67 - 70	+0.65±0.005	sum	+0.7±0.007	78 - 81	+1.65±0.03	sum	+1.03±0.015
		71	+0.18±0.001			82	+0.36±0.003
		72	ND			83	NS
71 - 72	-0.16±0.001	sum	ND			84	+0.12±0.001
						85	+0.86±0.033
						86	+0.62±0.013
				82 - 86	+2.7±0.09	sum	+1.96±0.033
						87	+0.42±0.003
						88	NS
						89	ND
						90	+0.54±0.001
				87 - 90	+0.26±0.003	sum	+0.96±0.003
						91	+0.84±0.01
						92	-0.14±0.001
						93	-0.03±0.0001
						94	-0.07±0.003
				91 - 94	+0.29±0.006	sum	+0.6±0.01

<sup>a</sup>Determined as the difference in free energies of TFIIIA binding by the mutant nucleic acid and the wild type nucleic acid. Average of three or more independent determinations. ND (not determined), NS (base pair not substituted in the clustered mutation;  $\Delta\Delta G_c=0$ ). <sup>b</sup>Relative binding affinities for the clustered substitution mutants were reported in You et al., 1991.

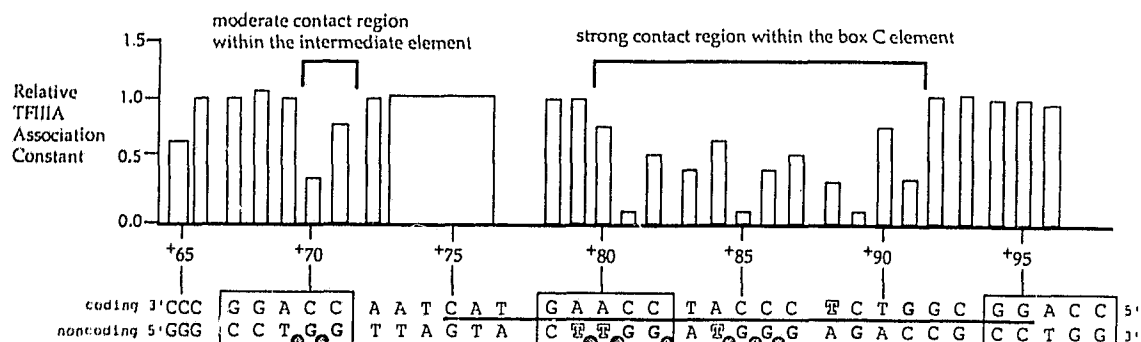


Figure 2.10 Summary of the base pair interactions within the intermediate and box C promoter elements of the 5S RNA gene. Sequence of the coding and noncoding strands from residues +65 to +98 of the 5S RNA gene are shown. The effects of point mutations on TFIIIA binding affinity were averaged for each base pair and are indicated above the DNA sequence. Results obtained using clustered substitution mutations within this region of the ICR are also shown (You et al., 1991). Guanine bases that interfere with TFIIIA binding when methylated are indicated by boldface letters and DNA backbone phosphates that contact TFIIIA are marked by gray circles (Sakonju & Brown, 1982). The sequence protected by an N-terminal peptide of TFIIIA containing the first three fingers is underlined (Liao et al., 1992). Thymine residues that closely approach TFIIIA are shown in outlined letters (Lee et al., 1991b). Repeated GC-box sequences within this promoter region are boxed (Huber et al., 1991b).

of TFIID with the noncoding strand of the ICR (Sakonju & Brown, 1982; Churchill et al., 1990; Lee et al., 1991b). Many of the GC base pairs within the 5S gene promoter that make a significant contribution to TFIID binding affinity correspond to positions at which methylation of the guanine base on the noncoding DNA strand reduces protein binding (positions +81, +82, +85, +86, +87, and +89) (Figure 2.10). Reduced TFIID binding to the methylated 5S RNA gene indicates a prominent role for the guanine N<sup>7</sup> atom in protein interaction. Strong selection, in the SAAB analysis, of guanine residues situated on the noncoding DNA strand at positions +81, +82, +85, +86, +87, and +89 also support a role for guanine-specific atoms in promoter recognition and binding by TFIID. The pattern of hydrogen bond contacts to individual guanine residues may vary significantly along the box C element, as suggested by the differential effects on TFIID binding affinity of point mutations at these critical GC base pairs. Selection of the wild type AT base pair sequence at other positions within the box C region (+80, +83, and +84) suggest they also contribute to the energy of TFIID binding, either by providing direct contacts or an optimal DNA conformation.

Contradictory evidence exists that the 5S RNA gene adopts an A-form DNA conformation (McCall et al., 1986; Rhodes & Klug, 1986), similar to 5S RNA, or a B-form conformation (Gottesfeld et al., 1987; Aboul-ela et al., 1988), similar to mixed sequence DNA. However, the 5S RNA gene may be structurally heterogeneous, with a conformation intermediate to both classical DNA structures (Fairall et al., 1989; Huber et al., 1991a; Huber et al., 1991b). This structural heterogeneity has been suggested to arise due to the periodic positioning of GC-rich sequence clusters along the ICR (Figure 2.2B) (Huber et al., 1991a; Huber et al., 1991b). Such conformational heterogeneity could provide additional information for the recognition and binding of

individual zinc fingers of TFIIIA to the 5S RNA gene. A sequence-dependent local DNA conformation required for zinc finger binding may explain selection of native 5S DNA base sequence within the region +79 to +94 at positions that do not provide energetically significant contributions to TFIIIA binding. AT base pairs selected at positions +79, +80 and +84, as well as CG base pairs at +92 and +94, could help form the local conformation necessary for correct TFIIIA zinc finger positioning in the major groove (Figure 2.9). Pyrimidines are also found at these latter positions on the non coding strand in the 5S DNA consensus sequence that includes the 5S RNA genes from highly divergent organisms, such as *Drosophila* and man (Erdmann et al., 1985). Thus, specific DNA conformation, as well as direct base-specific contacts, may help position individual zinc fingers within the box C binding site and align TFIIIA correctly along the ICR.

X-ray crystallographic structures of the zinc finger DNA binding domains of *Zif268* (Pavletich & Pabo, 1991), *GLI* (Pavletich & Pabo, 1993), and *Tramtrack* (Fairall et al., 1993) complexed with their respective target DNA sites have been determined. Comparison of individual zinc finger interactions with DNA suggests that no simple rules for determination of zinc finger-DNA contact patterns exist. However, from these few examples, structural parameters of both DNA and zinc finger proteins can be identified that contribute to binding site specificity and protein affinity. DNA subsites contacted by individual zinc fingers range from three to five bases and may overlap (*Tramtrack*), abut (*Zif268*), or be spaced apart (*GLI*). A single zinc finger can interact with bases on one (*Zif268*) or both (*GLI*) DNA strands within a subsite. DNA conformation can also play a major role in binding site specificity by adopting a unique structure that provides critical base and phosphate backbone contacts (Fairall et al., 1993; Pavletich & Pabo, 1993).

Amino acid residues at positions -1, +2, +3, +5, and +6, relative to the start of the  $\alpha$ -helical region within a zinc finger, can be used to contact DNA bases. Differences in the amino acid sequences of zinc fingers result in a variety of base contact patterns that may be difficult to predict through modeling studies. The pattern of phosphate contacts made by each finger can exhibit even greater variability. This allows zinc fingers to be functionally distinct, with individual fingers making predominantly phosphate or base contacts (Pavletich & Pabo, 1993). Thus, although the overall docking of zinc fingers onto DNA appears similar, individual differences in DNA subsite conformation and zinc finger amino acid sequence can result in marked differences in DNA binding specificity. The architecture of the TFIIA-box C interaction may also exhibit unique characteristics, as suggested by a long contact region and by footprinting studies with TFIIA 'broken finger' mutants (Del Rio et al., 1993a). Individual TFIIA finger recognition subsites within the box C element could include up to five base pair positions and/or include spacing segments.

Studies with truncated mutants of TFIIA suggest that binding of the N-terminal zinc fingers to the box C element provides a major contribution to the free energy of complex formation (Vrana et al., 1988; Liao et al., 1992; Theunissen et al., 1992). Interactions between the C-terminal zinc fingers and proximal promoter elements further contribute to the binding energy (Churchill et al., 1990; Clemens et al., 1992). This simple model does not take into account possible complex thermodynamic interactions between zinc finger interaction sites that could make it impossible to interpret the energetics of binding by any particular zinc finger considered in isolation. Nonetheless, it appears that the N-terminal zinc fingers of TFIIA provide nonequivalent contributions to the binding energy. Finger three is involved

in strong contacts to the box C element and its removal (Vrana et al., 1988; Liao et al., 1992; Theunissen et al., 1992) or disruption (Del Rio et al., 1993a) severely reduces TFIIIA-5S RNA gene binding activity. Finger two provides a moderate contribution to the free energy of binding and interacts with the center of the box C element (Del Rio et al., 1993a). Deletion of the N-terminal region or finger one has little effect on TFIIIA binding affinity. These apparent energetic differences in zinc finger association can be observed in the nonequivalent selection of wild type sequence along the box C element (Figure 2.9). Strong selection occurs from +80 to +87, perhaps corresponding to the region of finger two and three interaction. Weaker selection from +88 to +94 may involve finger one and amino terminal non-finger residues. It is interesting to note that three base pair positions within the box C region (+81, +85, and +89) provide the greatest individual contribution to the energy of binding; it is possible that these mutations identify three separate subsites for finger interaction. These positions were also identified as being energetically important in a missing nucleoside analysis of the TFIIIA-5S RNA gene complex (Hayes & Tullius, 1992).

In summary, TFIIIA interacts strongly with the box C element of the 5S gene promoter through multiple contacts to base pairs extending from positions +80 to +91. Base pairs flanking this region, at +79 and +92 to +94 also influence TFIIIA-5S gene association. It is likely that the N-terminal zinc fingers of TFIIIA are involved in this energetically important interaction. Local DNA conformation, as well as critical base contacts, are important in promoter recognition by TFIIIA and may function in the alignment of individual zinc fingers along the ICR.

## Chapter 3.0 Interaction of TFIIA with 5S RNA

### 3.1 Introduction

#### 3.1.1 General structure of 5S RNA

Since its discovery in 1963, ribosomal 5S RNA has proven to be a powerful tool in the study of cellular evolution and RNA-protein interaction. Sequence compilation demonstrates that 5S RNA averages about 120 nucleotides in length and contains few modified nucleotides, with the exception of some in eukaryotic species (Wolters & Erdmann, 1988). Comparison between the 5S RNA from diverse organisms has provided a means for the accurate phylogenetic characterization of many species (Hori & Osawa, 1986; Hori & Osawa, 1987). In addition, these studies provide support for the endosymbiotic origin of the eukaryotic cellular organelles (chloroplast and mitochondria) during evolution (Delihias et al., 1984; Hori & Osawa, 1986; Hori & Osawa, 1987).

A minimal secondary structural model was first proposed for eukaryotes by Nishikawa and Takemura (1974) and for eubacteria by Fox and Woese (1975). Further analysis of an increasing number of 5S RNA sequences indicated that characteristic structural differences exist which allows the identification of four general types; eukaryotic, mitochondrial, eubacterial (including plastids), and archaebacterial (or metabacteria) (Hori & Osawa, 1987). Sequence alignment, nuclease protection and chemical modification analyses suggest that the overall secondary structure for all 5S RNA has been strongly conserved throughout evolution (Delihias et al., 1984; Kjems et al., 1985; Mannella et al., 1987). The calculated free energies ( $\Delta G^\circ$ ) for most 5S RNAs are close to an average of  $-50$  Kcal/mol, with a few exceptions (Delihias & Andersen, 1982). These universal structures for 5S RNA each consist of

five helical regions and five loop domains arranged into three arms. For the sake of comparison and clarity, helical and loop designations for all 5S RNA species discussed will be based on the nomenclature reported by Romaniuk (1985) for *Xenopus* 5S RNA (Figure 3.4).

A number of highly conserved nucleotide sequences are found dispersed throughout the eukaryotic 5S RNA molecule (Delihias et al., 1984). These sequences are found predominantly in the loop domains and at the ends of helical regions (Delihias & Andersen, 1982; Küntzel et al., 1983; Delihias et al., 1984) (Figure 3.1). In contrast, base sequence within the helices can vary considerably between species. Base-pair interactions are found to be maintained between species by compensatory base changes of paired nucleotides lending further support for the existence of universal helical regions within 5S RNA. Greater variability exists in the secondary structure of helices IV and V of 5S RNA than in helices I, II, and III (Küntzel et al., 1983; Erdmann et al., 1986). Notable differences between eukaryotic and eubacterial 5S RNAs include the length of helix V and the possible existence of non-Watson-Crick base pairing within loop E and helix V (Stahl et al., 1981; de Wachter et al., 1982; Delihias & Andersen, 1982; Erdmann et al., 1986; Wolters & Erdmann, 1988).

Loop E is a region of considerable interest as it appears to be protected from nuclease and chemical attack by ribosomal protein L25 in prokaryotic 5S RNA (Huber & Wool, 1984) and by TFIIA and ribosomal protein L5 in eukaryotic 5S RNA (Pieler & Erdmann, 1983; Romaniuk, 1985; Huber & Wool, 1986). The conformation of loop E varies considerably between 5S RNA species. In *E. coli* 5S RNA this region exhibits little secondary structure (Zhang & Moore, 1989), while in spinach chloroplast 5S RNA loop E contains purine-purine base pairs and pyrimidine bases that face the solvent

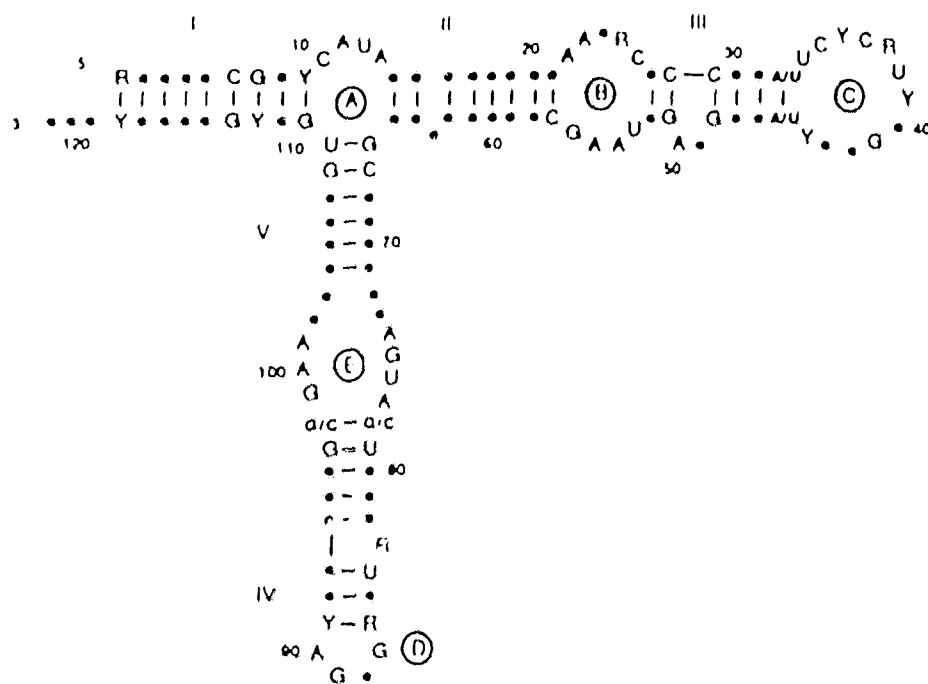


Figure 3.1 The general eukaryotic consensus sequence for 5S RNA (Delihias & Andersen, 1982).

(Romby et al., 1988). Non-Watson-Crick base pairing in loop E is also observed in yeast, *Xenopus*, and *Bacillus* 5S RNA (Kjems et al., 1985; Wimberly et al., 1993). Such differences in the observed secondary structures of 5S RNA may reflect magnesium-dependent stabilization of the helix IV-loop E-helix V arm (Delihias et al., 1984; Kjems et al., 1985; Zhang & Moore, 1989). Loop E of *Bacillus* 5S RNA in the absence of magnesium is selectively destabilized and appears in a single-stranded conformation (Pieler et al., 1984a). In the presence of the divalent ion, loop E is observed to be highly structured (Kjems et al., 1985). Magnesium binding sites within loops B and E of spinach chloroplast 5S RNA have been proposed (Romby et al., 1988). The structure of the loop E region of *Xenopus* 5S RNA is described in detail in section 3.1.2.

Bulged nucleotides occur at highly conserved positions in helix II and to a lesser extent helix IV of both eubacterial and eukaryotic 5S RNAs (Kjems et al., 1985). Chemical modification suggests that these nucleotides are in a dynamic equilibrium with adjacent positions (de Wachter et al., 1984; Kjems et al., 1985; Westhof et al., 1989). Such extra-helical positions are of interest as they may provide critical nucleation sites for the interaction of proteins with 5S RNA. The bulged nucleotide in helix II of *E. coli* 5S RNA has been implicated in protein L18 recognition (Peattie et al., 1981). Another bulged nucleotide critical for protein binding is found within the translational operator of the R17 replicase gene (Romaniuk et al., 1987b). A three nucleotide sequence within the loop and a bulged adenosine within the helical stem provide the necessary contacts for the binding of the R17 coat protein.

Tertiary interactions involving loops C and E have been suggested from chemical and nuclease studies of eubacterial (Hancock & Wagner, 1982;

Pieler & Erdmann, 1982; Kjems et al., 1985; Romby et al., 1988) and eukaryotic 5S RNAs (Böhm et al., 1981; Toots et al., 1982). Base positions 25 and 45 within loops B and C, respectively, of spinach chloroplast 5S RNA may be involved in local tertiary interactions that stabilized the loop structures (Romby et al., 1988). Additional studies suggest that loop D of yeast 5S RNA is involved in tertiary interactions, possibly with loop B sequence (Kjems et al., 1985). However, contradictory evidence for the lack of tertiary interactions within loops C and E of prokaryotic (Zhang & Moore, 1989) and eukaryotic (Westhof et al., 1989; Chow et al., 1992) 5S RNA also exists. The apparent discrepancies in the chemical and nuclease protection patterns obtained with different 5S RNA species may be due to differences in sequence and/or reaction conditions, such as the concentration of divalent cations (Romaniuk et al., 1988).

The structural similarity between 5S RNAs from diverse organisms is perhaps best illustrated by the ability of heterologous eubacterial, archaeobacterial, and eukaryotic 5S RNA to maintain *Bacillus* 50S ribosomal subunit activity in reconstitution experiments (reviewed in Erdmann et al., 1986). 5S RNA from *E. coli*, spinach chloroplast, and *Bacillus* are incorporated to produce ribosomes that exhibit wild type levels of activity (Vogel et al., 1984). The eukaryotic 5S RNA from *Saccharomyces* also incorporates into the eubacterial ribosome but with significantly reduced biological activity. Structural conservation is also suggested by the observation that *Xenopus* TFIIA has been found to recognize and bind to 5S RNAs from *Xenopus* and wheat germ and to a lesser extent from *E. coli* (Romaniuk, 1985).

Eukaryotic 5S RNA from somatic cells or the major 5S RNA species transcribed in lower eukaryotes differs from 5S RNA isolated from oocytes or minor 5S RNA species at several nucleotide positions (Delihias & Andersen,

1982). Some of these nucleotide substitutions are concentrated at sequence positions common to eukaryotic 5S RNA such as 12 and 53, while others are species-dependant (Figure 3.2). Such oocyte-specific substitutions in conserved sequence regions raises the possibility of their importance either in the differential expression of the 5S RNA genes or in 5S RNA structure and biological activity.

### 3.1.2 Structure of *Xenopus* 5S RNA

The secondary and tertiary structures of oocyte and somatic-type 5S RNA from *Xenopus* have been investigated by chemical and nuclease analysis alone (Romaniuk et al., 1988) and in combination with computer modeling (Westhof et al., 1989; de Stevenson et al., 1991) (Figure 3.3). Both *Xenopus* RNA molecules conform to the consensus secondary structure model of 5S RNA, containing five loop regions and five helices. However, local loop and helix conformations are strongly dependent on nucleotide sequence (de Stevenson et al., 1991). Bulged nucleotides are identified at positions A49, A50, C63, G75, and A83 (or U84 due to a dynamic equilibrium) within each RNA molecule (Romaniuk et al., 1988). Loop A is structurally similar in both RNAs and adopts essentially a single stranded conformation (Westhof et al., 1989). In contrast, loop A within spinach chloroplast 5S RNA is highly structured. Loop C is stabilized by hydrogen bonds formed between U35-A42 and C36-G41, as well as by base stacking of C34 and C44 inside the loop (Westhof et al., 1989). The pattern of protection of purine N<sup>7</sup> sites and backbone phosphates within loops C and D of *Xenopus* 5S RNA suggests that they may be stabilized by magnesium ion binding or be involved in tertiary interactions (Romaniuk et al., 1988). However, further analyses using chemical probes and computer modeling show a lack of tertiary interactions

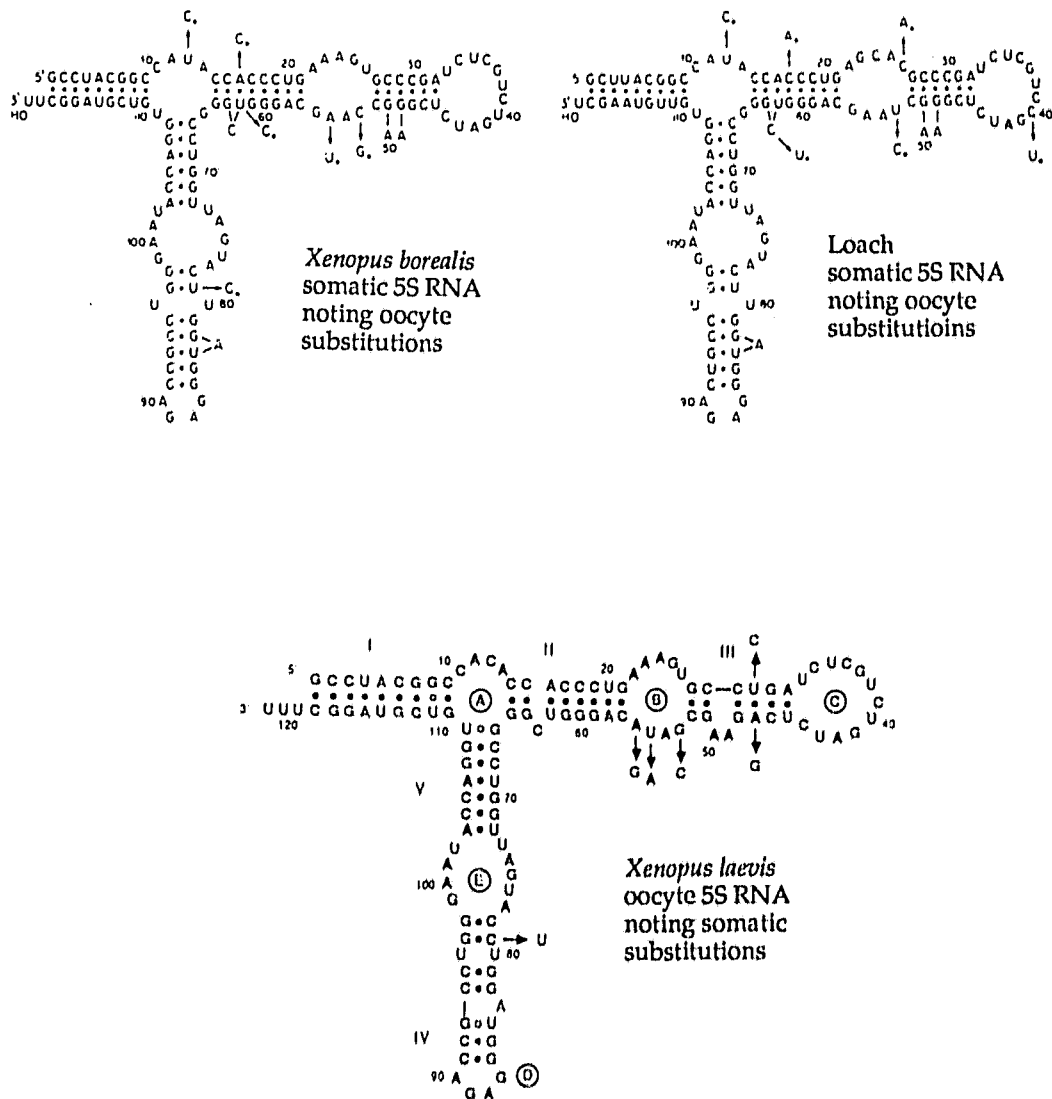


Figure 3.2 Secondary structure of eukaryotic 5S RNA with somatic and oocyte substitutions indicated (Delihias & Andersen, 1982; Romaniuk et al., 1988).

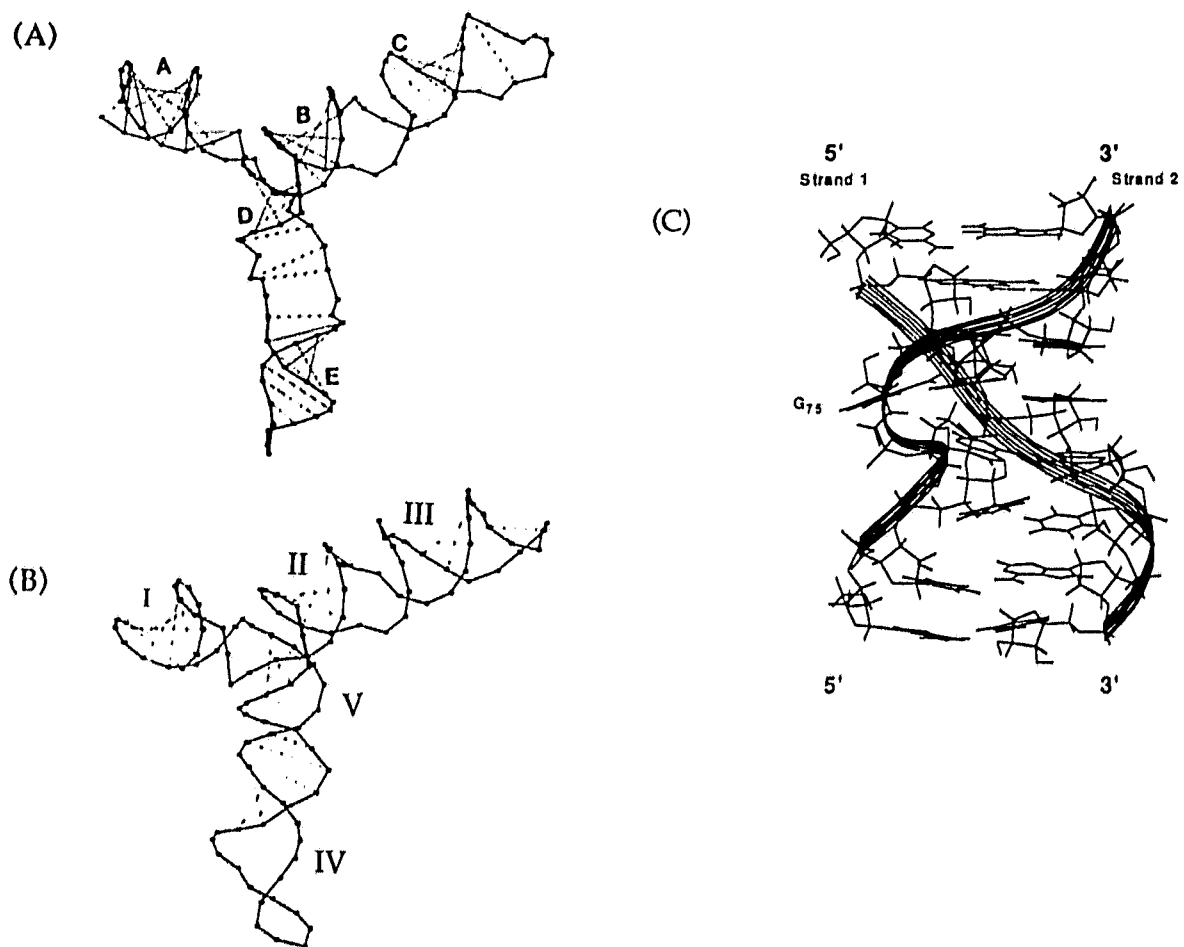


Figure 3.3 Three-dimensional models of (A) spinach chloroplast 5S RNA, (B) *Xenopus* oocyte-type 5S RNA (Westhof et al., 1989), and (C) loop E of *Xenopus* 5S RNA (Wierberly et al., 1993).

between loop C and the helix V-loop E-helix IV-loop D arm of the molecule (Christiansen et al., 1987; Westhof et al., 1989; de Stevenson et al., 1991; Chow et al., 1992). The conformation of loop D may be stabilized by hydrogen bond formation between G87 and A90 and by base stacking between G89 and A90 (Westhof et al., 1989; de Stevenson et al., 1991).

Important differences do exist between the two 5S RNA forms. There are six nucleotide substitutions between oocyte and somatic 5S RNA. Three of these substitutions reside within loop B, two within helix III (providing a base pair replacement), and one on helix IV (Figure 3.2) (Romaniuk et al., 1988). Loop B is less structured in oocyte-type 5S RNA than in the somatic-type molecule, with a Watson-Crick base-pair between G25 and C53 and base stacking by A55 and G56 identified in the latter molecule (Westhof et al., 1989). Loop E adopts an unusual quasi-helical secondary structure containing non-Watson-Crick base pairs and a bulged guanine residue at position 75 (Andersen et al., 1984; Romaniuk et al., 1988; Westhof et al., 1989; de Stevenson et al., 1991; Wimberly et al., 1993) (Figure 3.3). Under non-denaturing conditions, the only chemically reactive purine N<sup>7</sup> atom within loop E is G75 for both RNAs (Romaniuk et al., 1988). The conformation of loop E is similar between the 5S RNA forms, however, it is more stable in the oocyte-form than in the somatic-form (Romaniuk et al., 1988). The presence of magnesium ions may significantly stabilize the loop E conformation (de Stevenson et al., 1991). Increased stability is also observed for helices III and IV in the oocyte 5S RNA compared with the somatic RNA molecule. The increased structural stability of oocyte 5S RNA in the presence of 4M urea is conferred by the five nucleotide substitutions in loop B and helix III. In contrast, oocyte 5S RNA stability in the presence of the chelating agent, EDTA, is due to the single nucleotide substitution at position 79 within helix IV.

It has been suggested that both *Xenopus* 5S RNAs and spinach chloroplast 5S RNA adopt a distorted 'Y'-shape structure, with helices II and V stacked and nearly colinear (Westhof et al., 1989) (Figure 3.3). The orientation of helix I with respect to the two 'arms' of 5S RNA is controlled by 'hinge' nucleotides in loop A. The dimensions of this structural model are 118Å between residues 40 (loop C) and 90 (loop D), 109Å between residues 40 and the 5'-terminal nucleotide, and 84Å between residue 90 and the 3'-terminal nucleotide (Westhof et al., 1989). These measurements agree with those estimated by electron spectroscopic imaging (ESI) of the 7S RNP particle (Bazett-Jones, 1988).

### 3.1.3 Interaction of 5S RNA with *Xenopus* TFIIA

The 7S RNP particle is found in very large amounts in previtellogenic oocytes of *Xenopus laevis* (Picard & Wegnez, 1979; Miller et al., 1989). A similar particle is also observed in other amphibian species and in teleost species (Picard & Wegnez, 1979). The expression of TFIIA and 5S RNA and, hence, the levels of 7S RNP are developmentally regulated during oogenesis (Ford & Brown, 1976; Honda & Roeder, 1980). In oocytes the majority of accumulated 5S RNA is derived from the large population of oocyte-type genes. Following ovulation no 5S RNA synthesis is detectable in the egg. However, during embryonic development 5S RNA synthesis is reestablished from the somatic-type genes (Ford & Brown, 1976).

Immunological detection indicates that TFIIA is present in high concentration in immature oocytes, corresponding with maximal 5S RNA transcription (Honda & Roeder, 1980). In contrast, TFIIA is present in vastly reduced amounts in unfertilized eggs and in somatic cell extracts, correlating with reduced 5S RNA transcription (Honda & Roeder, 1980; Pelham et al.,

1981). The requirement of TFIIA for 5S RNA transcription and storage suggests that a feedback-inhibition mechanism may exist to control 5S RNA levels during oogenesis (Honda & Roeder, 1980). Strict regulation of the TFIIA intracellular concentration allows the regulation of 5S RNA gene transcription. The accumulation of large amounts of 5S RNA required for ribosome assembly requires the relative over production of TFIIA. During early oogenesis, the accumulated 5S RNA sequesters TFIIA in the 7S RNP particle (and p43 in the 42S RNP particle) which is subsequently transported to the cytoplasm. This reduction in the nuclear concentration of TFIIA may contribute to a reduced rate of 5S RNA synthesis (Guddat et al., 1990).

The structure of the 7S RNP has been probed using neutron scattering and sedimentation analyses (Timmins et al., 1988) and by electron spectroscopic imaging (ESI) (Bazett-Jones, 1988). The molecular weight of the complex ranges from  $95.7 \pm 10$  kD to  $86.7 \pm 9$  kD, depending on the method of analysis and has an overall width of  $59\text{\AA}$  and a length of  $140\text{\AA}$  (Timmins et al., 1988). Similar dimensions are obtained by ESI analysis of *Xenopus* 5S RNA bound by TFIIA with a length of approximately  $140\text{\AA}$  and a width of approximately  $80\text{\AA}$  (Bazett-Jones, 1988). Differences in the measured length of free 5S RNA and the 7S RNP complex (approximately  $15\text{\AA}$ ) may be due to TFIIA protein extending beyond the RNA molecule (Timmins et al., 1988). No significant alteration in 5S RNA conformation is observed in the 7S RNP particle (Timmins et al., 1988).

The *Xenopus* 7S RNP appears to undergo a reversible equilibrium of complex assembly at pH 7.5 and  $23^\circ\text{C}$  which is characterized by the formation of a 1:1 complex that self-aggregates to a dimer (Callaci et al., 1990). The presence of higher order ribonucleoprotein structures has also been observed in RNA exchange analyses (Andersen & Delias, 1986). Determination of the

dissociation kinetics for reconstituted *Xenopus* 7S RNP suggests the presence of two populations of complexes; a rapidly dissociating species, and a species which dissociates with a rate constant of approximately  $4.5 \times 10^{-4} \text{ s}^{-1}$  (Romaniuk, 1985). The biological significance of both higher order 7S RNP interactions and the presence of populations exhibiting different dissociation rates remains unknown.

A nitrocellulose filter binding assay has been employed to study the interaction of *Xenopus* TFIIA with 5S RNA (Romaniuk, 1985). The protein binds to oocyte-type 5S RNA with an association constant of  $1.4 \times 10^9 \text{ M}^{-1}$  at 0.1 M salt, pH 7.5 and 20°C. In comparison, non specific interaction of TFIIA with tRNA<sup>Phe</sup> exhibits a  $K_a$  of less than  $1.0 \times 10^7 \text{ M}^{-1}$ . Specific complex formation is favoured both by enthalpy and entropy ( $\Delta G_c = -12.1 \text{ kcal/mole}$ ,  $\Delta H = -8.3 \text{ kcal/mole}$ , and a  $\Delta S = +13.1 \text{ cal mol}^{-1} \text{ deg}^{-1}$ ) and exhibits a broad pH optimum ranging from pH 6.0 to pH 8.0. Analysis of the salt dependence of  $K_a$  indicates that as many as five lysine-phosphate type ionic bonds may be formed leaving approximately 68% of the free energy of complex formation to be contributed by non-electrostatic interactions (Romaniuk, 1985).

Regions within the 5S RNA molecule involved in TFIIA interaction have been studied extensively using chemical (Pieler & Erdmann, 1983; Pieler et al., 1986; Andersen, 1987; Christiansen et al., 1987; Baudin et al., 1989; Darsillo & Huber, 1991) and nuclease (Romaniuk, 1985; Huber & Wool, 1986; Pieler et al., 1986; Christiansen et al., 1987) protection analyses. The primary site of TFIIA-induced protection of 5S RNA appears to include the helix V-loop E-helix IV-loop D arm of the molecule. This region from position 64 to 116 is protected by TFIIA from ribonuclease cleavage (Huber & Wool, 1986). In particular, protection analyses and missing nucleoside studies suggest that loop E provides critical conformation and/or sequences necessary for TFIIA

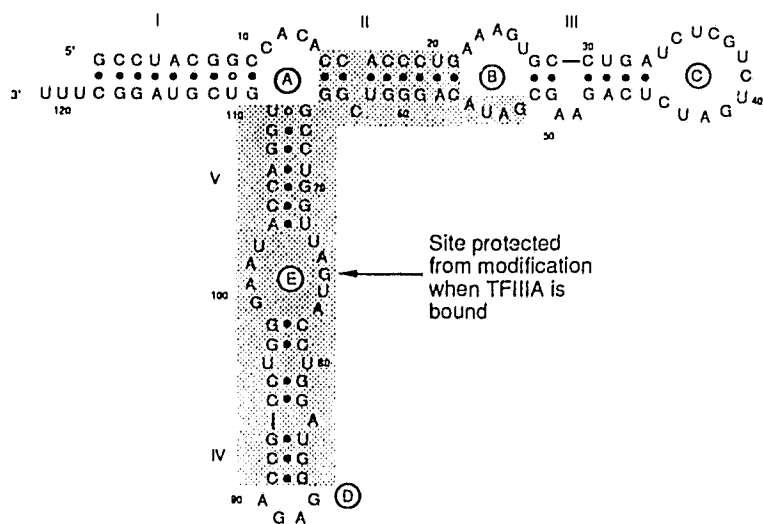


Figure 3.4 Secondary structure of *Xenopus* 5S RNA indicating the TFIIA footprint area (Romaniuk et al., 1989).

interaction (Pieler & Erdmann, 1983; Christiansen et al., 1987; Darsillo & Huber, 1991). A 5S RNA enzymatic fragment containing sequence from position 73 to 102 retains native 5S RNA structure and is sufficient to interact with TFIIA and inhibit transcription *in vitro* (Pieler et al., 1986). However, additional sites of protection are also observed along the other arm of 5S RNA (helix II-loop B-helix III-loop C) (Figure 3.4). TFIIA binding studies with truncation and internal deletion 5S RNA mutations define a minimum binding site that includes helices II, III, and V and loops A, B, and E (Sands & Bogenhagen, 1987; Romaniuk et al., 1987a; Bogenhagen & Sands, 1992). Removal of sequences within loop C and helix IV or substitution of base sequence in loop D did not significantly affect TFIIA binding. Mutations within helices II and III result in a more severe reduction in TFIIA association than mutation of helices IV and V (Sands & Bogenhagen, 1987; Romaniuk et al., 1987a). Thus, TFIIA interacts with both arms of the 5S RNA molecule, suggesting that the spatial arrangement of these helical structures may contribute to complex formation or stability.

The orientation of the helical arms of 5S RNA is established by the central hinge region (loop A) (Westhof et al., 1989). Indeed, the central hinge region (loop A) and surrounding structures (loop B and helices II and V) have been found by chemical crosslinking to be in close proximity to bound TFIIA (Baudin et al., 1989). The protein approaches within 7Å of loop A-helix II sequence from position 9 to 21 and helix II-helix V-loop B sequence from position 54 to 71 (Baudin et al., 1989). The conformation and, thus, the sequence of this central region would be expected to be critical for high affinity TFIIA binding. This is supported by the reduced association of TFIIA with 5S RNA mutants containing substitutions of residues within loop A (Romaniuk, 1989). The absence of direct contacts to the central hinge region

(loop A) is suggested by the lack of TFIIA-induced protection (Pieler & Erdmann, 1983; Huber & Wool, 1986; Christiansen et al., 1987; Romaniuk et al., 1987a).

5S RNA structures required for TFIIA interaction have been studied using deletion and substitution mutations within the RNA molecule (Sands & Bogenhagen, 1987; Romaniuk et al., 1987a; Baudin & Romaniuk, 1989; Romaniuk, 1989; Romaniuk et al., 1989; You & Romaniuk, 1990; You et al., 1991; Bogenhagen & Sands, 1992). None of the bulged nucleotides within 5S RNA were found to contribute direct bonding contacts and/or local conformations required for TFIIA binding (Pieler & Erdmann, 1983; Baudin & Romaniuk, 1989; Romaniuk et al., 1989; You et al., 1991). Nucleotide sequences within the single-stranded loop regions of *Xenopus* 5S RNA, that are found to be highly conserved in eukaryotic 5S RNA, do not provide strong contacts to TFIIA (Romaniuk, 1989; Romaniuk et al., 1989; Bogenhagen & Sands, 1992). However, as discussed above, nucleotide sequences within loop A are essential for coaxial stacking of the RNA helical arms and, thus, indirectly contribute to TFIIA binding. In addition, sequence differences between oocyte- and somatic-type 5S RNAs at positions 53, 55, and 56 within loop B are directly responsible for an enhanced affinity of TFIIA for somatic 5S RNA (Romaniuk et al., 1987a; Romaniuk et al., 1989). Perhaps, these somatic substitutions provide additional RNA-protein contacts that increase complex stability. The nucleotide sequence and quasi-helical conformation of loop E is apparently not required for high affinity interaction with TFIIA (Romaniuk, 1989). However, a potential purine N<sup>7</sup> contact is maintained at position 75 in the substitution mutants analyzed. Thus, the importance of this loop E nucleotide to TFIIA binding remains unknown. The most significant structural feature of 5S RNA required for TFIIA

interaction appears to be maintenance of helices II and V within the two arms of 5S RNA (Sands & Bogenhagen, 1987; Romaniuk et al., 1989; You & Romaniuk, 1990; You et al., 1991). The native conformations within loops B and C are not critical for their interaction with TFIIA (You & Romaniuk, 1990). The importance of 5S RNA conformation for TFIIA interaction is supported by binding studies with 5S RNAs isolated from different organisms (Hanas et al., 1984; Pieler et al., 1984b; Romaniuk, 1985; Andersen & Delihias, 1986). TFIIA can interact with eukaryotic, archaebacterial, and eubacterial 5S RNAs with varying affinities. Thus, the interaction of TFIIA with 5S RNA may occur through the recognition of the native RNA conformation and by forming a number of relatively weak contacts with extra-helical nucleotides and with the phosphoribose backbone.

It has been suggested that the interaction of TFIIA with 5S RNA and the 5S RNA gene may be similar by virtue of the protein binding region of the RNA stacking into a DNA-like conformation similar to the internal promoter of the gene (Huber & Wool, 1986; Christiansen et al., 1987) or by the 5S RNA gene adopting an RNA-like cruciform structure similar to 5S RNA helices IV and V (Andersen, 1987). In light of comparisons made of TFIIA interactions with 5S RNA and 5S RNA gene mutants, a similar mode of interaction between the protein and both nucleic acids seems unlikely (Baudin & Romaniuk, 1989; You et al., 1991; Veldhoen et al., 1994). TFIIA binding to the 5S RNA gene requires sequence-specific interactions within the internal promoter, with DNA conformation contributing less to the free energy of binding. In contrast, sequence identity of the helices within 5S RNA is apparently not as important as maintenance of native tertiary conformation for high affinity TFIIA interaction.

The contribution to RNA binding and the positioning of individual zinc fingers within the 7S RNP particle has been investigated using proteolytic fragments (Sands & Bogenhagen, 1991), proteolytic footprinting (Bogenhagen, 1993), truncation mutants (Darby & Joho, 1992; Clemens et al., 1993), and internal deletion mutants (Theunissen et al., 1992) of TFIIIA. Progressive N- and C-terminal truncation mutants of TFIIIA and proteolytic footprinting define the minimum region required for high affinity 5S RNA binding as zinc fingers four through seven (Bogenhagen, 1993; Clemens et al., 1993). An additional mutagenesis study of TFIIIA suggests that high affinity RNA binding requires fingers five through nine (Darby & Joho, 1992). A model has been proposed in which fingers five and seven are placed along helices V and II, respectively, similar to their proposed locations within the internal promoter of the 5S RNA gene (Clemens et al., 1993). This orientation is supported by nuclease protection studies using TFIIIA mutants lacking finger triplets (Theunissen et al., 1992). Zinc fingers one through three associate with helix IV, finger four through six interact with helices I, II, and V, and fingers seven through nine are oriented towards helix III. In contrast, RNA footprinting of TFIIIA fragments containing zinc fingers one through seven suggest that the C-terminal fingers eight and nine interact with the distal end of helix IV and that the boundary of finger seven interaction with 5S RNA resides near position 83 (Sands & Bogenhagen, 1991). TFIIIA may form structurally different complexes with somatic and oocyte forms of 5S RNA, as suggested by an increased accessibility of the linker sequence between fingers three and four to protease attack in the somatic 5S RNA complex (Bogenhagen, 1993).

Single amino acid substitutions in fingers four and six lead to a four-fold and 30-fold reduction in affinity for 5S RNA, respectively (Clemens et al.,

1993). This suggests non-equivalence in the contribution each zinc finger makes to the free energy of 5S RNA binding. However, an indirect immunoprecipitation assay, which also identified the RNA binding region of TFIIA as extending from fingers four through seven, indicates that each finger is functionally equivalent (Theunissen et al., 1992). It was noted, however, that removal of finger six resulted in a greater  $Mg^{2+}$ -dependent sensitivity for TFIIA interaction with 5S RNA.

In a collaborative study with the laboratory of Dr. Joel Gottesfeld, the interaction of TFIIA zinc fingers four through seven (zf4-7) with 5S RNA was investigated in an attempt to further identify finger positioning along the RNA molecule and specific RNA structures required for protein interaction. Dr. Gottesfeld and coworkers measured the affinity of zf4-7 for a series of 5S RNA mutants constructed in the laboratory of Dr. Romaniuk (refer to Romaniuk et al., 1987a; Romaniuk, 1989; You et al., 1991). I compared the interaction sites of TFIIA and zf4-7 on 5S RNA by ribonuclease footprinting.

## 3.2 Methods and Materials

### 3.2.1 Bacterial strains and DNA vectors

The 5S RNA gene-containing plasmid pXlo was constructed using a modification of the 'micro-scale ligation' method developed by Chambon et al. (1985) and described elsewhere (Romaniuk et al., 1987a). Plasmid pRK172 contains a PCR-generated truncation mutant of the TFIIA cDNA and expresses the zf4-7 polypeptide. This TFIIA truncation mutant was constructed in the laboratories of Dr. Joel Gottesfeld and Dr. Peter Wright at the Scripps Clinic and Research Institute and is described by Clemens et al. (1992; 1993). The identity of all PCR generated DNA fragments were confirmed by sequencing. Plasmids were maintained in *E. coli* JM105.

Expression of *zf4-7* from pRK172 was carried out using the *E. coli* B strain BL21(DE3) pLysS (F<sup>+</sup>ompTr<sup>-</sup>BM<sup>-</sup>B).

### 3.2.2 Transcription of *Xenopus* oocyte-type 5S RNA

*In vitro* run-off transcription of 5S RNA used in the RNA footprinting assay was performed as described by Romaniuk et al. (1987a). The pXlo plasmid was digested with *Dra* I, which cleaves the 5S RNA gene at position +121, relative to the first nucleotide position incorporated into RNA. The restriction digest was carried out at 37°C in a 50 µl reaction containing 10 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTE, 100 µg/ml BSA, 50 µg pXlo, and 100 units of *Dra* I (New England Biolabs). Completion of pXlo *Dra* I digestion was confirmed by electrophoresis of an aliquot on a one percent agarose gel. The digestion reaction was extracted with 100 µl phenol:CHCl<sub>3</sub> and DNA recovered by ethanol precipitation. The DNA pellet was dried *in vacuo*, resuspended in water, and stored at -20°C.

RNA synthesis was performed in a 200 µl reaction containing 40 mM Tris-HCl pH 8.1 at 37°C, 30 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM spermidine, 100 µg/ml BSA, 8% PEG 8000, 0.01% Triton X-100, 500 units/ml RNAGuard (Pharmacia), 5 mM each ATP, CTP, UTP, and GTP, 5 µg to 10 µg linearized pXlo template DNA, and 3 µl T7 RNA polymerase. The T7 RNA polymerase was purified from *E. coli* strain BL21/pAR1219 kindly provided by Dr. F.W. Studier using a published procedure (Davanloo et al., 1984). The transcription reaction was incubated for four hours at 37°C followed by the addition of 0.1 volumes of 0.5 M EDTA. The reaction was extracted with 200 µl of phenol:CHCl<sub>3</sub> and precipitated with two volumes of 6 M ammonium acetate and three volumes of ethanol. The 5S RNA sample was resuspended in water and stored at -20°C. The RNA concentration was estimated by

spectrophotometric analysis and agarose gel electrophoresis. Due to the presence of DNA template and unincorporated trinucleotides, however, this concentration determination was merely a crude estimate used to establish the amount of input RNA required for a subsequent radiolabeling reaction. Yields of *Xenopus* 5S RNA were between 30  $\mu\text{g}$  and 50  $\mu\text{g}$ .

### 3.2.3 Labeling of 5S RNA

*Xenopus* oocyte-type 5S RNA was radiolabelled with  $^{32}\text{P}$  at the 3' end for ribonuclease footprinting studies using standard methods (England & Uhlenbeck, 1978). The 20  $\mu\text{l}$  labeling reaction contained 50 mM Tris-HCl pH 7.8, 10 mM  $\text{MgCl}_2$ , 10 mM 2-mercaptoethanol, 1 mM ATP, 60  $\mu\text{Ci}$  [5'- $^{32}\text{P}$ ]-pCp (Dupont NEN), 3  $\mu\text{g}$  to 5  $\mu\text{g}$  5S RNA, and 30 units of  $\text{T}_4$  RNA ligase (New England Biolabs). Incubation was carried out at 4°C for 16 hours. End labeled RNA was purified by electrophoresis on 8 % denaturing polyacrylamide gels (29:1, acrylamide:bis; 16 x 16 cm x 0.75 mm) followed by elution of excised bands overnight and ethanol precipitation (Sambrook et al., 1989). The efficiency of radiolabel incorporation was measured using a LKB 1214 Rackbeta Scintillation counter and toluene with 0.4% (w/v) PPO (Sigma) added as scintillant.

### 3.2.4 Protein purification

Purification of recombinant TFIIA was performed as described in chapter 2, section 2.2.2. Purification of *zf4-7* was as described by Clemens et al. (1992) and by Hayes and Clemens (1992). A 100 ml volume of LB media containing 50  $\mu\text{g}/\text{ml}$  ampicillin was inoculated with 1 ml of an overnight culture of *E. coli* BL21(DE3) pLysS containing the *zf4-7* expression plasmid pRK172. The culture was grown at 37°C with shaking at 300 rpm to an O.D.<sub>600</sub>

of 0.6. *zf4-7* protein synthesis was induced with the addition of  $\text{ZnSO}_4$  to 50  $\mu\text{M}$  and IPTG to 1 mM final concentration. Cells were harvested following a further four hour incubation by centrifugation at 3800 x g for ten minutes in a Beckman JA-14 rotor, washed once with water, and stored overnight at  $-20^\circ\text{C}$ . Sonication was performed with addition of 4 ml of *zf* buffer (50 mM Tris-HCl pH 7.5, 1.0% glycerol, 5 mM DTT, 100 mM NaCl, and 50  $\mu\text{M}$   $\text{ZnCl}_2$ ) to the thawed cells. Sonication was for three 30 second pulses using a Heat Systems-Ultrasonics W-385 sonicator at a microtip setting of four and with one minute cooling intervals. The sonicate was centrifuged in a Beckman JA-20 at 12,000 x g for 10 minutes and the pellet was resuspended in 3 ml of *zf* buffer containing 5 M urea. The resuspension was mixed by inversion for one hour at  $4^\circ\text{C}$  and centrifuged in a Beckman JA-20 at 12,000 x g for 20 minutes. The supernatant was applied to a 1 ml heparin-sepharose column (Pharmacia) preequilibrated with 10 volumes of *zf* buffer containing 1 M NaCl and 5 M urea followed by 10 volumes of *zf* buffer containing 5 M urea. *zf4-7* was eluted with a NaCl step gradient in *zf* buffer (400 mM, 600 mM, 800 mM, 1 M NaCl) containing 5M urea. *zf4-7* eluted maximally in the 600 mM NaCl fraction with a purity of approximately 99%, as determined by SDS PAGE analysis (Figure 3.5). Aliquots of the purified protein were stored in the elution buffer at  $-20^\circ\text{C}$ . The final concentration of *zf4-7* was 75  $\mu\text{M}$ , as determined by the Bradford assay with bovine serum albumin as a standard (Bradford, 1976).

### 3.2.5 5S RNA footprinting analysis

Labeled 5S RNA was heated to  $95^\circ\text{C}$  in a renaturation buffer containing 20 mM Tris-HCl pH 7.5 and 100 mM KCl and allowed to slowly cool to room temperature. Binding reactions were prepared in a buffer containing 20 mM

Hepes pH 7.8, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 10% glycerol, 25 μM ZnCl<sub>2</sub>, 5 mM DTT, 100 mg/ml BSA, and 10 units RNAGuard (Pharmacia). RNAGuard was left out of reactions probed with RNase A. Each 20 μl footprinting reaction contained 100 nM of TFIIA or zf4-7 and 100,000 cpm of radioactively end labeled Xlo 5S RNA. Control reactions were also prepared that contained only the end labeled 5S RNA. The binding reactions were incubated for 20 minutes at ambient temperature to allow for the formation of protein-RNA complexes. Ribonuclease A (Sigma), T<sub>1</sub> (Pharmacia), and T<sub>2</sub> (Pharmacia) were added to final concentrations of  $2.3 \times 10^{-5}$  units/ml, 10 units/ml, and 5 units/ml, respectively. The reactions were incubated for a further two minutes followed by addition of 2 μg of tRNA<sup>phe</sup> (Boehringer) to each reaction. The quenched footprinting reactions were loaded onto an 8% non denaturing polyacrylamide gel (29:1, acrylamide:bis; 16 x 16 cm x 0.75 mm) prepared in 88 mM Tris-borate buffer. Electrophoretic separation of protein-bound and free 5S RNA was performed at 4°C. The gel was pre electrophoresed at 9 V/cm for 2 hours prior to loading. Footprinting reactions were electrophoresed at 15 V/cm for 6 hours. The separated bands were then resolved by autoradiography of the wet gel at 4°C for two hours with intensifying screens. Free and protein-bound 5S RNA bands were isolated and eluted overnight at ambient temperature in 250 μl of elution buffer (Sambrook et al., 1989). The eluted RNA was ethanol precipitated with 10 μg of *E. coli* tRNA (Sigma) carrier and resuspended in 15 μl of a urea-dye buffer containing 10 M urea, 1.5 mM EDTA, 0.04% xylene cyanol, and 0.04% bromophenol blue.

Footprinting reactions probed with ribonuclease CV<sub>1</sub> (Pharmacia) were prepared in TMK buffer containing 20 mM Tris-HCl pH 7.5, 1 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM DTT, and 100 mg/ml BSA. Each 200 μl

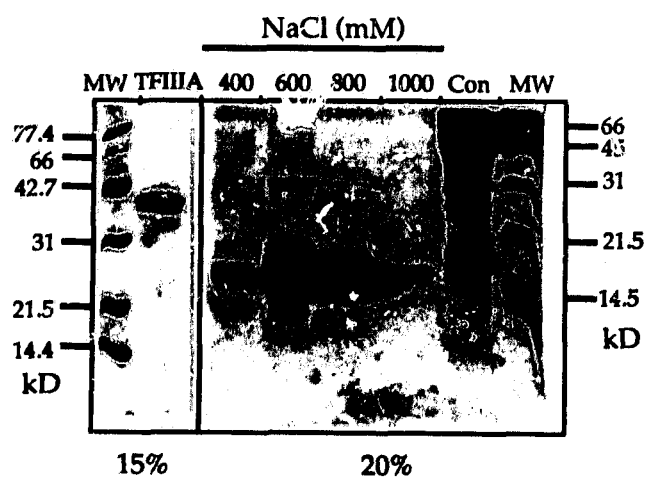


Figure 3.5 SDS-PAGE analysis of TFIIIA and zf4-7 proteins. TFIIIA was run on a 15% polyacrylamide gel and eluted fractions from the heparin-sepharose column containing zf4-7 were run on a 20% polyacrylamide gel. A control lysate from induced *E. coli* BL21(DE3) pLys S without the zf4-7 expression plasmid was also run.

binding reaction contained 100,000 cpm of labeled 5S RNA and 100 nM TFIIA or zf4-7 protein. The reactions were incubated for 20 minutes at ambient temperature and ribonuclease CV<sub>1</sub> was added to a final concentration of 0.06 units/ml and 0.012 units/ml. The binding reactions were incubated for a further two minutes and phenol:CHCl<sub>3</sub> extracted twice. Labeled 5S RNA was recovered by ethanol precipitation with 25 µg of *E. coli* tRNA (Sigma) as carrier and resuspended in 15 µl of urea-dye buffer.

The processed footprinting reactions were counted and normalized in preparation for loading onto 8% denaturing polyacrylamide sequencing gels. Ribonuclease T<sub>1</sub> (Pharmacia) and U<sub>2</sub> (Pharmacia) RNA sequencing reactions and a base ladder reaction were performed on 5S RNA. Labeled 5S RNA was dried *in vacuo* and resuspended in sequencing reaction buffers. The T<sub>1</sub> sequencing reaction contained 25 mM citrate pH 5, 0.8 µg *E. coli* tRNA, labeled 5S RNA, 5.6 M urea, 0.84 mM EDTA, 0.022% (v/v) xylene cyanol and bromophenol blue dyes, and 0.1 units of ribonuclease T<sub>1</sub>. The U<sub>2</sub> sequencing reaction contained 25 mM citrate pH 3.5, 0.8 µg *E. coli* tRNA, labeled 5S RNA, 5.6 M urea, 0.84 mM EDTA, 0.022% (v/v) xylene cyanol and bromophenol blue dyes, and one unit of ribonuclease U<sub>2</sub>. The base ladder reaction contained 50 mM NaHCO<sub>3</sub> pH 9.5, 1 µg *E. coli* tRNA, and labeled 5S RNA. Both ribonuclease sequencing reactions were incubated at 55°C for 15 minutes and the base ladder reaction was incubated at 95°C for three minutes. Footprinting and sequencing reactions were loaded onto sequencing gels pre-electrophoresed for 30 minutes and electrophoresed at 40 W until the bromophenol blue and xylene cyanol dyes were 9 cm from the bottom for the short and long runs, respectively.

### 3.3 Results

#### 3.3.1 Ribonuclease footprinting of TFIIIA and zf4-7 with *Xenopus* 5S RNA

In order to further understand the interaction of TFIIIA with 5S RNA, ribonuclease footprinting of TFIIIA:5S RNA and zf4-7:5S RNA complexes was performed. After the formation of stable 5S RNA-protein complexes using saturating amounts of protein, the complexes were probed with single strand specific ribonucleases A, T<sub>1</sub>, and T<sub>2</sub>, as well as with the structure specific ribonuclease CV<sub>1</sub>. The reactivity of these nucleases toward 5S RNA in the absence of protein was determined in parallel. The results of one such experiment are shown in Figure 3.6. Comparison of the reactivity patterns for the free 5S RNA and either of the protein-5S RNA complexes identifies three types of reactivity; nucleotide positions equally accessible to nuclease in free and protein-associated 5S RNA, nucleotide positions that become less accessible to nuclease when protein is bound to 5S RNA, and nucleotide positions that become more accessible when protein is bound to 5S RNA. Reactivities that fall into the last two classes are summarized in Figure 3.7 for both the TFIIIA:5S RNA and zf4-7:5S RNA complexes.

The region protected by zf4-7 is smaller than the region protected by full length TFIIIA. Both proteins protect loops A and E and helix V from nuclease attack and both proteins show enhanced reactivity in helix II. However, the two footprints differ significantly in the more distant regions of the helical arms of 5S RNA. TFIIIA protects loop B and helix III quite extensively from nuclease cleavage and to a lesser extent loop C. In contrast, the binding of zf4-7 with 5S RNA results in completely enhanced nuclease cleavage in helix III and loop C and reduced protection of loop B. A similar difference is observed in helix IV and loop D, where TFIIIA offers more protection from nuclease attack and zf4-7 binding promotes additional

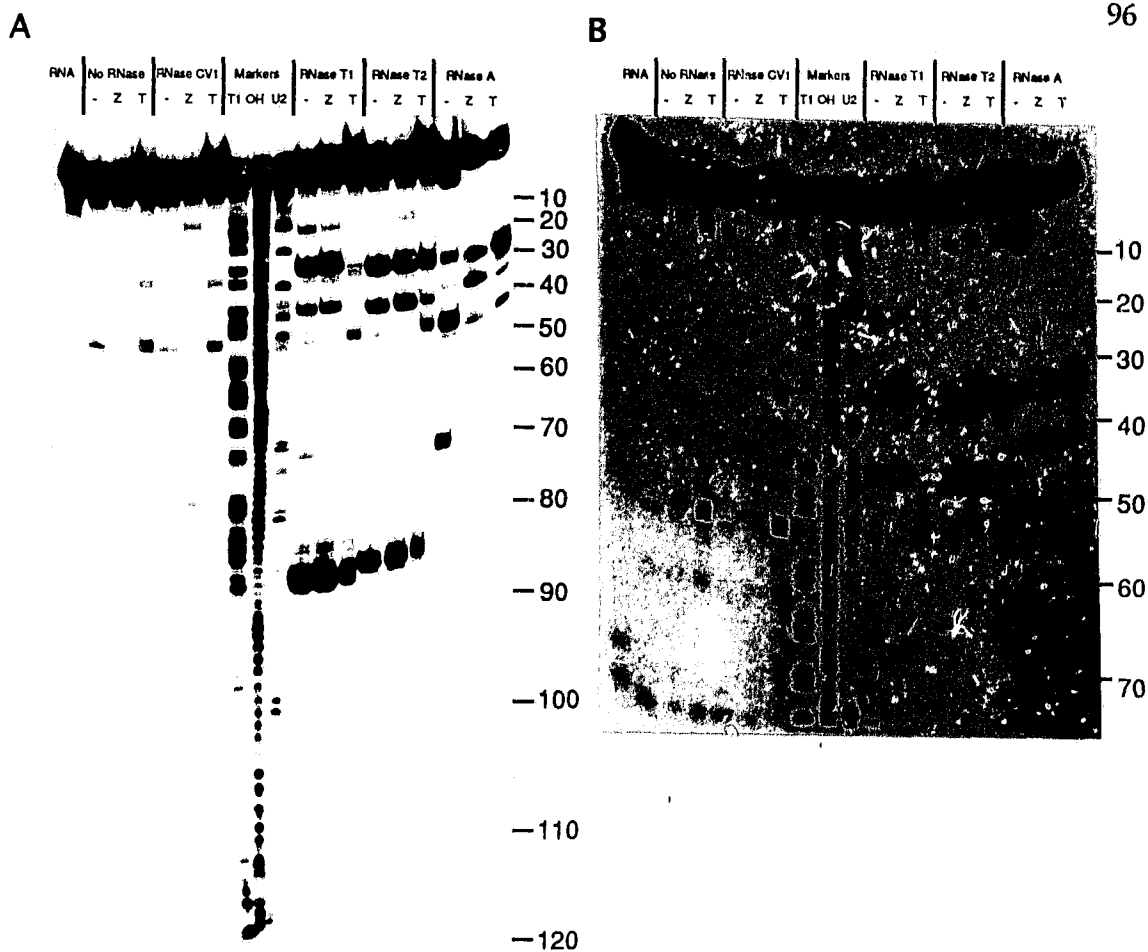


Figure 3.6 Ribonuclease footprinting of TFIIA:5S RNA and *zf4-7:5S* RNA complexes using (A) short and (B) long electrophoretic runs. RNase treatment is indicated above the autoradiogram; a '-' indicates the reactivity of free 5S RNA was assayed, a 'Z' indicates the reactivity of the *zf4-7:5S* RNA complex was assayed, and a 'T' indicates the TFIIA:5S RNA complex was analyzed. Markers are RNase T1 (guanine) and U2 (adenine) sequencing reactions and a base hydrolysis ladder. Nucleotide positions are indicated to the right of the autoradiograms. In this experiment, the RNase CV1 concentration was too low to provide reliable data. The CV1 footprinting summary shown in Figure 3.7 was derived from additional experiments conducted in TMK buffer at higher nuclease concentrations.

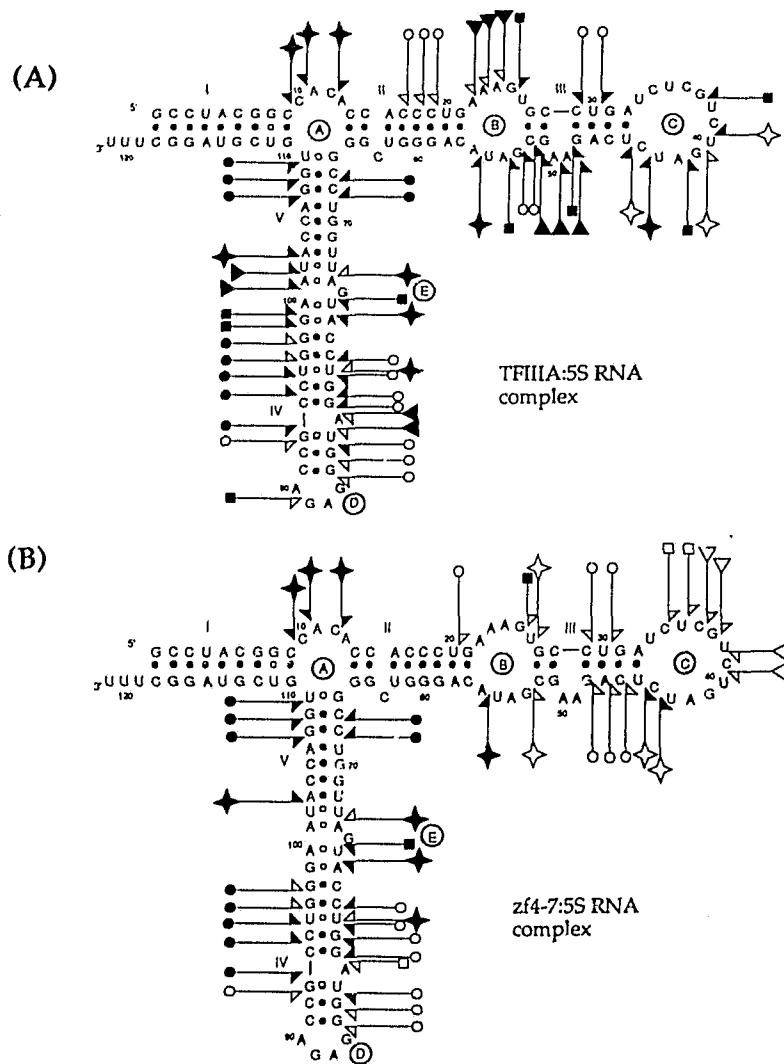


Figure 3.7 Summary of the footprinting results of (A) TFIIIA-5S RNA and (B) zf4-7-5S RNA complexes. Those nucleotide positions that displayed equal reactivity in the presence or absence of protein are not shown. Positions of ribonuclease cleavage are represented by a; circle (RNase CV<sub>1</sub>), square (RNase T<sub>1</sub>), triangle (RNase T<sub>2</sub>), and star (RNase A). Open symbols indicate those positions that had an enhanced reactivity in the complex, whereas solid symbols represent positions of reduced reactivity in the presence of protein relative to the reactivity of free 5S RNA. Open arrowheads represent positions of relatively weak nuclease reactivity, whereas positions of strong nuclease reactivity are denoted by solid arrowheads.

nuclease sensitivity. These data clearly indicate that zinc fingers four through seven of TFIIIA interact with the helix II-loop A and helix V-loop E regions of the 5S RNA, while the additional fingers of TFIIIA interact with helix IV-loop D and loop B-helix III-loop C of the RNA molecule.

### 3.4 Discussion

TFIIIA is rather a unique protein in that it binds to both the 5S DNA (Sakonju et al., 1981; Sakonju & Brown, 1982; Pieler et al., 1987; You et al., 1991; Veldhoen et al., 1994) and 5S RNA (Pieler & Erdmann, 1983; Romaniuk, 1985; Andersen & Delihias, 1986) molecules with high affinity. Although the nucleic acid binding domain of TFIIIA consists of nine zinc fingers, previous work has shown that fingers one through three provide the majority of the DNA binding affinity (Christensen et al., 1991; Liao et al., 1992), while fingers four through seven provide the majority of the RNA binding affinity (Clemens et al., 1993). It has been demonstrated that the zf4-7 polypeptide (consisting of fingers four through seven) exhibits a slightly higher affinity for 5S RNA (approximately 400 cal/mole) than full length TFIIIA (McBryant et al., 1995). Given the dual function of TFIIIA, perhaps it is not surprising that evolutionary selection resulted in a compromise protein that adequately fulfills both functions, or that a discrete portion of TFIIIA in fact exhibits a higher affinity for RNA than the complete protein.

In order to understand the structural requirements for the interaction of zf4-7 with 5S RNA, the affinity of zf4-7 for a series of sequence and structural mutants of 5S RNA was determined (McBryant et al., 1995) (Table 3.1). These same mutants had been assayed previously for their effects on the binding of TFIIIA (Romaniuk et al., 1987a; Baudin & Romaniuk, 1989; Romaniuk, 1989; You & Romaniuk, 1990; Baudin et al., 1991; You et al., 1991).

Comparison of the two proteins suggests that *zf4-7* interacts in the same manner with 5S RNA (but within a smaller region) as the full length TFIIIA protein (Figure 3.8). Mutations within 5S RNA that severely reduced *zf4-7* binding are located in loop A (positions 10 to 13), nucleotides that flank and possibly stabilize loop A (positions 14 to 15 and 64 to 65), helix II (positions 16 to 21 and 57 to 67), loop B (positions 22 to 26), helix V (positions 67 to 72 and 105 to 108), loop E (positions 73 to 76 and 99 to 101), and helix IV (positions 78 to 81, 83, and 96 to 101) (McBryant et al., 1995) (Table 3.1, Figure 3.8). Additional mutations within loop C (positions 41 to 44) are found to negatively affect TFIIIA binding.

There are a number of mutations of 5S RNA with slightly increased affinity for *zf4-7*, suggesting that the small, local changes in secondary or tertiary 5S RNA structure resulting from these mutations (Romaniuk et al., 1988; Brunel et al., 1990; Baudin et al., 1991; de Stevenson et al., 1991) can enhance the RNA-protein interaction. The gain in total free energy of complex formation is far less than might be expected from the gain of an additional hydrogen bond interaction and is consistent with a small conformational change in the RNA that energetically favors *zf4-7* interaction. It is interesting that many of these favorable mutations appear to be clustered at the presumed boundaries of the *zf4-7* interaction site on 5S RNA (Table 3.1, figure 3.8). This suggests that interactions of the RNA with finger four and/or finger seven of *zf4-7* are stabilized by local changes in RNA conformation.

The RNA footprinting and RNA mutagenesis results provide a fairly clear picture of the boundaries of the *zf4-7* interaction site within 5S RNA. These occur within helix IV on one arm and within loop B on the other arm

Table 3.1 Relative affinities for *zf4-7* and TFIIA binding to wild type and mutant *Xenopus* 5S RNAs.

5S RNA	Relative Affinity for <i>zf4-7</i>	Relative Affinity for TFIIA <sup>a</sup>	5S RNA	Relative Affinity for <i>zf4-7</i>	Relative Affinity for TFIIA <sup>a</sup>
wild type	1.00	1.00	67-70	0.75 ± 0.12	0.045 ± 0.008
10-13	0.08 ± 0.01	0.30 ± 0.01	67-70/105-108	1.25 ± 0.30	0.71 ± 0.01
14-15	0.04 ± 0.01	0.85 ± 0.22	71-72	0.13 ± 0.03	0.35 ± 0.21
14-15/64-65	0.12 ± 0.03	1.11 ± 0.32	71-72/103-104	2.64 ± 0.55	1.18 ± 0.32
16-21	0.17 ± 0.03	0.32 ± 0.15	73-76	0.05 ± 0.009	0.57 ± 0.02
16-21/57-62	1.09 ± 0.13	1.09 ± 0.48	ΔG75	0.04 ± 0.01	ND
22-26	0.017 ± 0.004	1.00 ± 0.02	78-81	0.068 ± 0.009	0.88 ± 0.01
27-32	1.18 ± 0.29	0.75 ± 0.02	78-81/95-98	2.14 ± 0.46	0.86 ± 0.01
33-39	1.45 ± 0.30	1.00 ± 0.02	82-86	5.00 ± 1.0	0.81 ± 0.31
41-44	1.65 ± 0.29	0.40 ± 0.20	82-86/91-94	1.29 ± 0.22	1.21 ± 0.35
45-52	1.42 ± 0.29	0.76 ± 0.12	ΔA83	0.44 ± 0.11	1.00 ± 0.02
ΔA49-ΔA50	0.78 ± 0.13	1.00 ± 0.02	87-90	2.03 ± 0.30	0.71 ± 0.10
53-56	0.75 ± 0.17	1.62 ± 0.12	96-101	0.048 ± 0.009	0.59 ± 0.01
57-62	0.017 ± 0.005	0.40 ± 0.15	99-101	0.07 ± 0.014	0.61 ± 0.08
ΔC63	0.88 ± 0.10	1.00 ± 0.02	103-104	4.19 ± 0.74	0.50 ± 0.23
64-65	0.022 ± 0.005	0.74 ± 0.24	105-108	0.12 ± 0.02	0.39 ± 0.06
G66C	0.032 ± 0.008	0.12 ± 0.01	U109C	0.045 ± 0.008	0.50 ± 0.03

<sup>a</sup>Data from You et al. (1991) and Romaniuk (1989) determined by nitrocellulose filter binding assays. Results obtained from the laboratories of Dr. Joel Gottesfeld and Dr. Peter Wright at Scripps Clinic and Research Institute.

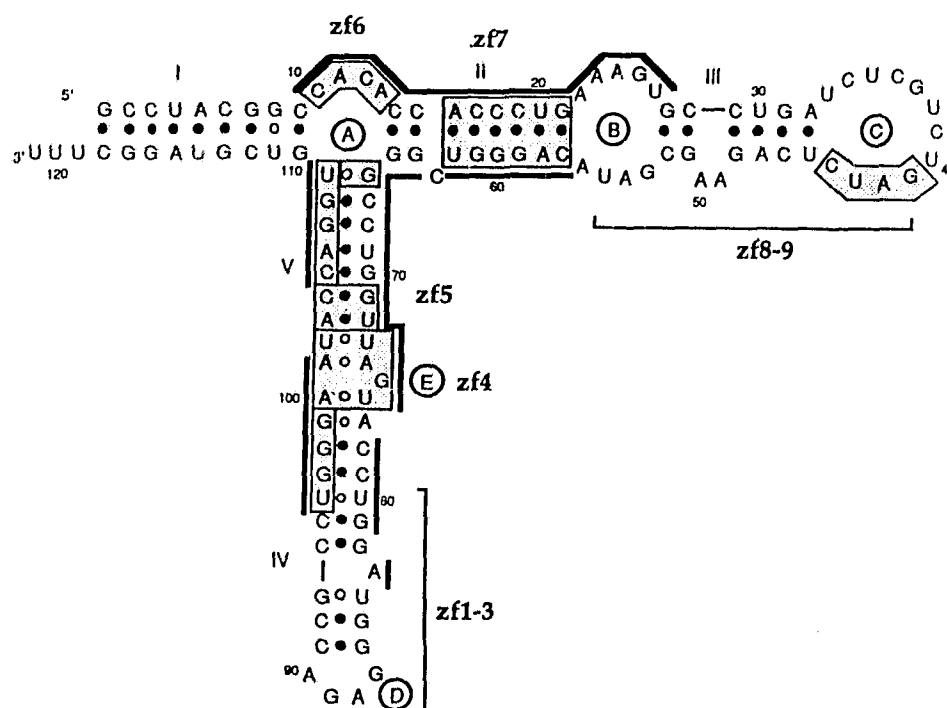


Figure 3.8 Summary of the effects that mutations in 5S RNA have on the binding of TFIIA and zf4-7. Nucleotide regions that when mutated result in a two-fold or greater reduction in TFIIA binding are boxed, while those critical for zf4-7 binding are denoted by a thick line. The proposed location of the nine zinc fingers of TFIIA are indicated to the side of the 5S RNA structure (McBryant et al., 1995).

of 5S RNA. Consistent with this identification of the *zf4-7* boundary, mutation of nucleotides 41-44 within loop C selectively decreased TFIIA binding affinity but did not affect *zf4-7* interaction (McBryant et al., 1995) (Table 3.1, Figure 3.8). This would be expected if the C-terminal domain and fingers of TFIIA are situated within this region of the 5S RNA molecule. The patterns of nuclease reactivity for the two proteins is very similar in the helix II-loopA-helix V-loop E portion of 5S RNA (Figure 3.7). However, for the *zf4-7* footprint, it is apparent that there is a loss of nuclease protection and a corresponding enhanced sensitivity in the helix III-loop C domain, relative to the TFIIA footprint. Differences between the two footprints in the lower region of helix IV and loop E are less striking. The *zf4-7* footprint lacks the nuclease protection observed at residues 82, 83, and 89 in the TFIIA footprint. Thus, zinc fingers four through seven of TFIIA interact with helix II, loop A, helix V, and loop E of 5S RNA.

Within this binding site for *zf4-7*, it appears that some structures and nucleotide residues are critical for high affinity binding of the polypeptide. Within helix II, the base pairs formed by residues 16 to 21 and 57 to 62 are a critical structural feature, although the sequence of the base pairs is not critical (McBryant et al., 1995). In contrast, both the pairing of nucleotides 14 to 15 with 64 to 65 and the identity of these nucleotides is critical for *zf4-7* binding (Table 3.1). The bulged cytosine residue at position 63 is not required for the binding of *zf4-7*. Loop A is a critical feature for the binding of TFIIA and *zf4-7*, and it has been proposed that nucleotide sequence within loop A may function to orient the two arms of the RNA binding site optimally for the interaction with TFIIA (Romaniuk, 1989; Baudin et al., 1991). Within helix V, the base paired structure is critical for *zf4-7* interaction but the wild type sequence is not required (McBryant et al., 1995).

Loop E plays a pivotal role in *zf4-7* binding. This region of 5S RNA is unable to form regular Watson-Crick base pairs, but the results of chemical and nuclease probing experiments clearly demonstrate that it is highly structured (Romaniuk et al., 1988). NMR analysis further supports a model in which loop E adopts a quasi-helical conformation that incorporates non-canonical hydrogen bonding arrangements between opposing pairs of nucleotides and a bulged guanine at position 75 (Wimberly et al., 1993) (Figure 3.3). Mutations within this region result in large reductions in *zf4-7* binding affinity, indicating that the unique structure of loop E, and particular nucleotides within this region, are critical interaction points for the protein (Table 3.1) (McBryant et al., 1995). The disruptive effect of deleting the bulged guanine residue suggests that this may represent an important structural feature for recognition by *zf4-7*. The availability of the N<sup>7</sup> group of this nucleotide for protein contact has been shown using chemical probes (Romaniuk et al., 1988). In addition, bulged residues have been implicated as the nucleation point for protein binding in other RNA-protein interactions (Peattie et al., 1981; Wu & Uhlenbeck, 1987).

The comparative nature of mutagenesis and footprinting analyses provides a detailed picture of the interaction site of zinc fingers four through seven of TFIIA with 5S RNA. An earlier model proposed by Clemens et al. (1993) suggests that finger four interacts with loop E of the 5S RNA, finger five with helix V, finger six with the loop A "hinge" region, and finger seven with helix II. This placement of fingers four through seven is consistent with previous 5S RNA footprinting results obtained with deletion mutants of TFIIA (Theunissen et al., 1992). It must be stressed, however, that the results presented here provide no direct information on the binding sites of

individual zinc fingers on 5S RNA, but do provide a clear indication of the location of fingers four through seven on the RNA molecule.

Truncated RNA molecules were assayed by the laboratories of Dr. Joel Gottesfeld and Dr. Peter Wright at Scripps Clinic and Research Institute in order to determine the minimal protein-RNA complex that retains a binding affinity comparable to full length TFIIIA with 5S RNA (McBryant et al., 1995) (Figure 3.9). Bogenhagen and Sands (1992) have shown that TFIIIA binds with high affinity to RNA molecules lacking up to twelve nucleotides at either loop C or the loop E-helix IV area of 5S RNA. Thus, nucleotides beyond loop E in the 63-mer RNA (containing helix II, loop A, helix V, and loop E) should be adequate to preserve high affinity TFIIIA binding in this region. However, such an RNA molecule retains only five percent of the binding affinity of wild type 5S RNA (McBryant et al., 1995).

In comparison with the mutant 5S RNA binding results, it is apparent that part of helix IV and loop B are essential for zf4-7 binding. A 75-mer RNA that incorporates these two features interacts with zf4-7 with close to wild type binding affinity. These findings suggest that the complex of zf4-7 with the 75-mer represents most, if not all, of the energetically important contacts in the TFIIIA-5S RNA complex.

Thus, the specific site on 5S RNA that interacts with zinc fingers four through seven of TFIIIA has been identified. Within this site exist critical structures and nucleotides, which may form direct contacts with amino acid residues within the zinc fingers of TFIIIA. Combining this information with that of previous studies, a model is derived that juxtaposes specific RNA binding fingers of TFIIIA with specific structures in 5S RNA. This model may help to direct further investigations into the precise nature of specific contacts formed between 5S RNA and TFIIIA.

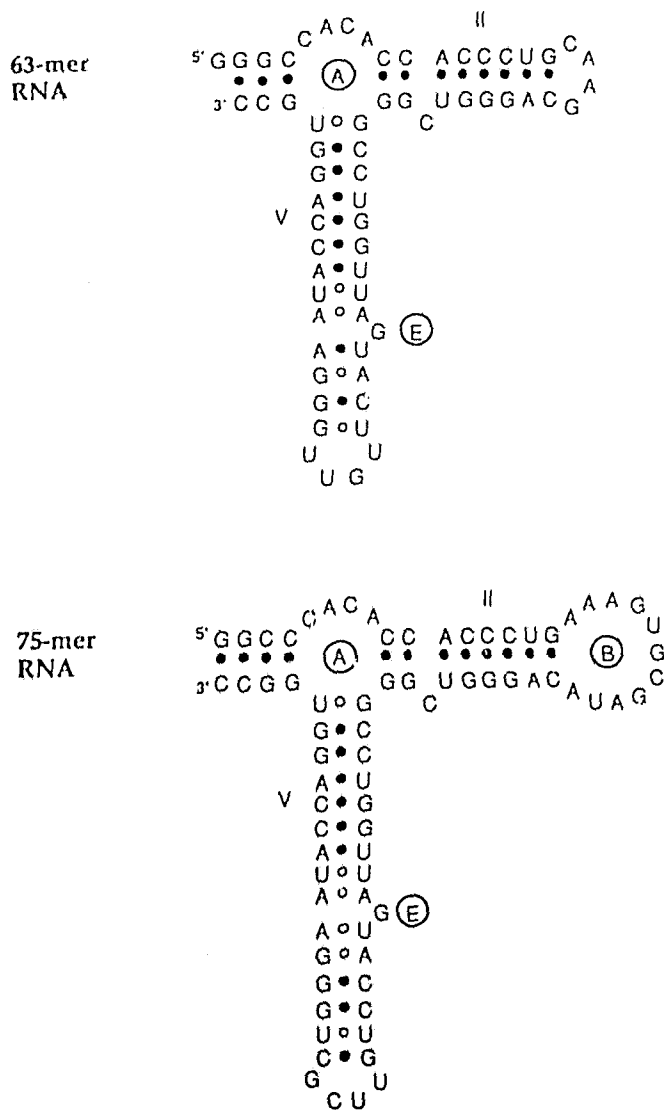


Figure 3.9 Proposed secondary structure of the truncated 5S RNA molecules analyzed for zf4-7 binding affinity (McBryant et al., 1995).

## Chapter 4.0 Characterization of the Nucleic Acid Binding Domain of TFIIIA

### 4.1 Introduction

#### 4.1.1 The C<sub>2</sub>H<sub>2</sub> zinc finger domain

The DNA binding zinc finger appears to be the most commonly used motif for protein-nucleic acid interaction and exists as tandem repeats ranging from two (ADR1) to 37 (Xfin) fingers in length within a single protein. Transcription regulatory proteins containing zinc fingers are found throughout the eukaryotic kingdom (Pellegrino & Berg, 1991) and play a role in such diverse functions as: sex determination [ZFY (human), SWI5 (yeast)], developmental gene regulation [*mKr2*, *Krox-20*, and *Zif268* (mouse), *Krüppel* and *Tramtrack* (*Drosophila*), *Xfin* and *TFIIIA* (*Xenopus*)], differential gene expression [MBP-1 (human), ADR1 (yeast)], and general gene expression [*Sp1* (human)]. The functioning of zinc finger proteins as oncoproteins (GLI in glioblastomas; Kinzler et al., 1988) or tumor suppressors (WT1 in Wilms' tumor; Call et al., 1990) in certain human cancers identifies these regulatory proteins as important targets of study.

The C<sub>2</sub>H<sub>2</sub> zinc finger motif was originally identified as a sequence repeat in the *Xenopus* transcription factor, TFIIIA (Brown et al., 1985; Miller et al., 1985). This 30 amino acid sequence contains two pairs of invariant cysteine and histidine residues that associate with a single zinc cation and three conserved hydrophobic framework residues (Miller et al., 1985; Diakun et al., 1986; Gibson et al., 1988) (Figure 4.1A). More recently, two groups of C<sub>2</sub>H<sub>2</sub> class finger sequences have been identified that differ primarily in the positioning of hydrophobic framework residues and in the number of residues between the zinc-binding histidines (Kochoyan et al., 1991a; Kochoyan et al., 1991b; Kochoyan et al., 1991c) (Figure 4.1B). These finger

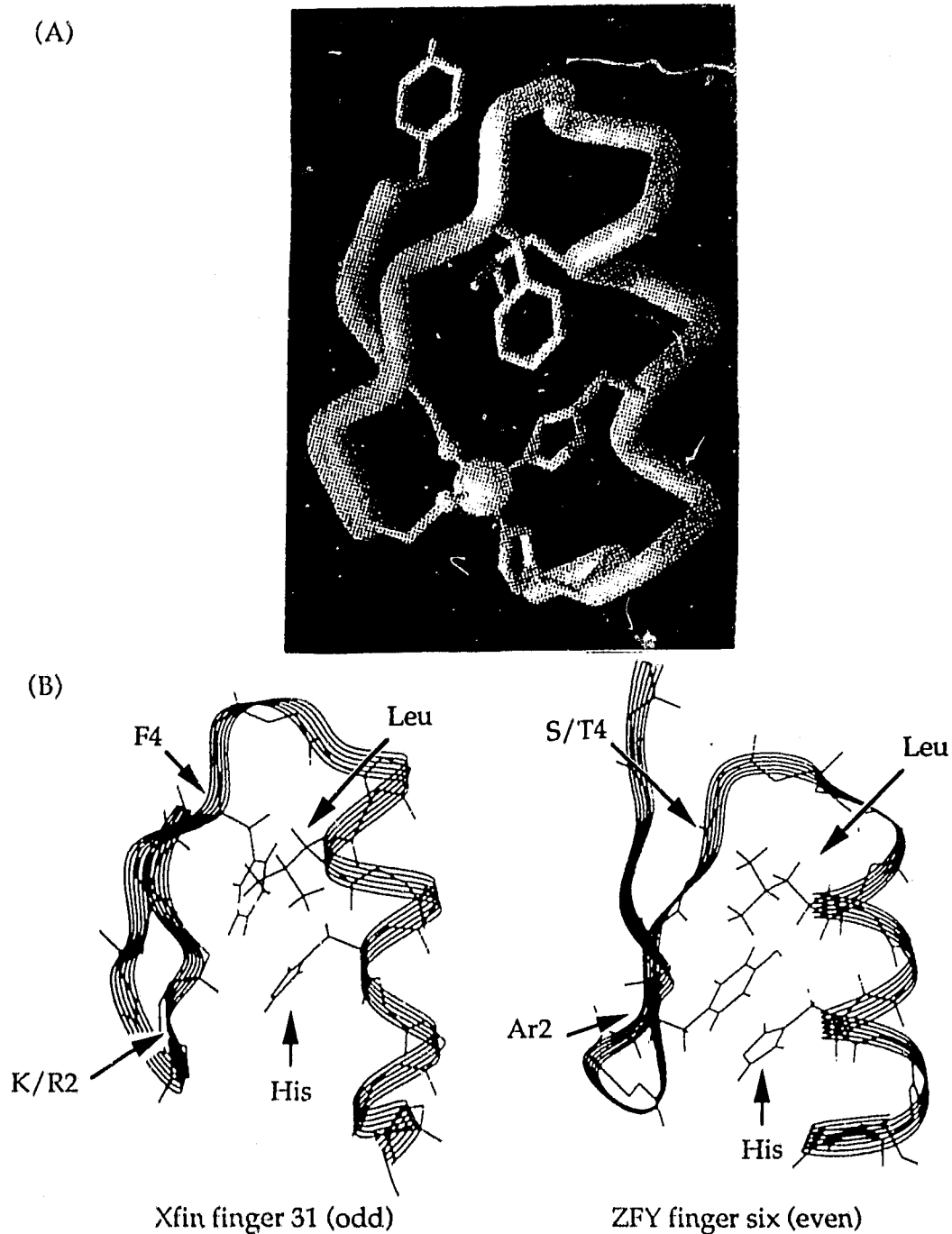


Figure 4.1 Tertiary structure of the  $C_2H_2$  zinc finger. (A) Xfin finger 31 (Lee et al., 1989a), (B) Comparison of odd (Xfin finger 31) and even (ZFY finger six) zinc finger conformations (Kochoyan et al., 1991b).

domains are designated 'odd' and 'even' due to their alternating positions in the 13-finger ZFY protein (Kochoyan et al., 1991d). The consensus C<sub>2</sub>H<sub>2</sub> zinc finger sequence represents the odd group and can be found in multiple repeats in proteins such as Xfin, Zif268, ZFY (odd fingers), TFIIIA, and Sp1 (Kochoyan et al., 1991a; Kochoyan et al., 1991b; Kochoyan et al., 1991c). In contrast, even fingers have not been found in multiple repeats and are generally interspersed among odd fingers in proteins like TFIIIA, ZFY, and MBP-1 (Kochoyan et al., 1991a; Kochoyan et al., 1991b; Kochoyan et al., 1991c) (Figure 4.2). The classification of C<sub>2</sub>H<sub>2</sub> zinc fingers may become more complex as individual characteristics within the odd and even finger groups are identified.

In general, metal ions within proteins are surrounded by hydrophilic atomic groups enclosed within a larger shell of hydrophobic atomic groups (Yamashita et al., 1990). The C<sub>2</sub>H<sub>2</sub> class of zinc fingers binds zinc(II) by tetrahedral coordination to conserved histidine N $\epsilon$ <sup>2</sup> atoms at 2 Å distance and to cysteine S $\gamma$  atoms at 2.3 Å distance (Diakun et al., 1986). The dissociation constant (K<sub>d</sub>) for zinc binding varies from 10<sup>-9</sup> M to 10<sup>-12</sup> M, depending on individual finger sequence. A consensus finger peptide has been observed to bind zinc by three amino acid ligands with the second histidine dissociated from the metal center (Krizek et al., 1991). It was suggested that a water molecule occupies the fourth coordination position. This second histidine could also be replaced with a cysteine residue without loss of zinc finger tertiary structure (Krizek et al., 1991). The resulting finger structure is similar to a number of CCHC zinc binding structures observed in retroviral DNA-binding proteins (Green & Berg, 1989). Cobalt(II) can also tetrahedrally associate with the metal binding ligands of the finger sequence (Frankel et al., 1987; Krizek et al., 1991). However, this association may result

Finger	Finger Number	Sequence
overall consensus	-	r-C---•CGK-E---S-L--H-RT••HTGEKP
odd consensus	-	r-C---•C-K-E----EL--H-----H-A/G
ADR1	1	FVCEV••CTRAFARQEH <u>L</u> KR <u>H</u> YRS••HTNEKP
ADR1	2	YPCGL••CNRCE <u>T</u> RRD <u>L</u> LIR <u>H</u> AQKI•HSGNL
Xfin	31	YKCGL••CERSE <u>V</u> EKS <u>A</u> LSR <u>H</u> QRV••HKNE <u>S</u> P
<i>mKr2</i>	7	YECTE••CGKA <u>F</u> SQSA <u>L</u> IE <u>H</u> RRRI••HTGEK
MBP-1	C-term	YHCSY••CN <u>F</u> SE <u>K</u> TKGN <u>L</u> TK <u>H</u> MKSKA <u>H</u> SKK
SW15	1	FECLFP <u>G</u> CTK <u>T</u> E <u>K</u> RRY <u>N</u> IR <u>S</u> <u>H</u> IQT••HLED <u>R</u> P
SW15	2	YSCDHP <u>G</u> CDKA <u>F</u> VRN <u>H</u> D <u>L</u> IR <u>H</u> KKS••HQEKA
SW15	3	YACP•••CGK <u>K</u> ENRED <u>A</u> <u>L</u> V <u>V</u> <u>H</u> RSR <u>M</u> IC <u>S</u> G
TFIIIA	1	YIC <u>S</u> FADCGA <u>A</u> Y <u>N</u> KN <u>N</u> W <u>K</u> <u>L</u> QA <u>H</u> LCK••HTGEKP
TFIIIA	2	FPCKEEG <u>C</u> E <u>K</u> GET <u>S</u> LS <u>H</u> HL <u>T</u> R <u>H</u> SLT••HTGE <u>K</u> N
TFIIIA	3	FTCDSD <u>G</u> CD <u>L</u> RE <u>T</u> TKAN <u>M</u> K <u>K</u> <u>H</u> FN <u>R</u> F•HN <u>I</u> K <u>I</u> CV
even consensus	-	rQC-Y/H•••C- <u>E</u> -S/T----EL--H-----H <u>Z</u> K
ZFY	4	HKCKF•••CE <u>Y</u> ETAEO <u>Q</u> LL <u>N</u> R <u>H</u> LLA <u>V</u> HSK <u>N</u> FP
ZFY	6	YQCQY•••CE <u>Y</u> RSADSS <u>N</u> L <u>K</u> T <u>H</u> IKTK <u>H</u> SK <u>E</u> MP
TFIIIA	6	YPC <u>K</u> KDD <u>S</u> C <u>S</u> E <u>V</u> GT <u>W</u> T <u>L</u> Y <u>L</u> K <u>H</u> VA <u>E</u> CH <u>Q</u> DLA
hb	4	YRCAD•••CD <u>Y</u> AT <u>K</u> Y <u>C</u> HS <u>F</u> KL <u>H</u> LR <u>K</u> Y <u>G</u> <u>H</u> K <u>P</u> GM <u>V</u>

Figure 4.2 Sequence alignment of odd and even C<sub>2</sub>H<sub>2</sub> zinc fingers. An 'r' indicates an aromatic residue position, an 'h' identifies a hydrophobic amino acid position, '-' denotes any amino acid, and '.' indicates alignment spacing.

in a net loss in energy as the cobalt ion is bound by water molecules in an octahedral coordination in solution (Regan & Clarke, 1990). Thus, the preference of the metal binding site for zinc over other metal ions may reflect the electronic environment within the finger and the coordination chemistry of different metal ions (Frankel et al., 1987; Krizek et al., 1991). It is interesting to note that an Sp1 protein derivative, in which zinc(II) was replaced with cadmium(II) in the three finger motifs, retained its specific DNA binding activity (Kuwahara & Coleman, 1990).

Metal binding is essential for the folding of a number of individual finger sequences (Frankel et al., 1987; Párraga et al., 1988; Lee et al., 1989b; Kuwahara & Coleman, 1990; Lee et al., 1991a; Michael et al., 1992; Eis & Lakowicz, 1993) and for DNA binding by the intact proteins TFIIIA, Sp1, and SWI5 (Hanas et al., 1983a; Nagai et al., 1988; Kuwahara & Coleman, 1990). The isolated finger seven of *mKr2* is exceptional as it is observed to adopt a compact structure in the absence of bound metal (Carr et al., 1990). Isolated fingers associate non-specifically with DNA but exhibit no specific sequence recognition activity (Frankel et al., 1987; Lee et al., 1991a). Once folded, the individual zinc finger is surprisingly resistant to thermal denaturation (Frankel et al., 1987) and protease digestion (Miller et al., 1985).

The tertiary structure of individual C<sub>2</sub>H<sub>2</sub> zinc fingers have been probed by NMR using protein sequences originating from the yeast transcription factors ADR1 (finger two; Párraga et al., 1988; Xu et al., 1991) and SWI5 (finger two; Nakaseko et al., 1992; Neuhaus et al., 1992), the *Xenopus* protein Xfin (finger 31; Lee et al., 1989a; Lee et al., 1989b; Palmer et al., 1991), the mouse *mKr2* protein (finger seven; Carr et al., 1990), and the human enhancer binding protein, MBP-1 (C-terminal finger; Omichinski et al., 1990). Additional information on finger structure has been obtained from X-ray

crystallography of the mouse immediate early protein, *Zif268* (Pavletich & Pabo, 1991), the *Drosophila* developmental protein, *Tramtrack* (Fairall et al., 1993), and the human oncoprotein, *GLI* (Pavletich & Pabo, 1993). These studies suggest that the general architecture is very similar for fingers isolated from a wide range of proteins. The zinc finger adopts a highly compact structure in which an N-terminal irregular two-stranded  $\beta$ -sheet is packed against a C-terminal three-turn  $\alpha$ -helix (Figure 4.1A). Once folded into its tertiary conformation, the backbone structure of the zinc finger is quite rigid, displaying little internal motion (Palmer et al., 1991). A third  $\beta$ -strand has been observed in the N-terminal fingers of peptides derived from *SWI5* (Neuhaus et al., 1992) and *Tramtrack* (Fairall et al., 1993) and plays a role in finger stability (Figure 4.3). Studies using a 26 amino acid minimalist finger peptide containing 16 alanine residues suggests that the tertiary fold is maintained almost entirely through zinc binding to the conserved cysteine and histidine ligands and by hydrophobic interactions between the framework residues (Michael et al., 1992). However, additional amino acid residues may contribute to zinc finger stability (Krizek et al., 1991). The structural differences that do occur are generally localized to the site of sequence alteration and are not global. Such differences in finger architecture have been observed within the irregular  $\beta$ -sheet, the C-terminus of the  $\alpha$ -helix, the hydrophobic core, and the interfinger linker region (Kochoyan et al., 1991a; Kochoyan et al., 1991b; Kochoyan et al., 1991c; Kochoyan et al., 1991d; Bernstein et al., 1994). These differences may play an important role in DNA binding.

The packing of the central hydrophobic core is essential for correct zinc ion coordination and creation of a functional DNA-binding surface. Comparison of odd and even zinc fingers suggests that this packing can be

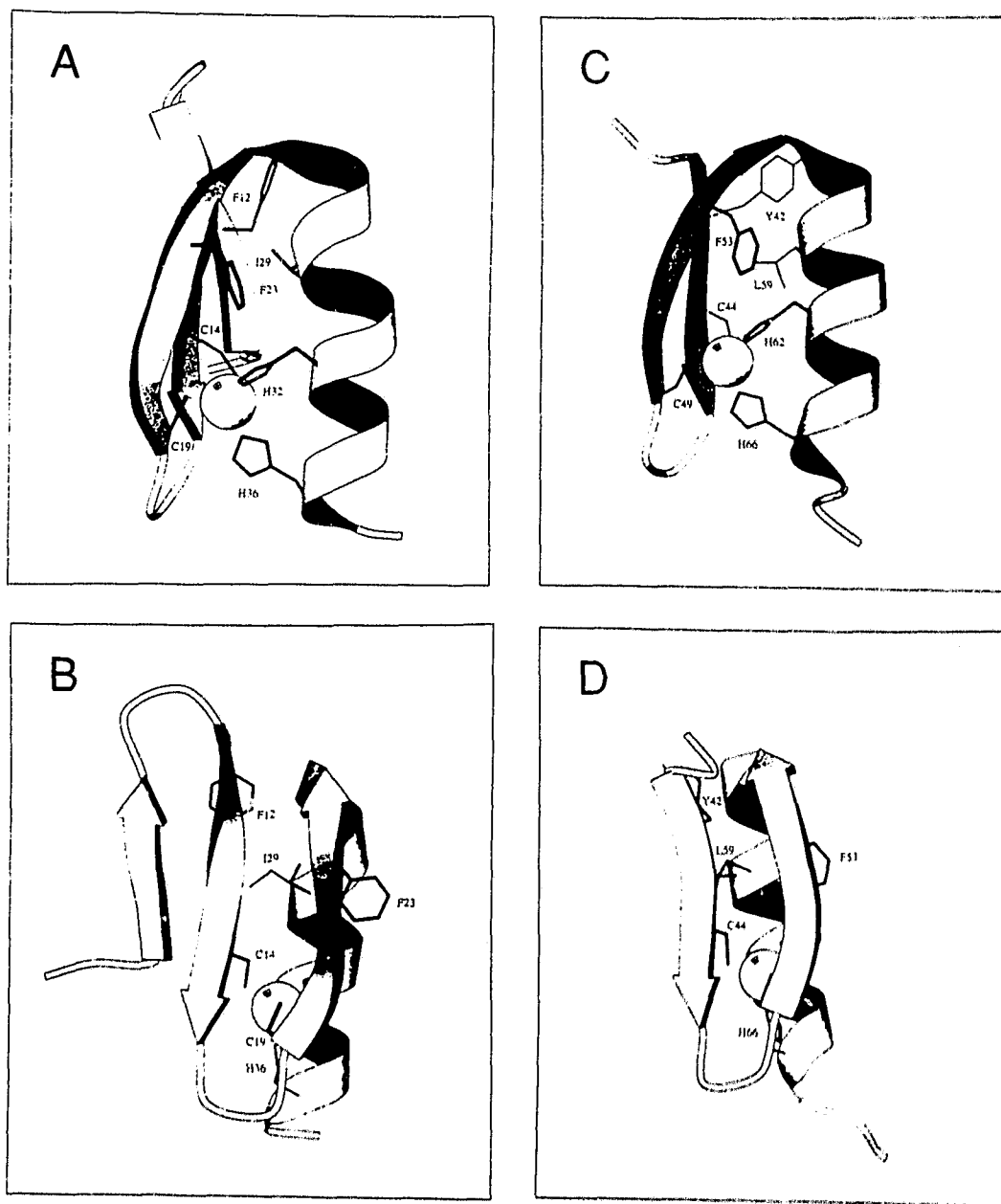


Figure 4.3 Tertiary structure of SWI5 zinc fingers one and two. Panels (A) and (B) depict finger one with a 90° rotation, while panels (C) and (D) show finger two in similar orientations (Neuhaus et al., 1992).

obtained using hydrophobic 'framework' residues at different positions within the second strand of the  $\beta$ -sheet and conserved leucine and histidine residues in the  $\alpha$ -helix (Kochoyan et al., 1991a; Kochoyan et al., 1991b). Odd zinc fingers maintain a phenylalanine four positions from the second coordinating cysteine (F4) and a basic residue at the second position (K/R2), while even fingers have an aromatic residue (H, F, or Y) two positions from the second cysteine (Ar2) and a serine or threonine at the fourth position (S/T4) (Figure 4.2). The resulting central finger cores are similar in stability but exhibit different orientations of these alternate hydrophobic residues (Kochoyan et al., 1991b) (Figure 4.1B). Other less conserved residues throughout the finger sequence form hydrophobic 'patches' on either side of the finger structure and help shield the zinc binding site from the solvent (Lee et al., 1989a; Omichinski et al., 1990; Pavletich & Pabo, 1991).

There exists functional non equivalence between hydrophobic amino acid residues at the F4 position in odd fingers (Jasanoff & Weiss, 1993). Replacement of phenylalanine with tyrosine at this position maintains hydrophobic interactions with conserved leucine and histidine residues but results in displacement of the phenolic ring towards the solvent (Jasanoff & Weiss, 1993). This placement of the p-hydroxyl group on the DNA-binding surface of the zinc finger may provide an additional hydrogen bond contact to the DNA phosphate backbone (Pavletich & Pabo, 1993; Fairall et al., 1993). These relatively rare odd fingers (which contain a phenylalanine to tyrosine switch at F4) are observed in proteins such as TFIIA (fingers one and eight), Sp1 (finger one), GLI (fingers three and five), and Tramtrack (finger one) (Gibson et al., 1988; Pavletich & Pabo, 1993; Fairall et al., 1993). Similar DNA contacts may not be made by tyrosine at the Ar2 position in even fingers as

the p-hydroxyl group appears to participate in an intrafinger hydrogen bond with serine or threonine at position S/T4 (Kochoyan et al., 1991a).

Variation also exists between C<sub>2</sub>H<sub>2</sub> zinc fingers in HX<sub>*n*</sub>H loop spacing, where 'X' is any amino acid residue and '*n*' ranges from three to five amino acid residues (Gibson et al., 1988; Omichinski et al., 1990; Kochoyan et al., 1991c). In general, even fingers exhibit a spacing of HX<sub>4</sub>H, while the spacing in odd fingers is predominantly HX<sub>3</sub>H (Gibson et al., 1988) (Figure 4.2). A few odd fingers have been identified with HX<sub>4</sub>H spacing, such as fingers three and eight from TFIIIA (Miller et al., 1985), finger two from ADR1 (Bernstein et al., 1994), and fingers one, four, and five from GLI (Pavletich & Pabo, 1993). In addition HX<sub>5</sub>H spacing has been observed in the C-terminal finger of MBP-1 (Omichinski et al., 1990) and finger three of SWI5 (Neuhaus et al., 1992). NMR and X-ray crystallographic analyses have suggested that the helical region containing a HX<sub>3</sub>H spacing tightens up from a classic  $\alpha$ -helix in the N-terminal region to a  $3_{10}$ -type helix in the C-terminus (Lee et al., 1989a; Lee et al., 1989b; Carr et al., 1990; Pavletich & Pabo, 1991) (Figure 4.1B). This change in helical conformation is reflected by a change in internal hydrogen bonding patterns within the protein backbone from *i*-contact-(*i*+4) for an  $\alpha$ -helix (where a given residue position is designated as *i*) to *i*-contact-(*i*+3) for the  $3_{10}$ -helix (Kochoyan et al., 1991c). The formation of a  $3_{10}$ -helix may be due to positioning constraints placed on the second zinc coordinating histidine (Pavletich & Pabo, 1991). The even finger HX<sub>4</sub>H spacing tends to distort the  $\alpha$ -helix into a wider turn just before the second coordinating histidine (Kochoyan et al., 1991c; Pavletich & Pabo, 1993; Bernstein et al., 1994; Liao et al., 1994) (Figure 4.1B). However, the orientation of putative DNA-binding and zinc coordinating residues within the HX<sub>4</sub>H and HX<sub>3</sub>H zinc fingers appears to be very similar (Bernstein et al., 1994). The helical regions of

fingers with HX<sub>5</sub>H spacing terminate midway between the histidines. The remaining finger sequence forms a loop-like structure that positions the second coordinating histidine in the correct orientation for zinc binding. This structure is seen in finger three of SWI5 (Neuhaus et al., 1992) and in the C-terminal finger of MBP-1 (Omichinski et al., 1990).

Differences in spacing between the zinc coordinating cysteine residues have also been observed in fingers from TFIIA (Miller et al., 1985), SWI5 (Neuhaus et al., 1992), GLI (Pavletich & Pabo, 1993), Sp1 (Gibson et al., 1988), and Zif268 (Pavletich & Pabo, 1991). A CX<sub>2</sub>C spacing is found in the majority of odd and even fingers, while a CX<sub>4</sub>C spacing is observed less often (Gibson et al., 1988). As with the different HX<sub>*n*</sub>H spacing elements, coordination of the zinc atom dictates the orientation of the cysteine residues and not the number of intervening amino acid residues. Both regions adopt irregular  $\beta$ -turns, with a conserved glycine positioned just before the second coordinating cysteine in CX<sub>4</sub>C-loop fingers (Miller et al., 1985; Omichinski et al., 1990; Pavletich & Pabo, 1991) and immediately after the second cysteine in many CX<sub>2</sub>C-loop fingers (Gibson et al., 1988; Bernstein et al., 1994). TFIIA contains seven fingers exhibiting CX<sub>4</sub>C spacing (Gibson et al., 1988).

The linker regions between zinc fingers have been proposed to play a role in finger orientation and/or DNA binding (Kochoyan et al., 1991d; Pavletich & Pabo, 1991). The odd finger consensus sequence for this region is; HX<sub>3</sub>HTGEKPF/YXCX<sub>*n*</sub>C (where X is any residue) (Gibson et al., 1988). Proline is found conserved three residue positions before the first coordinating cysteine and may form a hydrophobic interaction with an adjacent conserved aromatic amino acid (underlined) (Gibson et al., 1988; Pavletich & Pabo, 1991). This weak interaction and the restricted structure of proline could help to stabilize a preferred linker conformation and orient adjacent fingers for

optimal DNA binding along the major groove. Even fingers have been proposed to contain a C-terminal helix-linker motif of the sequence  $\text{HX}_4\text{HX}_3\text{-hydrophobic-PX}_2\text{CX}_n\text{C}$  that allows crossing over of the minor groove and placement of the adjacent finger in the major groove of DNA (Kochoyan et al., 1991d). The hydrophobic residue (underlined) conserved four positions before the adjacent  $\text{CX}_n\text{C}$ -loop may enter the minor groove and provide stabilizing hydrophobic interactions (Kochoyan et al., 1991d). This hydrophobic residue is replaced by a conserved lysine in odd fingers (Gibson et al., 1988; Kochoyan et al., 1991d). Such a 'jumping-linker' conformation is suggested to occur between even and odd fingers of ZFY and between fingers three and four of TFIIA.

Interactions between adjacent  $\text{C}_2\text{H}_2$  zinc fingers have been investigated by NMR of MBP-1 (Omichinski et al., 1992) and SWI5 (Nakaseko et al., 1992) derived two-finger peptides and by X-ray crystallography of the DNA-binding domains of *Zif268* (Pavletich & Pabo, 1991) and GLI (Fairall et al., 1993) complexed with their respective DNA target sequences. No general structural rules are apparent for finger-finger interactions. Interfinger contacts are not observed for the SWI5 peptide, with the two fingers connected by a flexible linker sequence (Nakaseko et al., 1992). In contrast, the two fingers derived from MBP-1 interact through amino acid-mediated hydrophobic and hydrogen bond contacts between the C-terminal helix-linker region of one finger and the finger 'tip' region of the adjacent zinc finger (Omichinski et al., 1992) (Figure 4.4). Similar hydrogen bonding schemes are also observed between fingers one through three of *Zif268* (Pavletich & Pabo, 1991) and two through five of GLI (Pavletich & Pabo, 1993). More extensive contacts are observed between fingers one and two of GLI, with backbone-mediated hydrogen bond contacts and a tryptophan-tryptophan hydrophobic interface.

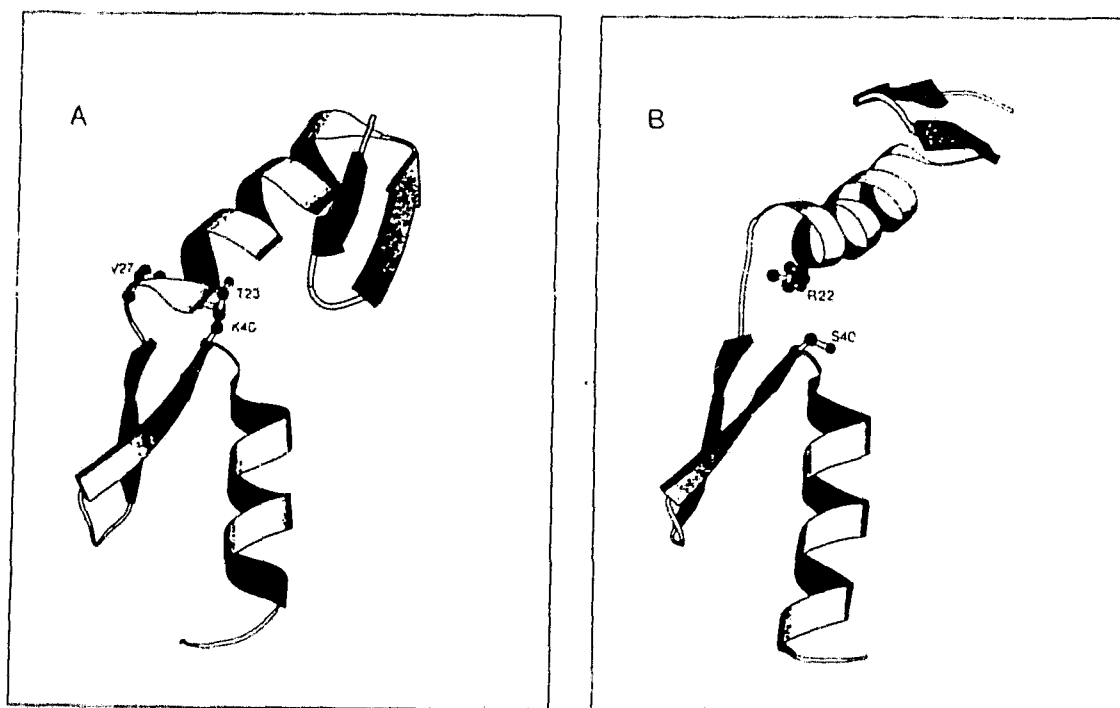


Figure 4.4 Schematic representation of the interfinger orientations of (A) MBP-1 zinc fingers and (B) *Zif268* fingers one and two. The C-terminal finger of MBP-1 and finger two of *Zif268* are shown at the bottom of each panel (Omichinski et al., 1992).

It is important to note that the absence of interfinger interactions does not necessarily indicate the absence of a specific orientation of zinc fingers in solution. Other regions of the trans-acting factors not present in the experimental peptides may play a role in zinc finger spatial orientation and enhance nucleic acid interactions.

#### 4.1.2 DNA sequence recognition by C<sub>2</sub>H<sub>2</sub> zinc finger domains

Zinc fingers are proposed to bind to regions of DNA that contain an enlarged major groove (Nekludova & Pabo, 1994). Such DNA conformations may exist within free DNA at junctions between B-form and A-like DNA sequences (Huber et al., 1991) or may be induced through protein binding (Nekludova & Pabo, 1994). Widening of the major groove may allow access of the zinc finger  $\alpha$ -helix to bases on the DNA.

Comparison of the X-ray crystallographic structures of *Zif268* (Pavletich & Pabo, 1991), *GLI* (Pavletich & Pabo, 1993), and *Tramtrack* (Fairall et al., 1993) complexed with their respective DNA recognition sequences have helped to identify conserved hydrogen bond contacts between odd C<sub>2</sub>H<sub>2</sub> zinc fingers and the DNA backbone. A conserved basic amino acid (K/R2) two positions from the second coordinating cysteine in fingers one through three of *Zif268*, finger one of *Tramtrack*, and finger two of *GLI* is observed to make contact with DNA phosphates. A second interesting DNA backbone contact by odd fingers is a zinc-histidine-phosphate 'charge relay' interaction established by the first coordinating histidine. This contact is observed for fingers one through three of *Zif268*, fingers two through three of *GLI*, and finger two of *Tramtrack*. Studies on *Tramtrack* (Fairall et al., 1993) and *ADR1* (Bernstein et al., 1994) suggest that zinc fingers with both HX<sub>3</sub>H and HX<sub>4</sub>H spacing within the helical region can form this important DNA backbone interaction. Thus,

this histidine residue participates in three critical functions; zinc coordination, hydrophobic interactions in the finger core, and stabilization of finger docking into the major groove of DNA. It must be stressed that additional non conserved residues within each finger contribute to non-specific DNA binding and that the conserved amino acid positions mentioned above do not contribute equally to protein-DNA interaction within all odd zinc fingers.

Specific amino acid-base contacts between C<sub>2</sub>H<sub>2</sub> zinc fingers and DNA have been identified by sequence analysis (Jacobs, 1992), X-ray crystallography of *Zif268*, *GLI*, and *Tramtrack* (Pavletich & Pabo, 1991; 1993; Fairall et al., 1993), phage display of *Zif268* (Jamieson et al., 1994; Rebar & Pabo, 1994; Choo & Klug, 1994a), and differential selection of DNA binding sites with *Zif268*, *Krox 20*, and *Sp1* derivatives and with an artificial finger protein, *CP-1* (Nardelli et al., 1991; Desjarlais & Berg, 1992; Desjarlais & Berg, 1993; Desjarlais & Berg, 1994; Choo & Klug, 1994b). The combination of these methods have shown that the relationship between contacting amino acid to bound DNA base pair is essentially one to one for *Zif268*, *Sp1*, *Krox20*, and *ADR1*. Thus, a single amino acid at a conserved position within the finger helix is responsible for contacting one base pair within the DNA binding site (Pavletich & Pabo, 1991; Choo & Klug, 1994a; Choo & Klug, 1994b) (Figure 4.5A). Each individual zinc finger associates with a three base pair subsite based on an interaction 'code' established using *Zif268* (Choo & Klug, 1994b) (Figure 4.5B). This code may be generally applicable to a family of zinc finger proteins that interact with their binding sites in a similar fashion (*Zif268*, *Sp1*, *Krox20*, *ADR1*, and *Tramtrack*). However, the interaction of *GLI* with DNA suggests that this interaction code is not universal and may be subject to variation (Pavletich & Pabo, 1993).

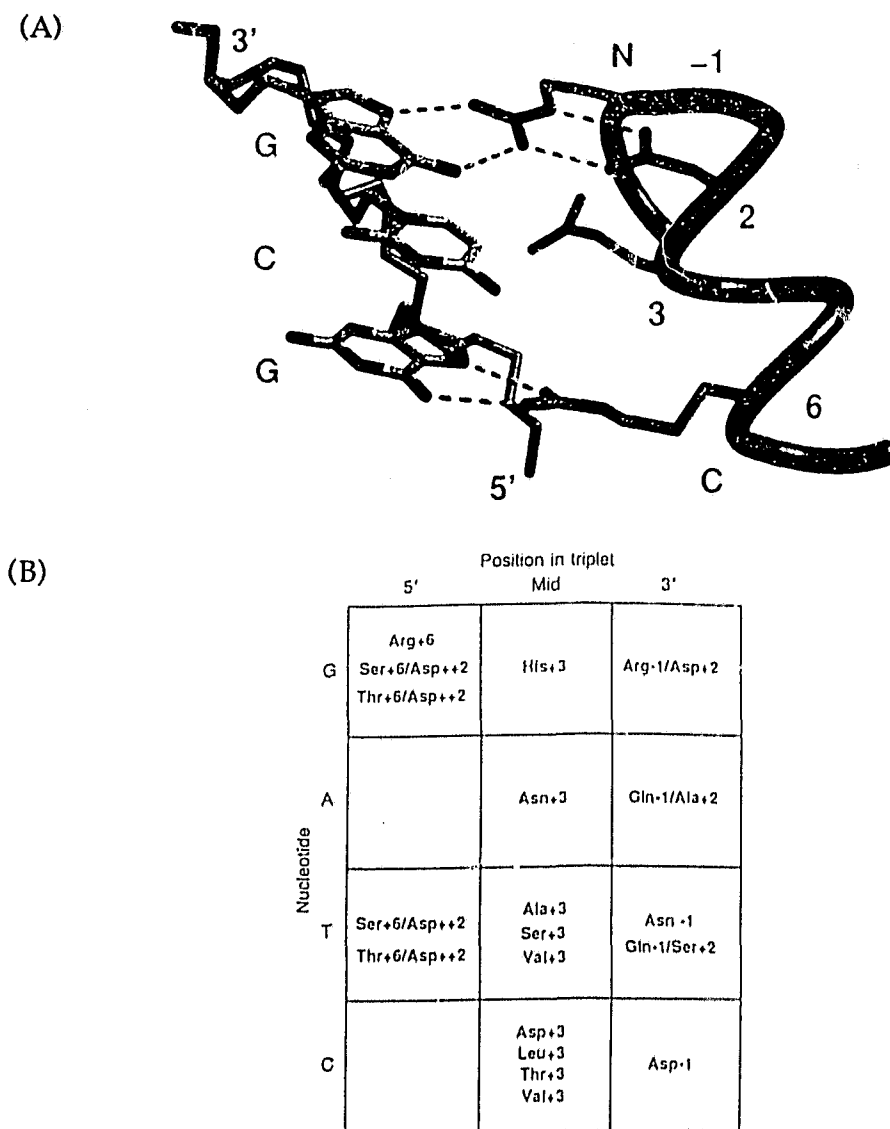


Figure 4.5 DNA sequence recognition by zinc finger domains. (A) The interaction of amino acid residues within the *Zif268* finger one helical region with the DNA subsite 5'GCG3' is shown (Jamieson et al., 1994). (B) The recognition code between core amino acid residues within the zinc finger and three base pair subsites within the DNA binding site is depicted (Choo & Klug, 1994b). Auxiliary residues within the same finger at position +2 and within the adjacent finger at position ++2 influence base contacts by amino acid residues at positions -1 and position +6, respectively.

The core contact amino acid residues within the zinc finger  $\alpha$ -helix are found at the conserved positions -1, +3, and +6, where +1 refers to the first helical residue (Choo & Klug, 1994a; Rebar & Pabo, 1994) (Figure 4.5A). The finger residue at position +6 contacts the 5' base pair, position +3 contacts the central base pair, and position -1 interacts with the 3' base pair of the DNA subsite. The zinc finger  $\alpha$ -helix is, therefore, positioned anti-parallel to the DNA binding site (N- to C-terminus of the finger helix oriented 3' to 5' along the DNA) (Pavletich & Pabo, 1991). Auxiliary amino acid residues at position +2, and to a lesser extent at other positions, can influence the type and strength of contacts established by adjacent core residues and thereby affect DNA binding site recognition (Choo & Klug, 1994b; Rebar & Pabo, 1994; Jamieson et al., 1994) (Figure 4.5A). These studies have also indicated that it is the amino acid complement at core and auxiliary helical positions that results in different contributions by individual zinc fingers to the free energy of DNA binding (Desjarlais & Berg, 1992; Kriwacki et al., 1992; Bernstein et al., 1994). Thus amino acid sequence context within the zinc finger  $\alpha$ -helix as well as sequence context within the DNA binding site function in establishing high affinity complex formation.

#### **4.1.3 The TFIIIA nucleic acid binding domain**

A number of mutagenesis studies of TFIIIA have been carried out in order to understand the function of zinc fingers in DNA binding and organization of the TFIIIA-5S RNA gene complex. It appears that the contributions made to promoter binding are non equivalent between the nine zinc fingers of TFIIIA. Energetically favorable interactions between TFIIIA and the 5S RNA gene promoter may be established by clusters of zinc fingers that contribute to the binding energy in an inter-dependent manner

(Del Rio et al., 1993a; Clemens et al., 1994). The first three zinc fingers provide 95 percent of the DNA binding energy and interact with the box C element of the 5S RNA gene promoter (Vrana et al., 1988; Christensen et al., 1991; Clemens et al., 1992; Hayes & Clemens, 1992; Liao et al., 1992; Hansen et al., 1993; Del Rio et al., 1993a; Clemens et al., 1994; Veldhoen et al., 1994). A polypeptide containing fingers one through three of TFIIIA binds to the 5S RNA gene with a dissociation constant of  $5.6 \pm 0.9$  nM, representing only a two-fold lower affinity compared with the full length protein (Liao et al., 1992; Clemens et al., 1994). The remaining zinc fingers and the C-terminal domain critical for maximal transcription interact with the intermediate and box A elements within the promoter and are oriented towards the 5' end of the gene (Vrana et al., 1988; Del Rio et al., 1993a). Finger five is proposed to contact the intermediate element, while fingers seven through nine interact with the box A promoter element (Clemens et al., 1992; Clemens et al., 1994). Single amino acid substitutions within the helical regions of fingers four and six reduced DNA binding of a seven-finger TFIIIA polypeptide eight-fold and 17-fold, respectively (Clemens et al., 1994). Substitution of the second and first zinc coordinating cysteine residues within fingers four and six, respectively, results in protection of base pair positions +78 through +96 within the 5S RNA gene promoter from DNase I attack (Smith et al., 1991). Therefore, these fingers may provide additional contacts to the 5S RNA gene promoter that increase the rate of complex formation or stabilize the TFIIIA-promoter interaction. Zinc fingers eight and nine of TFIIIA may also contribute directly to transcription activation *in vivo* (Rollins et al., 1993; Del Rio & Setzer, 1993b). In contrast, mutations that destabilize the folded conformation of zinc fingers four and six increase the transactivation activity of the mutant TFIIAs above that of the wild type protein (Rollins et al., 1993). Thus

different functional roles exist for the zinc fingers within TFIIIA, resulting in a cooperative and polarized interaction with the 5S RNA gene by the multi-finger nucleic acid binding domain and activation of 5S RNA transcription.

Within the N-terminal three-finger cluster of TFIIIA there is non equivalence in contributions made to DNA binding. Studies using *in vitro* translation (Vrana et al., 1988) or bacterial expression (Fiser-Littell et al., 1988; Smith et al., 1991) systems suggest that removal of finger one significantly reduces TFIIIA DNA-binding activity. In contrast, finger one truncation (Theunissen et al., 1992) and destabilization (Del Rio et al., 1993a) mutants purified to homogeneity exhibit DNA binding activity similar to wild type TFIIIA. Disruption of finger two conformation reduces DNA binding approximately seven-fold, while similar disruption of finger three reduces TFIIIA-5S RNA gene interaction 26-fold (Del Rio et al., 1993a). Polypeptides containing fingers one and two or two through four do not interact specifically with the 5S RNA gene (Liao et al., 1992). The N-terminal 12 amino acid residues of TFIIIA that are not within the zinc finger nucleic acid binding domain do not contribute to DNA binding (Vrana et al., 1988; Clemens et al., 1992).

The first three fingers of TFIIIA are connected by the linker sequence, TGEKP/N, found highly conserved in many zinc finger proteins (Figure 1.7). The other linkers of TFIIIA vary considerably from this consensus. Substitution mutation of these two N-terminal linkers with heterologous linkers from *Xenopus* p43 protein or with non conserved amino acids indicates that the native sequence is essential for efficient DNA binding and/or complex stability (Smith et al., 1991; Choo & Klug, 1993; Clemens et al., 1994). Swapping the finger one-two linker for the finger three-four linker of TFIIIA also reduces DNA binding considerably (Choo & Klug, 1993). This

suggests that individual linker sequences are functionally distinct in their contributions to TFIIIA-DNA complex formation. Single amino acid substitution of threonine, lysine, or proline within the first linker of TFIIIA reduced DNA binding between seven and 13-fold, while substitution of the glycine residue resulted in a 24-fold reduction in DNA interaction (Choo & Klug, 1993). Linker sequences between fingers three and four and between fingers six and seven are suggested to adopt a conformation that allows exit of the downstream finger from the major groove of DNA (Kochoyan et al., 1991d; Hansen et al., 1993). Thus, individual linker sequence appears to be important for TFIIIA-DNA complex assembly and/or stability and may function in the correct orientation of adjacent finger domains.

While fingers one through three of TFIIIA are required for high affinity DNA binding, the central zinc fingers four to seven are sufficient for high affinity RNA binding (Darby & Joho, 1992; Bogenhagen, 1993; Clemens et al., 1993). Single amino acid substitutions at position Q121 within finger four and positions T176, W177, T178 within finger six reduce 5S RNA binding four-fold to 30-fold (Clemens et al., 1993). These residues may be located on the surface of each finger at the N-terminal end of the  $\alpha$ -helix, suggesting that base-specific contacts could be formed with 5S RNA similar in architecture to zinc finger-DNA interactions that occur in the major groove of the 5S RNA gene.

To identify amino acid residues within the first three zinc fingers of TFIIIA critical for nucleic acid interaction, a set of finger swap and scanning substitution mutants of TFIIIA were generated by PCR-mediated site-directed mutagenesis. The donor sequence used in this study originates from zinc finger 31 of the *Xenopus* protein, Xfin. Xfin contains 37 C<sub>2</sub>H<sub>2</sub> zinc fingers and is expressed in both the oocyte and highly differentiated cells of *Xenopus*

(Ruiz i Altaba et al., 1987). Xfin does not exhibit significant DNA binding activity, however, its interaction with RNA suggests that this protein may function in post-transcriptional regulation of mRNA (Andreazzoli et al., 1993). Wild type and mutant TFIIIA proteins were expressed in a bacterial system and purified to homogeneity (Del Rio & Setzer, 1991; Veldhoen et al., 1994). The relative association constants of these mutants with 5S RNA and the 5S RNA gene were determined using a nitrocellulose filter binding assay.

The present TFIIIA mutagenesis analysis was part of a collaborative study with Wei Qing Zang from Dr. Romaniuk's research group. Wei Qing Zang constructed and assayed TFIIIA mutants containing substitutions of *Xenopus* p43 protein sequence (Zang et al., in preparation). The effects of TFIIIA mutants containing Xfin 31 sequence will be compared with the TFIIIA-p43 mutants in the discussion.

## **4.2 Methods and Materials**

### **4.2.1 Bacterial strains and DNA vectors**

Plasmid pT7-TF containing the TFIIIA cDNA (Ginsberg et al., 1984) was kindly provided by J. Tso. The *Nde* I/*Bam* HI fragment of pT7-TF was cloned into the same sites within pET-11b (Studier et al., 1990) to yield pTF4 (Figure 4.6). Plasmid pXlo contains a synthetic oocyte-type *Xenopus* 5S RNA gene cloned into the *Eco* RI and *Bam* HI sites of pUC18 (Romaniuk et al., 1987a). All plasmids were maintained in *E. coli* strain JM109 and protein expression was carried out in *E. coli* strain BL21(DE3).

### **4.2.2 Construction of mutant TFIIIA expression vectors**

TFIIIA mutant TX1 was produced using a combination of shotgun ligation followed by extension of the mutant sequence by PCR-mediated site-

directed mutagenesis. Xfin finger 31 sequence was introduced into the TFIIIA cDNA on the 5' side of a *Pst* I restriction site within finger one by shotgun ligation of overlapping oligonucleotides (Romaniuk et al., 1987a). The resulting mutant construct, TX1.1, was extended using PCR-mediated site-directed mutagenesis to generate the full Xfin finger 31 swap mutant of TFIIIA finger one (Nelson & Long, 1989).

The TFIIIA finger two mutant, TX2, was generated using an oligonucleotide overlapping two-stage PCR technique (Dillon & Rosen, 1990). Four long partially overlapping oligonucleotide primers were used to PCR amplify DNA fragments encoding fingers one to three of TFIIIA that incorporated the designated mutant sequence as well as flanking *Pst* I and *Bam* HI/*Bgl* II sites for insertion into pUC19 and pTF4 (Figure 4.7). The amplified DNA product was initially cloned into the *Pst* I and *Bam* HI restriction sites of pUC19 and identified by sequence analysis. The resulting subclone was digested by *Pst* I and *Bgl* II and the purified DNA fragment was cloned into the same sites in pTF4.

The first stage reaction (20  $\mu$ l) contained; 10 mM Tris-HCl pH 8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 50  $\mu$ g/ml gelatin, 1 nmol each of dTTP, dGTP, dATP, and dCTP, 40 pmol external primers, 4 pmol internal primers, and 0.5 units Taq DNA polymerase. Eight rounds of thermal cycling were carried out using a denaturation temperature of 94°C (1.5 minutes), an annealing temperature of 50°C (1.5 minutes), and an extension temperature of 72°C (two minutes). This PCR amplification generated the template DNA containing the desired TFIIIA substitution mutation. The second stage reaction (100  $\mu$ l) contained; 10 mM Tris-HCl pH 8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 50  $\mu$ g/ml gelatin, 5 nmol each of dTTP, dGTP, dATP, and dCTP, 1  $\mu$ l stage one reaction product, 20 pmol M13 forward and reverse universal

primers, and 2.5 units Taq DNA polymerase. Thirty rounds of thermal cycling were carried out using a denaturation temperature of 94°C (1.5 minutes), an annealing temperature of 55°C (1.5 minutes), and an extension temperature of 72°C (two minutes).

The scanning substitution mutants within fingers one to three of TFIIIA (TX1.2 in finger one, TX2.1 to TX2.7 in finger two, and TX3.1 to TX3.7 in finger three) were constructed using a PCR-mediated site-directed mutagenesis procedure (Nelson & Long, 1989) (Figure 4.8). pUC-TF1-3, which contains the *Nde* I/*Bgl* II fragment of TFIIIA cDNA cloned into the *Kpn* I and *Bam* HI sites of pUC19, was used as the PCR template. In the first stage, the mutagenic sequence (MP) and a unique flanking priming site (FUMP) were incorporated into the wild type template DNA. This PCR product was agarose gel purified using Mermaid (Bio 101) to remove unincorporated primers and resuspended in 30 µl water. An extension step was performed in order to extend the first stage PCR product along wild type template DNA and incorporate a second priming site. Finally, this extended product was PCR amplified in the second stage to produce the full length mutant DNA. Each mutant DNA was initially subcloned into pUC19 (*Eco* RI and *Pst* I sites) for sequence verification and then introduced back into pTF4 using the unique *Nde* I and *Bgl* II restriction sites.

The first stage reaction (50 µl) contained; 10 mM Tris-HCl pH 8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 50 µg/ml gelatin, 2.5 nmol each of dTTP, dGTP, dATP, and dCTP, 10 pmol FUMP and mutagenic primer, 1 ng linearized pUC-TF1-3 template, and 1.25 units Taq DNA polymerase. Twenty-six rounds of thermal cycling were carried out using a denaturation temperature of 94°C (1.5 minutes), an annealing temperature of 37 °C to 45°C (1.5 minutes), and an extension temperature of 72°C (two minutes).

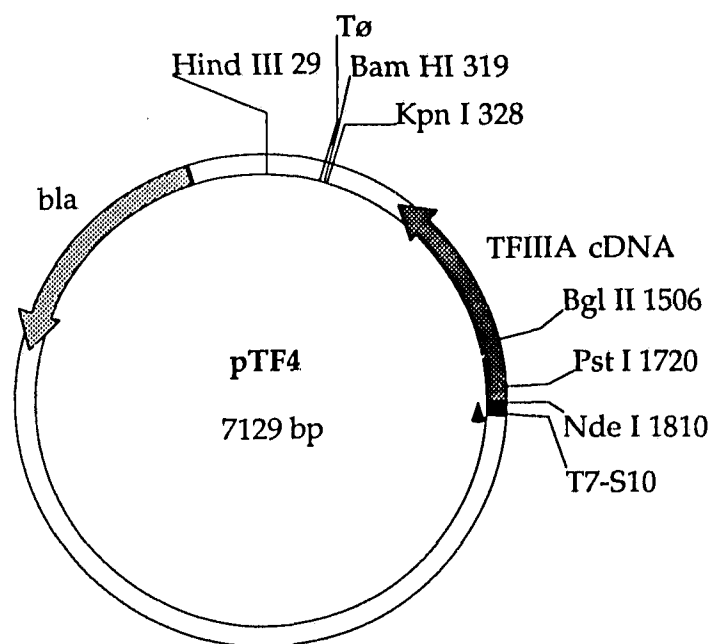


Figure 4.6 Schematic representation of the recombinant TFIIIA expression plasmid, pTF4. Only unique restriction enzyme sites used in construction of the TFIIIA mutants are shown

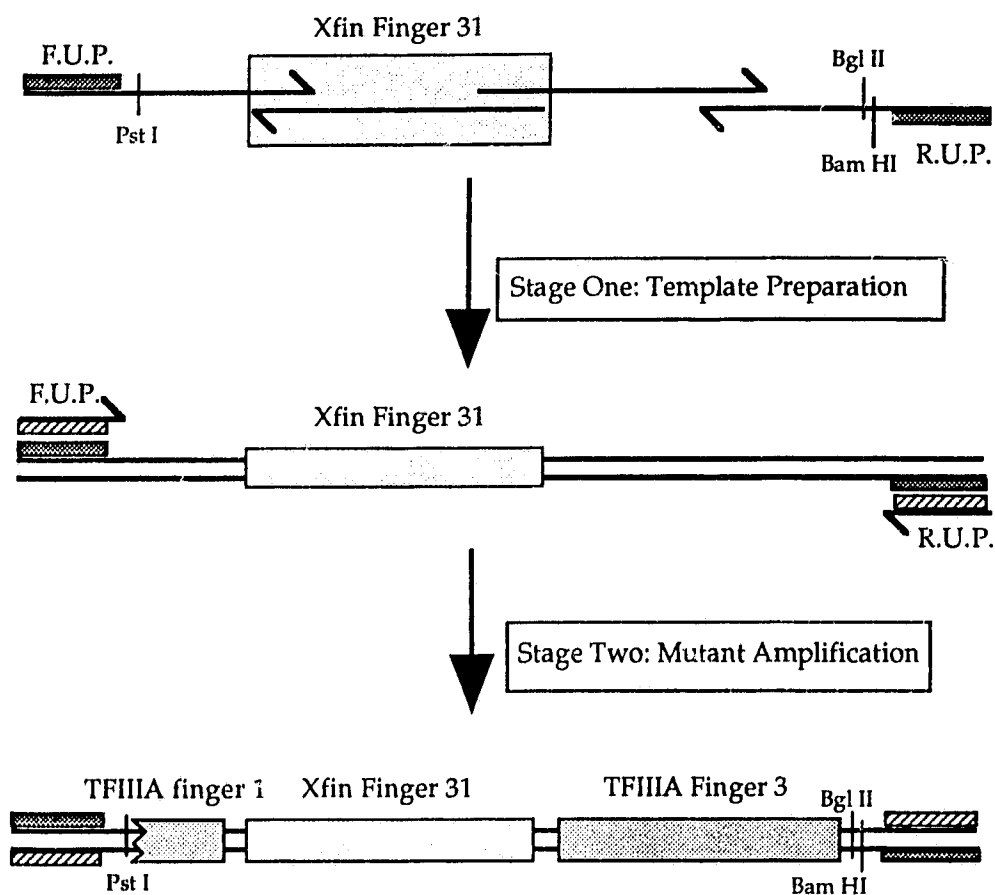


Figure 4.7 Schematic representation of the template-independent PCR mutagenesis procedure used to create the TFIIIA finger two substitution mutant, TX2 (Dillon & Rosen, 1990).

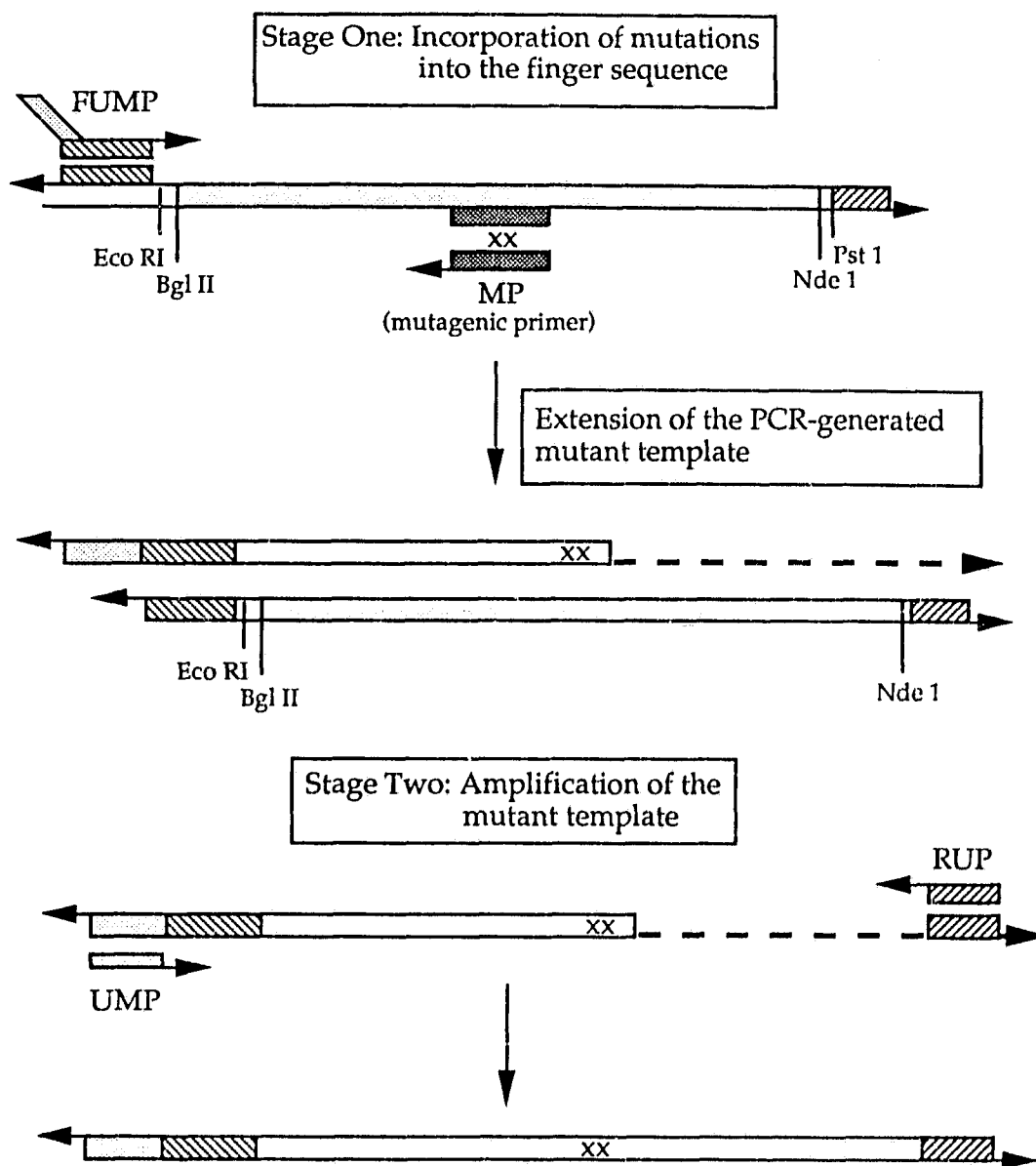


Figure 4.8 Schematic representation of the PCR-mediated site-directed mutagenesis procedure used in construction of scanning substitution mutations within TFIIA (Nelson & Long, 1989).

The extension reaction (80  $\mu$ l) contained; 10 mM Tris-HCl pH 8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 50  $\mu$ g/ml gelatin, 5 nmol each of dTTP, dGTP, dATP, and dCTP, 1 ng linearized pUC-TF1-3 template, 1  $\mu$ l to 3  $\mu$ l stage one PCR product, and 2.5 units Taq DNA polymerase. Five rounds of thermal cycling were carried out using a denaturation temperature of 94°C (1.5 minutes), an annealing temperature of 45°C (1.5 minutes), and an extension temperature of 72°C (two minutes). The mutant DNA was amplified with the addition of 20 pmol universal mutagenic (UMP) and M13 reverse universal (RUP) primers in 20  $\mu$ l reaction buffer. Thermocycling temperatures and times were identical to those used in the first stage.

#### **4.2.3 Expression and purification of recombinant wild type and mutant TFIIIA proteins**

Preparation of wild type and mutant TFIIIA proteins were carried out as described in section 2.2.2 and by (Veldhoen et al., 1994). Protein purity was confirmed by SDS-PAGE analysis (Figure 4.9) and the concentration of each protein preparation was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

#### **4.2.4 Synthesis and radiolabeling of the 5S RNA gene and 5S RNA**

The 5S RNA gene was released from plasmid pXlo using the restriction endonucleases *Eco* RI and *Hind* III and end-labeled with [ $\alpha$ -<sup>32</sup>P]-dATP and the Klenow fragment of *E. coli* DNA polymerase I (Sambrook et al., 1989). The 40  $\mu$ l reaction contained; 50 mM Tris-HCl pH 7.2, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 50  $\mu$ g/ml BSA, 0.2 mM each of dCTP, dGTP, dTTP, 40  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]-dATP, 1  $\mu$ g digested pXlo, and 9 units of Klenow (New England Biolabs). The labeling reaction was incubated at ambient temperature (22°C) for 30 minutes and

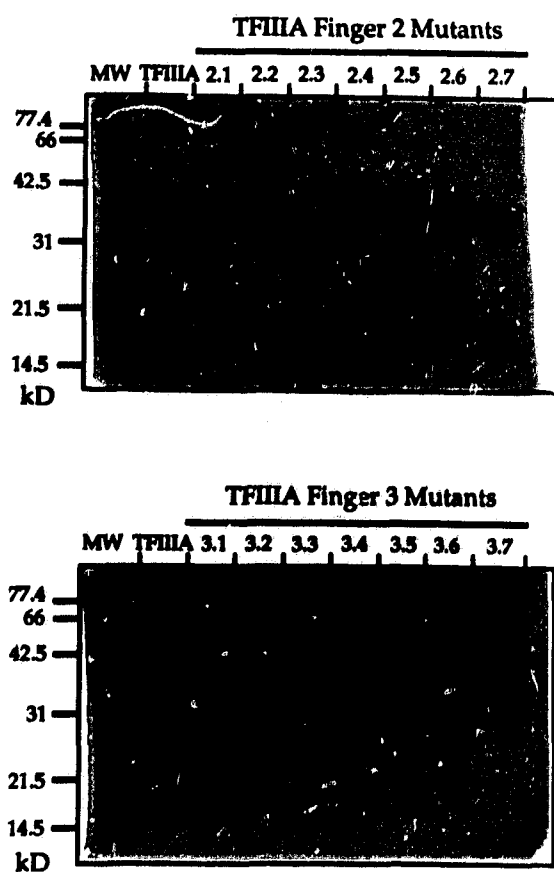


Figure 4.9 SDS-PAGE analysis of TFIIIA mutants containing scanning substitution mutations within fingers two and three. TFIIIA mutants were purified by ion exchange chromatography from induced *E. coli* cell extracts.

chased with the addition of 0.2 mM dATP for five minutes. Labeled DNA containing the 5S RNA gene was purified by electrophoresis on a 8% non-denaturing polyacrylamide gel (29:1, acrylamide:bis; 16 x 16 cm x 0.75 mm), eluted for 16 hours, and ethanol precipitated.

*Xenopus* oocyte-type 5S RNA was produced by *in vitro* transcription and end-labeled at the 3' terminus with [5'-<sup>32</sup>P]-pCp and T4 RNA ligase as described in section 3.2.3 and by Romaniuk (1985); Romaniuk et al., (1987a). The efficiency of radiolabel incorporation into both 5S RNA and the 5S RNA gene was measured using a LKB 1214 Rackbeta Scintillation counter and toluene with 0.4% (w/v) PPO (Sigma) added as scintillant.

#### **4.2.5 Equilibrium binding of the 5S RNA gene and 5S RNA to TFIIIA substitution mutants**

The apparent association constants for the binding of radiolabeled 5S RNA and the 5S RNA gene to wild type and mutant TFIIAs were determined using a nitrocellulose filter binding assay as described in section 2.2.4 and by Romaniuk (1985; 1990). Nitrocellulose filter binding reactions of end-labeled 5S RNA did not contain poly dI-dC as a competitor nucleic acid.

### **4.3 Results**

#### **4.3.1 Substitution mutagenesis of TFIIIA**

To determine the basis of zinc finger-DNA interaction, a series of amino acid substitution mutants of TFIIIA were generated that incorporated Xfin finger 31 sequence into the first three fingers of TFIIIA. The 5S RNA gene and 5S RNA binding affinities for mutant TFIIAs were assayed using a nitrocellulose filter binding assay. This method identifies amino acid

sequences within the N-terminal zinc fingers of TFIIIA that contribute to the free energy of TFIIIA-nucleic acid complex formation.

The substitution of Xfin zinc finger sequence in place of TFIIIA finger domains is advantageous in that all the structural determinants required to maintain the finger conformation are retained (Michael et al., 1992). This includes the metal ion coordinating residues as well as the hydrophobic core amino acids. Effects on nucleic acid binding activity can then be interpreted as a loss or gain of contacts due to amino acid substitution rather than to drastic conformational changes in zinc finger structure. The results of thermolysin protease digestion analysis of the TFIIIA mutant proteins suggests that none of the substitution mutations introduced major conformational changes from that of wild type TFIIIA. However, in many cases residues other than the "structural" amino acids are found to be conserved between TFIIIA and the donor Xfin finger 31. This conservation of amino acid residues may lead to the retention of critical amino acid-DNA contacts by chimaeric TFIIIA mutants. This effect is observed for Xfin sequence substitutions at positions 62R (mutant TX2.5), where the DNA binding affinity is similar to wild type TFIIIA (position one denotes the N-terminal methionine of TFIIIA) (Figure 4.11). In contrast, alanine substitution at this position severely affects DNA binding ability. Thus, substitution mutagenesis has the advantage of retaining overall zinc finger structure but may also result in certain critical DNA contact amino acids evading detection.

#### **4.3.2 Effects of finger swap mutations of TFIIIA on nucleic acid binding activity**

As a first step towards the identification of zinc finger domains important for the interaction of TFIIIA with the 5S RNA gene, full finger

Table 4.1 The effects of TFIIIA finger substitution mutations on binding activity to the 5S RNA gene and 5S RNA.

Mutant <sup>a</sup>	TFIIIA fingers replaced	Relative K <sub>a</sub> for binding to the 5S RNA Gene <sup>b</sup>	Relative K <sub>a</sub> for binding to 5S RNA <sup>b</sup>
TX 1	1	0.91 ± 0.10	1.17 ± 0.12
TX 2	2	0.28 ± 0.08	1.15 ± 0.12

<sup>a</sup>The nomenclature 'TX' refers to TFIIIA substitution mutations made using X<sub>1</sub>in protein finger 31 as the donor. <sup>b</sup>Relative K<sub>a</sub> is determined as the ratio of the apparent association constant for the mutant protein to the apparent association constant for the wild type protein. Average of three or more independent determinations.

substitution mutants of TFIIIA fingers one and two were constructed and purified. The results of nucleic acid binding analysis indicate that substitution of finger two of TFIIIA reduced 5S RNA gene binding affinity (Table 4.1). In contrast, the finger one substitution mutant retained wild type DNA binding activity. These results agree with studies performed using truncated forms of TFIIIA (Christensen et al., 1991; Liao et al., 1992; Hansen et al., 1993) and "broken finger" mutants (Del Rio et al., 1993a) that identify fingers two, three, and four as important for TFIIIA-DNA complex formation. Substitution of TFIIIA finger three with the corresponding finger from the *Xenopus* p43 protein has also been shown to reduce TFIIIA-5S RNA gene interaction over 100-fold (Zang et al., in preparation). Finger one and two substitution mutants exhibit 5S RNA binding activity similar to wild type TFIIIA. These results are not surprising as it has been demonstrated that fingers four to seven of TFIIIA are required for high affinity 5S RNA binding (Clemens et al., 1993; McBryant et al., 1995).

#### **4.3.3 Effects of scanning substitution mutations of TFIIIA on nucleic acid binding activity**

Several groups have found that fingers one to three of TFIIIA bind to the box C element of the 5S RNA gene promoter and that this interaction provides the majority of the free energy for high affinity binding of TFIIIA to the 5S RNA gene (Vrana et al, 1988; Christensen et al., 1991; Liao et al., 1992; Del Rio et al., 1993a). In order to identify amino acid sequences within the first three zinc fingers of TFIIIA involved in contacts to DNA, a series of scanning substitution mutants were constructed (Figure 4.10). Examples of binding curves generated from analysis of various TFIIIA mutants with the 5S RNA gene and 5S RNA are shown in Figures 4.11 and 4.12, respectively.

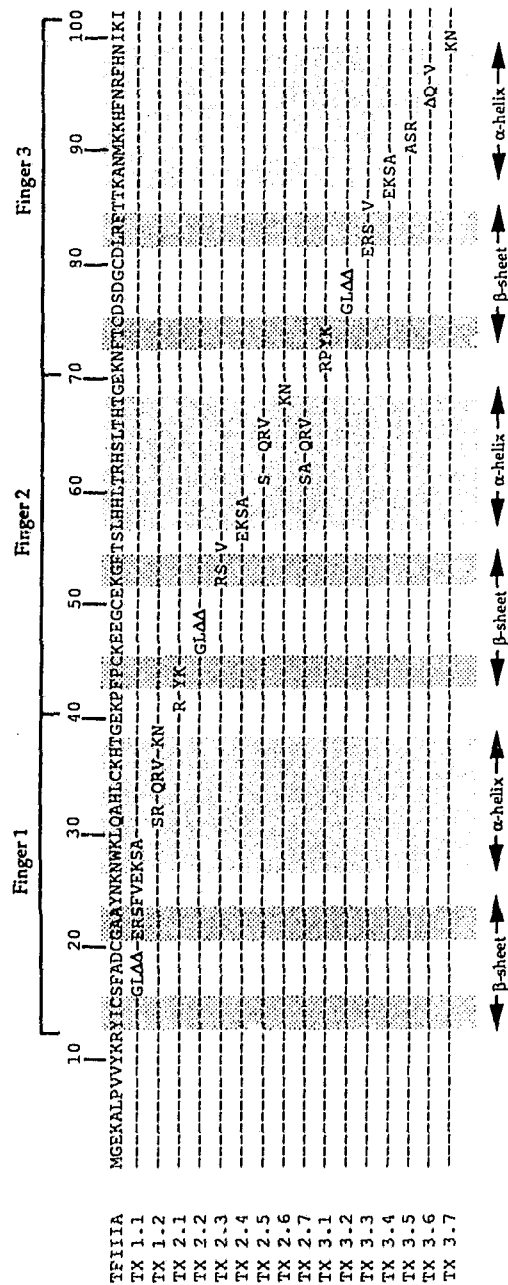


Figure 4.10 Location of scanning substitution mutations within the first three zinc fingers of TFIIIA. For each mutant, a dash indicates that an amino acid at that position is identical with that of wild type TFIIIA. A ( $\Delta$ ) symbol indicates that an amino acid has been deleted at that position in the mutant protein. The nomenclature 'TX' refers to mutants containing sequence from finger 31 of the Xfin protein.

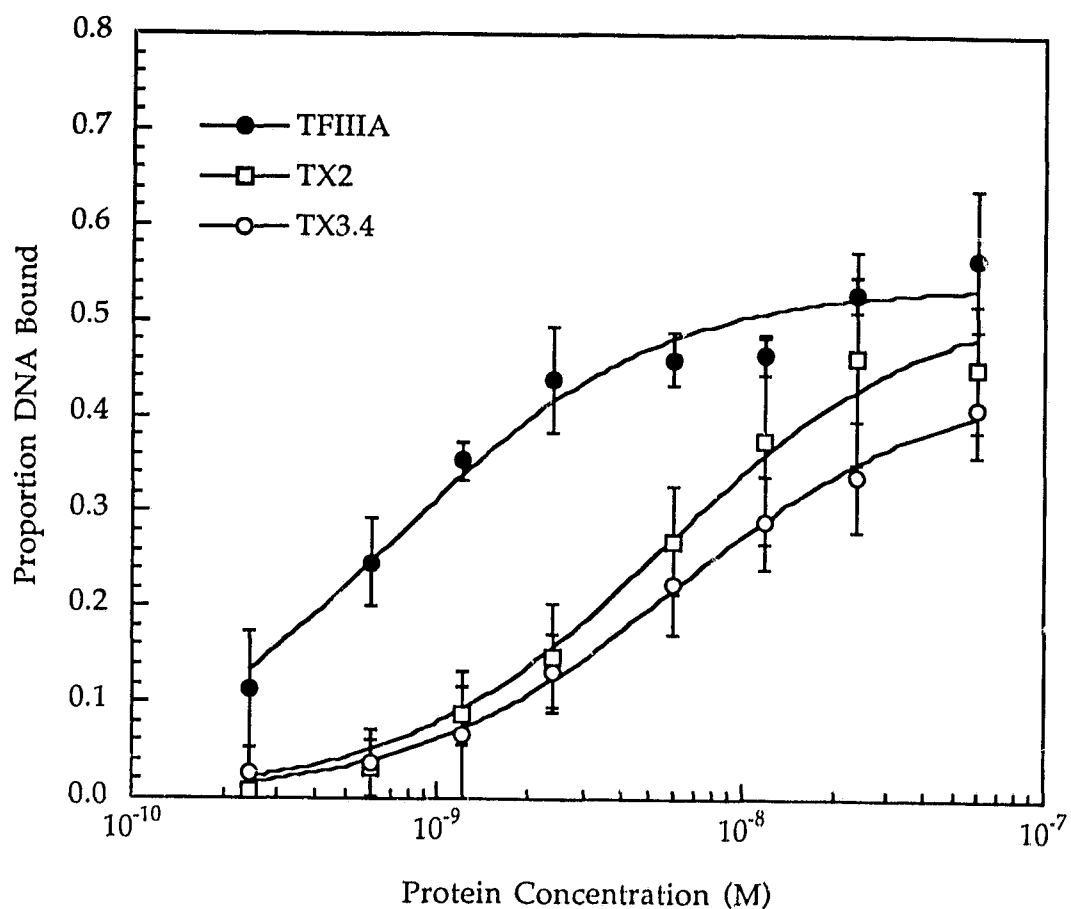


Figure 4.11 Sample nitrocellulose filter binding curves of TFIIIA mutants with the oocyte-type 5S RNA gene indicating the average of three or more determinations.

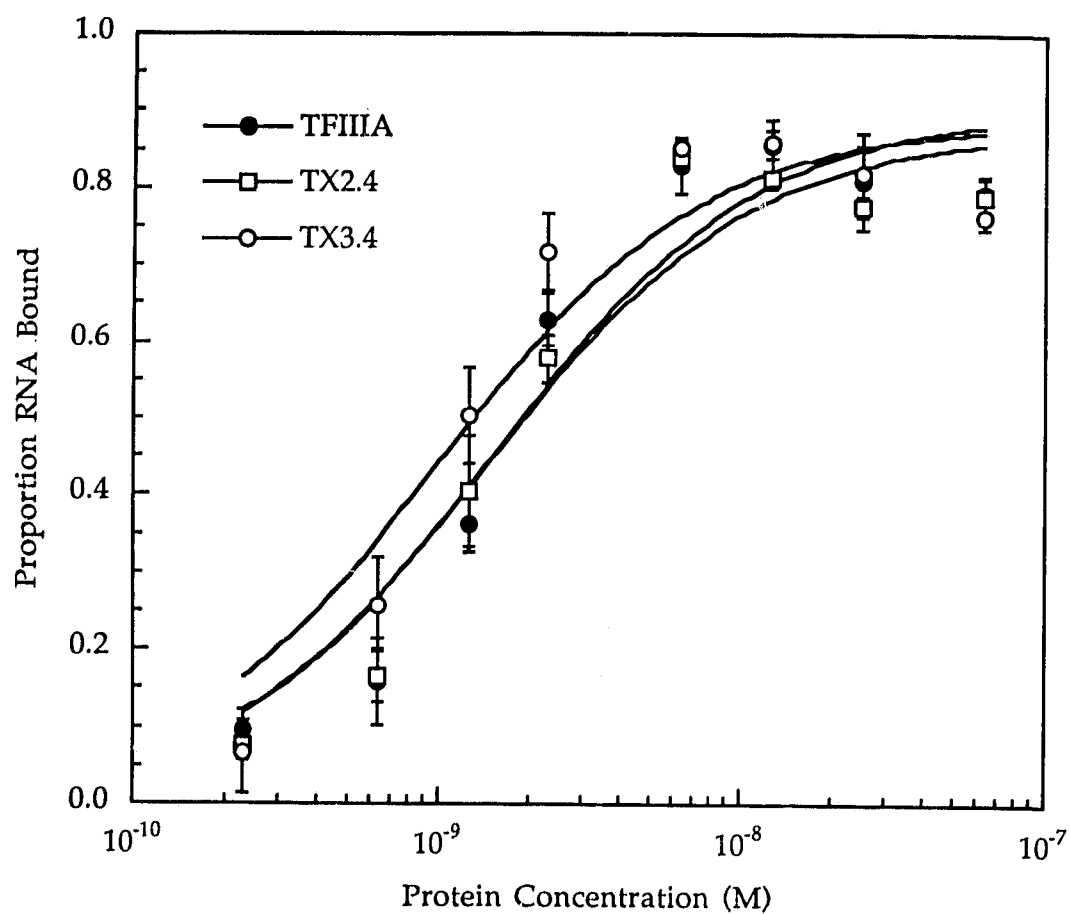


Figure 4.12 Sample nitrocellulose filter binding curves of TFIIIA mutants with oocyte-type 5S RNA indicating the average of three or more determinations.

Table 4.2 The effects of TFIIIA scanning substitution mutations on binding activity to the 5S RNA gene and 5S RNA.

Mutant <sup>a</sup>	Relative $K_a$ for binding to the 5S RNA Gene <sup>b</sup>	Relative $K_a$ for binding to 5S RNA <sup>b</sup>
TX 1.1	0.94 ± 0.06	0.97 ± 0.11
TX 1.2	0.84 ± 0.06	1.23 ± 0.05
TX 2.1	0.88 ± 0.12	1.00 ± 0.02
TX 2.2	0.77 ± 0.05	1.12 ± 0.22
TX 2.3	0.89 ± 0.10	1.16 ± 0.12
TX 2.4	0.34 ± 0.04	1.07 ± 0.10
TX 2.5	0.75 ± 0.17	0.87 ± 0.08
TX 2.6	0.72 ± 0.19	0.96 ± 0.01
TX 2.7	0.22 ± 0.07	0.92 ± 0.07
TX 3.1	1.25 ± 0.23	0.97 ± 0.02
TX 3.2	0.51 ± 0.17	0.96 ± 0.06
TX 3.3	0.65 ± 0.18	1.01 ± 0.13
TX 3.4	0.16 ± 0.03	1.07 ± 0.15
TX 3.5	1.23 ± 0.21	1.10 ± 0.07
TX 3.6	0.33 ± 0.02	1.22 ± 0.17
TX 3.7	0.67 ± 0.10	1.32 ± 0.10

<sup>a</sup>The nomenclature 'TX' refers to TFIIIA substitution mutations made using Xfin protein finger 31 sequence as the donor. <sup>b</sup>Relative  $K_a$  is determined as the ratio of the apparent association constant for the mutant protein to the apparent association constant for the wild type protein. Average of three or more independent determinations.

The effects of these scanning substitution mutations of TFIIIA on nucleic acid binding activity are summarized in Table 4.2.

The "finger tip" region of finger two and the  $\alpha$ -helical regions of both fingers two and three of TFIIIA are involved in DNA binding. These  $\alpha$ -helical regions contain a number of polar and basic amino acid residues that are in a position suitable for contacting the 5S RNA gene (Figures 4.13 and 4.14). A double mutant designated TX2.7, containing an alanine substitution within the sequence context of the scanning mutant TX2.5, was constructed. The mutant TX2.7, when compared with mutant TX2.5, suggests that the arginine residue at position 62 within finger two is essential for specific recognition of the 5S RNA gene promoter. Finger three provides a critical region within the  $\alpha$ -helix required to maintain high affinity DNA binding (Table 4.2, Figure 4.10). The use of  $\alpha$ -helical residues within the zinc finger to contact DNA is supported by X-ray crystallographic studies of co-crystals containing the DNA binding domains of Zif268 (Pavletich & Pabo, 1991), GLI (Pavletich & Pabo, 1993) and Tramtrack (Fairall et al., 1993) associated with their respective DNA recognition sites.

#### 4.4 Discussion

TFIIIA performs two essential functions in immature oocytes of *Xenopus*. It acts as a positive 5S rRNA gene-specific transcription factor in the nucleus and interacts with 5S RNA to form a stable 7S RNP storage complex in the cytoplasm of immature oocytes. The characterization of TFIIIA structure and function is necessary for understanding the developmental regulation of 5S rRNA genes. *In vitro* transcription-translation of a TFIIIA cDNA (Vrana et al., 1988) and the high yield purification of active recombinant TFIIIA expressed in *E. coli* (Del Rio & Setzer, 1991) have made

mutagenesis studies feasible. In this study, a series of substitution mutants of *Xenopus* TFIIIA were successfully expressed in *E. coli* and purified to homogeneity. The apparent association constants were measured using a nitrocellulose filter binding assay. None of the mutants analyzed exhibited a significant increase in DNA or RNA binding above that of wild type TFIIIA. This suggests that the reduction in DNA binding activity of individual TFIIIA mutants reflects either the loss of wild type DNA base or backbone contacts or a localized change in finger conformation that may affect finger docking.

Studies using deletion mutants of TFIIIA revealed that the N-terminal fingers form specific contacts with the box C element of the 5S rRNA gene promoter, while C-terminal zinc fingers interact with the box A promoter element (Vrana et al., 1988; Fiser-Littel et al., 1988). The locations of contacts between individual zinc fingers of TFIIIA and the 5S rRNA gene promoter have been elucidated using nuclease and chemical protection analyses of truncated TFIIIA mutants (Hanas et al., 1989; Christensen et al., 1991; Hayes & Clemens, 1992; Clemens et al., 1992; Hansen et al., 1993). These studies provide a model for the interaction of TFIIIA with the 5S RNA gene promoter in which fingers one to three, finger five, and fingers seven to nine contact the box C, intermediate, and box A elements, respectively (Clemens et al., 1992; Clemens et al., 1994).

Quantitative binding analysis of truncated TFIIIA mutants suggests that the first three fingers of TFIIIA are sufficient for high affinity interaction with the 5S RNA gene (Liao et al., 1992). In addition, fingers two and three provide a major contribution to the free energy of TFIIIA-5S DNA complex formation (Vrana et al., 1988; Del Rio et al., 1993a). Association of TFIIIA with the 5S RNA gene may be initiated by these N-terminal fingers, as the remaining C-terminal six zinc fingers can not bind to DNA independently.

DNA binding analysis of "broken finger" TFIIIA mutants indicate that zinc fingers three and four provide energetically critical promoter contacts, while fingers one and two are dispensable for high affinity TFIIIA-DNA interaction (Del Rio et al., 1993a). This apparent variability in determination of the energetic contributions made by individual zinc fingers may arise due to differences in TFIIIA mutant protein conformation and methods of analysis. The use of *in vitro* translated TFIIIA places restrictions on the available quantity of protein for analysis (Vrana et al., 1988). In this case, a reduction in DNA binding activity of four-fold or more may be outside the range of available *in vitro* translated protein required to accurately determine the association constant. In addition, the use of truncated or "broken finger" mutants of TFIIIA may result in long range effects on protein-DNA interaction. Local destruction of finger conformation could lead to a misalignment of downstream fingers within the DNA major groove of the 5S RNA gene. Thus, the effect of this local mutation on the DNA binding energy may be amplified by an overall alteration in TFIIIA positioning on the 5S gene promoter.

The lack of energetically significant contacts between the 5S RNA gene and TFIIIA finger one is supported by the present results using Xfin finger 31 substitution. Substitution of Xfin sequence for TFIIIA finger two results in a four-fold reduction in DNA binding activity. This decrease in the association constant may represent the loss of a small number of sequence-specific contacts within the box C promoter element (Vrana et al., 1988; Liao et al., 1992). The importance of finger three in the association of TFIIIA with the 5S RNA gene is suggested by the severe reductions in DNA binding activity shown for Xfin finger 31 substitutions. The critical role finger three plays in

TFIIIA-DNA interaction has been observed in previous TFIIIA mutagenesis studies.

Recently, amino acid sequences within the linker segments separating zinc fingers have been shown to contribute to high affinity DNA binding by TFIIIA (Choo & Klug, 1993; Clemens et al., 1994). These results were obtained in the context of a polypeptide containing zinc fingers one through three of TFIIIA. Substitutions of the conserved TGEKP/N sequence between fingers one and two and between two and three resulted in a significant reduction in DNA binding. In contrast, replacement of the first (TGEKP) and second (TGEKN) linker sequences, with the corresponding Xfin finger 31 sequence (KNERP) did not significantly affect DNA binding of full length TFIIIA (Table 4.2, Figure 4.10). Partial substitution of the third linker sequence (NIKI to KNKI) results in a modest reduction of less than two-fold. Similar results were obtained with substitutions of p43 protein linker sequences (Zang et al., in preparation). Thus, in the context of the full length TFIIIA protein, the contribution made to DNA binding of the wild type N-terminal linker sequences is minimal. Possible changes in finger orientation that could alter TFIIIA interaction with the 5S RNA gene may be compensated for by favorable DNA-finger interactions towards the C-terminus. This indicates that linker sequences do display some versatility and can be swapped between zinc finger proteins. In contrast, substitution of 'natural' linker sequences with irrelevant amino acid residues may abolish their normal function in finger orientation and DNA binding.

Structural comparison between metalloproteins (Berg, 1988) and amino acid sequence analysis of over 150 different  $C_2H_2$  zinc fingers (Gibson et al., 1988) suggest that each finger consists of an N-terminal two-stranded  $\beta$ -sheet and a C-terminal  $\alpha$ -helix stabilized by four zinc binding residues and a

hydrophobic core (Figure 4.1A). Structural analyses of individual zinc fingers by NMR support this model of the zinc finger motif (Párraga et al., 1988; Lee et al., 1989b). Determination of the X-ray crystallographic structure of *Zif268* (Pavletich & Pabo, 1991), *GLI* (Pavletich & Pabo, 1993), and *Tramtrack* (Fairall et al., 1993) complexed with their respective DNA binding sites indicate that amino acids within the zinc finger  $\alpha$ -helix provide the majority of base contacts to DNA. The results of scanning mutagenesis of *TFIIIA* suggests that the  $\alpha$ -helical regions of both fingers two and three are required for high affinity interaction with the 5S RNA gene. However, the contributions made to *TFIIIA*-DNA interaction are not equivalent between the N-terminal zinc fingers. Replacement of finger three sequence reduces DNA binding activity up to six-fold (mutant TX3.4), while finger two substitution results in a four-fold (mutant TX2.7) reduction in DNA binding. Non equivalence in the contribution to the free energy of DNA binding by individual zinc fingers is also observed for "broken finger" (Del Rio et al., 1993a) and truncated (Vrana et al., 1988) mutants of *TFIIIA* and may reflect differences in finger docking and/or the number of contacts made to DNA. The importance of the "finger tip" region in high affinity DNA binding is also observed for finger two of *TFIIIA*. Substitution of this region with the corresponding *Xfin* finger 31 sequence reduces DNA binding activity three-fold (Table 4.2).

Highly conserved amino acid residues within zinc finger domains are expected to be either structurally important or involved in conserved non-specific interactions with the phospho-ribose DNA backbone. In contrast, less conserved residue positions on the external face of the the  $\alpha$ -helix may contribute to DNA binding affinity and specificity (Jacobs, 1992). The position of the  $\alpha$ -helix within the zinc finger domain suggests two functional regions exist: residues involved in internal contacts that serve to stabilize the zinc

finger structure and residues participating in external interactions with the DNA binding site (Figure 4.13). Substitution of either residue type may lead to loss of DNA contacts and a reduction in complex stability. Thus, the  $\alpha$ -helix within each finger may display two functionally distinct groups of amino acid residues.

Based on sequence alignment, amino acids 57 to 68 within finger two and 87 to 99 within finger three of TFIIIA are predicted to form  $\alpha$ -helices (Figure 4.14). Helical wheel representations of these zinc finger regions indicate that, in general, hydrophilic residues are located on one side of the  $\alpha$ -helix and hydrophobic residues are positioned on the opposite side facing toward the internal core of the zinc finger domain (Figure 4.13). The solvent exposed face of the  $\alpha$ -helix within each zinc finger contains core recognition amino acids at positions  $-1$ ,  $+3$  and  $+6$  (relative to the first  $\alpha$ -helical amino acid residue) that were initially identified for *Zif268* (Rebar & Pabo, 1994; Choo & Klug, 1994b) (Figure 4.13). These residue positions are occupied by serine 56, histidine 59, and arginine 62 within finger two and by threonine 86, asparagine 89, and lysine 92 within finger three of TFIIIA (Figures 4.13 and 4.14). Substitution of 56S and 59H could contribute to the three-fold reduction in DNA binding activity for mutant TX2.4 (Table 4.2). In addition, comparison of the effects on DNA binding activity of mutants TX2.5 and TX2.7 suggests a role for 62R in providing energetically important contacts to DNA (Table 4.2).

Substitution of amino acid residues from position 86 through 89 within finger three of TFIIIA results in the greatest reduction in DNA binding (mutant TX3.4; Table 4.2). The most significant changes in amino acid identity are replacement of threonine with glutamic acid at position 86 and asparagine with alanine at position 89 (Figure 4.10). Both of these residues are

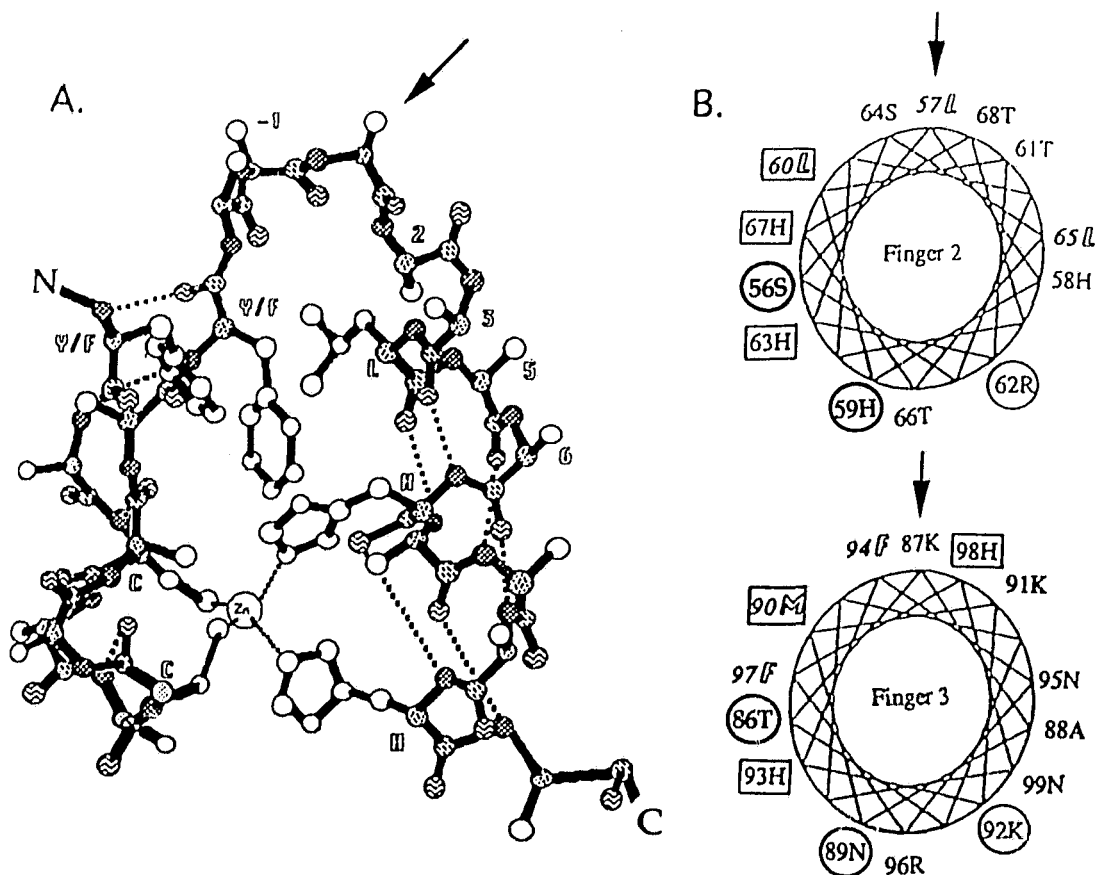


Figure 4.13 Structural representation of a  $C_2H_2$  zinc finger. (A) The zinc finger structure is based on a model proposed by Berg (1988) and shows the polypeptide backbone conformation stabilized by zinc ion binding and a hydrophobic core. (B) Helical wheel diagrams of TFIIIA zinc fingers two and three are shown. Amino acids involved in stabilization of the finger structure are boxed, hydrophobic residues are outlined, and putative core recognition positions are circled (Rebar & Pabo, 1994; Choo & Klug, 1994b). Arrows in Panels (A) and (B) depict the putative first amino acid residue in the helical regions of fingers two and three.

Protein	Finger	Sequence
human GLI	1	TDCRWDGCSQE---FDSQEQLVHHINSEHI <sup>1</sup> GERK
	2	FVCHWGGCSRELRFKAQ <sup>2</sup> YMLVVMRR-HTGEKP
	3	HKCTFEGCRKS---YS <sup>4</sup> RLENLKT <sup>6</sup> HLRS-HTGEKP
	4	YMC <sup>8</sup> EH <sup>10</sup> EGCSKA---FSN <sup>12</sup> ASDRAK <sup>14</sup> QNR <sup>14</sup> THSNE <sup>14</sup> KP
	5	YVCKLP <sup>1</sup> GC <sup>2</sup> TKR---Y <sup>3</sup> TD <sup>4</sup> PS <sup>5</sup> SL <sup>6</sup> R <sup>7</sup> K <sup>8</sup> HVKT <sup>9</sup> VHGPDA
<i>Drosophila</i> TTK	1	YRCKV--CSR <sup>1</sup> V---Y <sup>2</sup> TH <sup>3</sup> IS <sup>4</sup> N <sup>5</sup> FC <sup>6</sup> R <sup>7</sup> HYVTS <sup>8</sup> HKRN <sup>9</sup> VKV
	2	YPCPF--CF <sup>1</sup> KE---FTR <sup>2</sup> K <sup>3</sup> DN <sup>4</sup> MTA <sup>5</sup> VK <sup>6</sup> II <sup>7</sup> HKI
murine Zif268	1	YACPVESCDRR---FSR <sup>1</sup> S <sup>2</sup> DEL <sup>3</sup> TR <sup>4</sup> IRI-HTGQKP
	2	FQCRI--CMRN---FSR <sup>1</sup> S <sup>2</sup> D <sup>3</sup> HL <sup>4</sup> TT <sup>5</sup> IRI-HTGEKP
	3	FACDI--CGRK---FAR <sup>1</sup> S <sup>2</sup> DER <sup>3</sup> K <sup>4</sup> R <sup>5</sup> TKI-HLRQKD
murine Krox20	1	YCPAEGCDRR---FSR <sup>1</sup> S <sup>2</sup> DEL <sup>3</sup> TR <sup>4</sup> IRI-HTGHKP
	2	FQCRI--CMRN---FSR <sup>1</sup> S <sup>2</sup> D <sup>3</sup> HL <sup>4</sup> TT <sup>5</sup> IRI-HTGEKP
	3	FACDY--CGRK---FAR <sup>1</sup> S <sup>2</sup> DER <sup>3</sup> K <sup>4</sup> R <sup>5</sup> TKI-HLRQKER
<i>Xenopus</i> TFIIIA	2	FPCKEEGCEKG---FTSLHHLTR <sup>1</sup> MSLT-HTGEKN
	3	FTCDSDGCDLR---FTTKANM <sup>1</sup> KK <sup>2</sup> KN <sup>3</sup> RF <sup>4</sup> NN <sup>5</sup> IKI

Figure 4.14 Alignment of zinc finger sequences indicating the location of amino acid residues that contact DNA. Protein-DNA contacts were identified by X-ray crystallography for GLI (Pavletich & Pabo, 1993), Tramtrack (Fairall et al., 1993), and Zif268 (Pavletich & Pabo, 1991) proteins. Residues that interact with the phosphate backbone are shown with circles, while those that contact base pairs are boxed. Single amino acid residues within Krox20 that contribute to high affinity DNA binding are indicated with diamonds (Nardelli et al., 1991). Amino acid sequence within fingers two and three of TFIIIA that result in a two-fold or greater reduction in DNA binding activity when substituted are shaded. The amino acid positions within the  $\alpha$ -helix are numbered above the alignment, where one denotes the first  $\alpha$ -helical residue. The amino acid residues comprising the recognition code are shown below the alignment (Choo & Klug, 1994b). Open triangles denote core residue positions, while closed triangles identify auxiliary residue positions.

predicted to occur at positions within the  $\alpha$ -helix involved in DNA base recognition (Rebar & Pabo, 1994; Choo & Klug, 1994b) (Figure 4.14). The third putative core recognition amino acid within finger three of TFIIIA is lysine 92. This position is substituted with an arginine residue in mutant TX3.5 which exhibits wild type DNA binding activity. However, single amino acid substitution of this basic residue with alanine reduces DNA binding substantially (Zang et al., in preparation). Thus, lysine and arginine may be functionally equivalent at this position and maintain the same base and/or phosphate contacts to DNA. The functional equivalence of these basic residues is also observed at the same helical position in finger two of TFIIIA (mutants TX2.5 and TX2.7). The wild type DNA binding activity of substitution mutant TX3.5 also suggests that methionine 90 is not required to stabilize the internal packing or orientation of the  $\alpha$ -helical structure within zinc finger three. In contrast, deletion and/or replacement of the phenylalanine residues at positions 94 and 97 within the  $\alpha$ -helix of finger three may destabilize the central hydrophobic core and contribute to the reduced DNA binding of mutant TX3.6. However, the three-fold reduction in DNA binding affinity for this mutant indicates that only one hydrogen bond contact equivalent is lost suggesting the presence of a more localized effect on finger structure and function. Perhaps, deletion of phenylalanine 94 results in a positional shift of arginine 96 and loss of DNA contacts by this basic residue. Single residue replacement mutations have identified 96R as important for DNA binding and the location of this residue within the C-terminal region of the  $\alpha$ -helix suggests a role in contact to the DNA phosphoribose backbone (Zang et al., in preparation) (Figure 4.14).

The positioning of TFIIIA zinc fingers along the ICR has been the subject of extensive investigation. The N-terminal first three fingers of

TFIIIA protect a region extending from +77 to +96 within the box C element of the ICR from nuclease attack (Christensen et al., 1991; Liao et al., 1992; Clemens et al., 1994). Hydroxyl radical footprinting and SAAB analysis identifies a smaller region within the box C element from +79 to +91 as critical for high affinity TFIIIA-DNA interaction (Clemens et al., 1992; Hayes & Clemens, 1992; Veldhoen et al., 1994). The linker protein sequence between fingers two and three is adjacent to base pair TA84, as determined using photoreactive crosslinking (Lee et al., 1991b). The reduction in DNA binding activity observed for scanning substitution mutants TX2.7, TX3.5 and TX3.6 of TFIIIA suggest that the basic amino acid residues at positions 62, 92 and 96 provide direct contacts to base sequence and/or phosphate groups within the box C element.

Recently, a code for zinc finger-DNA recognition was established using phage display variants of *Zif268* (Rebar & Pabo, 1994; Choo & Klug, 1994a, Choo & Klug, 1994b) (Figure 4.5B). This code may apply to a substantial subset of zinc finger proteins that recognize triplet base pair subsites along the DNA major groove. However, the role that TFIIIA plays in both DNA and RNA binding and the apparent complexity of its promoter binding site suggest that it may utilize a recognition code considerably different from that of the zinc finger subgroup that includes *Zif268*. Although the *Zif268*-derived code may not successfully predict the possible DNA binding sites of all multi-finger proteins, it does indicate that recognition of a DNA binding site can be carried out by a relatively small group of amino acids conserved in their positions (-1, +3, and +6, relative to the first  $\alpha$ -helical residue) within the  $\alpha$ -helix of each zinc finger. The interaction of these core residues with DNA can be modulated by auxiliary amino acids (such as position +2) which may increase DNA sequence discrimination on the basis of sequence-context. By

application of the present code to TFIIIA fingers two and three and comparing this with results obtained from amino acid substitution mutagenesis of TFIIIA and base pair analysis within the ICR (Sakonju & Brown, 1982; Veldhoen et al., 1994), a model may be obtained for the interaction of these fingers with the promoter sequence. The "core" residues within finger two are occupied by 56S, 59H, and 62R. These residues are predicted to recognize a three-base subsite containing the sequence GGN (where N denotes an unknown base identity). This subsite exists within the box C promoter element at base pair positions GC85, GC86, and GC87. It is interesting to note that GC87 provides a small contribution to the free energy of complex formation, suggesting a non optimal amino acid residue at position 56 (serine) within TFIIIA for guanine (or cytosine) contact. This is supported by the recognition code, which predicts a basic residue at this location. In contrast, 62R is predicted to make a strong contact to the guanine base of GC85, which may be reflected by the severe reduction in complex formation upon mutation of either of these moieties. The "core" positions within finger three of TFIIIA are occupied by 86T, 89N, and 92K, while the "auxiliary" position contains alanine (residue 88A). The predicted DNA subsite contacted by this finger includes the sequence GAN. Mutagenesis of the ICR indicate that finger three may interact with a region of the box C sequence that includes GC81, GC82, and AT83 (Veldhoen et al., 1994). The strongest of these contacts occurs with GC81. The recognition code predicts a strong contact to this guanine by amino acid residue 92K, which can be functionally replaced with arginine.

In summary, zinc fingers two and three of TFIIIA are required for high affinity DNA binding. The  $\alpha$ -helix of both fingers is involved in direct contacts to the box C element within the ICR. Basic amino acids that function

in DNA binding are found at conserved positions within the zinc fingers of TFIID and may form contacts with guanine bases on the non coding strand or the phospho-ribose backbone of the 5S RNA gene.

## 5.0 Conclusions

TFIID is a multi-functional protein that has evolved specialized structural domains for interacting with nucleic acids and with other proteins involved in 5S RNA synthesis. The interactions of TFIID with 5S RNA and the 5S RNA gene promoter have been the subject of intensive study over the past fifteen years. These studies have been predominantly qualitative in nature, relying on nuclease and chemical probing of protein-nucleic acid complexes established with wild type or deletion variants of TFIID, 5S RNA, or the 5S RNA gene. The few quantitative studies that have been performed have relied on truncation or substitution mutations within TFIID that may result in gross changes in protein structure. The present TFIID-5S RNA gene analyses have utilized a series of zinc finger sequence substitution mutants as well as single base pair substitution mutants of the 5S RNA gene promoter in an attempt to minimize changes in overall protein or DNA conformation. Changes in binary complex formation were quantified by nitrocellulose filter binding.

Results of these binding studies suggest that TFIID contacts a 13 base pair region of the 5S RNA gene promoter termed the box C element. Guanine-cytosine base pairs within this binding site provide the majority of the free energy of DNA binding with flanking sequence influencing TFIID binding in a context-dependent manner. Zinc fingers two and three are responsible for high affinity interaction with the 5S RNA gene box C

promoter element. Energetically important contacts are mediated through amino acid residues within the  $\alpha$ -helical region of each zinc finger. These results indicate that, although the nine zinc fingers of TFIID interact with a region of the internal promoter extending over 50 base pairs, high affinity protein-DNA complex formation is established by a few strong contacts between amino acids within zinc fingers two and three and DNA base pairs within the box C promoter element. It is likely that the N-terminal fingers of TFIID also make non-specific contacts to the DNA phospho-ribose backbone within the box C element which further increase binary complex stability.

Recently, it has become evident that the RNA binding activity of TFIID is separable from the N-terminal DNA binding activity. The interaction of TFIID with 5S RNA is mediated primarily through zinc fingers four through seven. The present study mapped the location of a truncated polypeptide encompassing these zinc fingers of TFIID using a ribonuclease protection assay. The RNA-binding central fingers of TFIID were found to contact the central core of the 5S RNA molecule and protect helices II, IV, and V as well as loops A and E from ribonuclease attack. These results support a model in which the DNA and RNA binding activities of TFIID are separable within the nucleic acid binding domain. However, all zinc fingers contribute to some degree towards the interactions of TFIID with 5S RNA and the 5S RNA gene.

## 6.0 Literature Cited

Aboul-ela, F., Varani, G., Walker, G. T. and Tinoco, I., Jr., (1988). "The TFIIIA recognition fragment d(GGATGGGAG):d(CTCCCATCC) is B-form in solution." Nucleic Acids Research **16**: 3559-3572.

Allison, L. A., Romaniuk, P. J. and Bakken, A. H. (1991). "RNA-Protein Interactions of Stored 5S RNA with TFIIIA and Ribosomal Protein L5 During *Xenopus* Oogenesis." Developmental Biology **144** (1): 129-144.

Andersen, J., Delihias, N., Hanas, J. S. and Wu, C.-W. (1984). "5S RNA structure and interaction with transcription factor A. 1. Ribonuclease probe of the 7S particle from *Xenopus laevis* immature oocytes and RNA exchange properties of the 7S particle." Biochemistry **23**: 5752-5759.

Andersen, J. and Delihias, N. (1986). "Characterization of RNA-protein interactions in 7S RNP from *Xenopus laevis* oocytes." Journal of Biological Chemistry **261**: 2912-2917.

Andersen, J. (1987). "A model for the interaction of nucleic acids with transcription factor IIIA." FEBS Letters **217**: 197-202.

Andreazzoli, M., Delucchini, S., Costa, M. and Barsacchi, G. (1993). "RNA binding properties and evolutionary conservation of the *Xenopus* multifinger protein Xfin." Nucleic Acids Research **21** (18): 4218-4225.

Andrews, M. T. and Brown, D. D. (1987). "Transient activation of oocyte 5S RNA genes in *Xenopus* embryos by raising the level of the trans-acting factor TFIIIA." Cell **51**: 445-453.

Baudin, F. and Romaniuk, P. J. (1989). "A difference in the importance of bulged nucleotides and their parent base pairs in the binding of transcription factor IIIA to *Xenopus* 5S RNA and 5S RNA genes." Nucleic Acids Research **17** (5): 2043-2056.

Baudin, F., Romby, P., Romaniuk, P. J., Ehresmann, B. and Ehresmann, C. (1989). "Crosslinking of transcription factor TFIIIA to ribosomal 5S RNA from *X. laevis* by trans-diamminedichloroplatinum(II)." Nucleic Acids Research **17**: 10035-10046.

Baudin, F., Romaniuk, P. J., Romby, P., Brunel, C., Westhof, E., Ehresmann, B. and Ehresmann, C. (1991). "Involvement of Hinge Nucleotides of *Xenopus laevis* 5S rRNA in the RNA Structural Organization and in the Binding of Transcription Factor TFIIA." Journal of Molecular Biology 218 (1): 69-81.

Bazett-Jones, D. P. (1988). "Phosphorus imaging of the 7S ribonucleoprotein particle." Journal of Ultrastructure and Molecular Structure Research 99: 59-69.

Bazett-Jones, D. P. and Brown, M. L. (1989). "Electron Microscopy reveals that transcription factor TFIIA bends 5S DNA." Molecular and Cell Biology 9 (1): 336-341.

Berg, J. M. (1988). "Proposed structure for the zinc-binding domains from transcription factor IIIA and related proteins." Proceeding of the National Academy of Sciences of the United States of America 85: 99-102.

Berg, J. M. (1990). "Zinc Finger Domains: Hypotheses and Current Knowledge." Annual Review of Biophysics and Biophysical Chemistry 19: 405-421.

Bernstein, B. E., Hoffman, R. C., Horvath, S., Herriott, J. R. and Klevit, R. E. (1994). "Structure of a Histidine-X(4)-Histidine zinc finger domain - insights into ADR1-UAS1 Protein-DNA recognition." Biochemistry 33 (15): 4460-4470.

Bieker, J. J. and Roeder, R. G. (1984). "Physical properties and DNA-binding stoichiometry of a 5S gene-specific transcription factor." Journal of Biological Chemistry 259: 6158-6164.

Bieker, J. J. and Roeder, R. G. (1986). "Characterization of the nucleotide requirement for elimination of the rate-limiting step in 5S RNA gene transcription." Journal of Biological Chemistry 261: 9732-9738.

Birkenmeier, E. H., Brown, D. D. and Jordan, E. (1978). "A nuclear extract of *Xenopus laevis* oocytes that accurately transcribes 5S RNA genes." Cell 15: 1077-1086.

Bisbee, C. A., Baker, M. A., Wilson, A. C., Hadji-Azini, I. and Fischberg, M. (1977). "Albumin phylogeny for clawed frogs (*Xenopus*)." Science 195: 785-787.

Blackwell, T. K. and Weintraub, H. (1990). "Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection." Science 250: 1104-1110.

Blanco, J., Millstein, L., Razik, M. A., Dilworth, S., Cote, C. and Gottesfeld, J. (1989). "Two TFIIIA activities regulate expression of the *Xenopus* 5S RNA gene families." Genes & Development 3: 1602-1612.

Bogenhagen, D. F., Sakonju, S. and Brown, D. D. (1980). "A control region in the center of the 5S RNA gene directs specific initiation of transcription: II. The 3' border of the region." Cell 19: 27-35.

Bogenhagen, D. F. and Brown, D. D. (1981). "Nucleotide sequences in *Xenopus* 5S DNA required for transcription termination." Cell 24: 261-270.

Bogenhagen, D. F., Wormington, W. M. and Brown, D. D. (1982). "Stable transcription complexes of *Xenopus* 5S RNA genes: a means to maintain the differentiated state." Cell 28: 413-421.

Bogenhagen, D. F. and Sands, M. S. (1992). "Binding of TFIIIA to Derivatives of 5S RNA Containing Sequence Substitutions or Deletions Defines a Minimal TFIIIA Binding Site." Nucleic Acids Research 20 (11): 2639-2645.

Bogenhagen, D. F. (1993). "Proteolytic footprinting of transcription Factor-TFIIIA reveals different tightly binding sites for 5S-RNA and 5S-DNA." Molecular and Cellular Biology 13 (9): 5149-5158.

Böhm, S., Fabian, H., Venyaminov, S. Y., Mateev, S. V., Lucius, H., Welfle, H. and Filimonov, V. V. (1981) "Structural analysis of the A and B conformers of *Escherichia coli* 5S ribosomal RNA by infrared spectroscopy." FEBS Letters 132: 357-361.

Bouvet, P., Dimitrov, S. and Wolffe, A. P. (1994). "Specific regulation of *Xenopus* chromosomal 5S rRNA gene transcription *in vivo* by histone H1." Gene & Development 8 (10): 1147-1159.

Bradford, M. M. (1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." Analytical Biochemistry 72: 248-254.

Braun, B. R., Riggs, D. L., Kassavetis, G. A. and Geiduschek, E. P. (1989). "Multiple states of protein-DNA interaction in the assembly of transcription complexes on *Saccharomyces cerevisiae* 5S ribosomal RNA genes." Proceedings of the National Academy of Sciences of the United States of America 86: 2530-2534.

Brendel, V. and Karlin, S. (1989). "Association of Charge Clusters with Functional Domains of Cellular Transcription Factors." Proceeding of the National Academy of Sciences of the United States of America 86 (15): 5698-5702.

Brown, D. D. and Dawid, I. B. (1968). "Specific gene amplification in oocytes." Science 160: 272-280.

Brown, D. D., Carroll, D. and Brown, R. D. (1977). "The isolation and characterization of a second oocyte 5S DNA from *Xenopus laevis*." Cell 12: 1045-1056.

Brown, D. D. and Gurdon, J. B. (1978). "Cloned single repeating units of 5S DNA direct accurate transcription of 5S RNA when injected into *Xenopus* oocytes." Proceedings of the National Academy of Sciences of the United States of America 75 (6): 2849-2853.

Brown, R. S., Sander, C. and Argos, P. (1985). "The primary structure of transcription factor TFIIA has 12 consecutive repeats." FEBS Letters 186: 271-274.

Brunel, C., Romby, P., Westhof, E., Romaniuk, P. J., Ehresmann, B. and Ehresmann, C. (1990). "Effect of Mutations in Domain-2 on the Structural Organization of Oocyte 5S rRNA from *Xenopus laevis*." Journal of Molecular Biology 215 (1): 103-111.

Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., Jones, C. and Housman, D. E. (1990). "Isolation and characterization of a Zinc Finger polypeptide gene at the Human Chromosome 11 Wilm's Tumor Locus." Cell 60: 509-520.

Callaci, T. P., Cai, G.-Z., Lee, J. C., Daly, T. J. and Wu, C.-W. (1990). "Assembly of *Xenopus* transcription factor IIIA-5S RNA complex." Biochemistry 29: 4653-4659.

Campbell, F. E. and Setzer, D. R. (1991). "Displacement of *Xenopus* Transcription Factor-III<sub>A</sub> from a 5S rRNA-Gene by a Transcribing RNA Polymerase." Molecular and Cellular Biology **11** (8): 3978-3986.

Campbell, F. E. and Setzer, D. R. (1992). "Transcription Termination by RNA Polymerase-III - Uncoupling of Polymerase Release from Termination Signal Recognition." Molecular and Cellular Biology **12** (5): 2260-2272.

Carr, M. D., Pastore, A., Gausepohl, H., Frank, R. and Roesch, P. (1990). "NMR and Molecular Dynamics Studies of the *mKr2* Zinc Finger." European Journal of Biochemistry **188** (2): 455-461.

Chan, Y.-L., Lin, A., McNally, J. and Wool, I. G. (1987). "The primary structure of rat ribosomal protein L5." Journal of Biological Chemistry **262**: 12879-12886.

Chipev, C. C. and Wolffe, A. P. (1992). "Chromosomal Organization of *Xenopus laevis* Oocyte and Somatic 5S rRNA Genes *In vivo*." Molecular Cellular Biology **12** (1): 45-55.

Choo, Y. and Klug, A. (1993). "A role in DNA binding for the linker sequences of the first three zinc fingers of TFIII<sub>A</sub>." Nucleic Acids Research **21** (15): 3341-3346.

Choo, Y. and Klug, A. (1994a). "Toward a code for the interactions of zinc fingers with DNA: selection of randomized fingers displayed on phage." Proceedings of the National Academy of Sciences of the United States of America **91**: 11163-11167.

Choo, Y. and Klug, A. (1994b). "Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions." Proceedings of the National Academy of Sciences of the United States of America **91**: 11168-11172.

Chow, C. S., Hartmann, K. M., Rawlings, S. L., Huber, P. W. and Barton, J. K. (1992). "Delineation of Structural Domains in Eukaryotic 5S rRNA with a Rhodium Probe." Biochemistry **31** (13): 3534-3542.

Christensen, J. H., Hansen, P. K., Lillelund, O. and Thogersen, H. C. (1991). "Sequence-Specific Binding of the N-Terminal Three-Finger Fragment of *Xenopus* Transcription Factor-III A to the Internal Control Region of a 5S RNA Gene." FEBS Letters 281 (1-2): 181-184.

Christiansen, J., Brown, R. S., Sproat, B. S. and Garrett, R. A. (1987). "*Xenopus* transcription factor IIIA binds primarily at junctions between double helical stems and internal loops in oocyte 5S RNA." EMBO Journal 6: 453-460.

Churchill, M. E. A., Tullius, T. D. and Klug, A. (1990). "Mode of interaction of the zinc finger protein TFIIIA with a 5S RNA gene of *Xenopus*." Proceeding of the National Academy of Sciences of the United States of America 87 (7): 5528-5532.

Ciliberto, G., Raugei, G., Costanzo, F., Dente, L. and Cortese, R. (1983). "Common and interchangeable elements in the promoters of genes transcribed by RNA polymerase III." Cell 32: 725-733.

Clark, D. J. and Felsenfeld, G. (1992). "A nucleosome core is transferred out of the path of a transcribing polymerase." Cell 71: 11-22.

Clemens, K. R., Liao, X. B., Wolf, V., Wright, P. E. and Gottesfeld, J. M. (1992). "Definition of the binding sites of individual zinc fingers in the transcription factor IIIA-5S RNA gene complex." Proceedings of the National Academy of Sciences of the United States of America 89 (22): 10822-10826.

Clemens, K. R., Wolf, V., McBryant, S. J., Zhang, P. H., Liao, X. B., Wright, P. E. and Gottesfeld, J. M. (1993). "Molecular basis for specific recognition of both RNA and DNA by a zinc finger protein." Science 260 (5107): 530-533.

Clemens, K. R., Zhang, P., Liao, X., McBryant, S. J., Wright, P. E. and Gottesfeld, J. M. (1994). "Relative contributions of the zinc fingers of transcription factor IIIA to the energetics of DNA binding." Journal of Molecular Biology 244: 23-35.

Cozzarelli, N. R., Gerrard, S. P., Schlissel, M., Brown, D. D. and Bogenhagen, D. F. (1983). "Purified RNA Polymerase III Accurately and Efficiently Terminates Transcription of 5S RNA Genes." Cell 34: 829-835.

Daly, T. J. and Wu, C. W. (1989). "Cooperative DNA Binding by *Xenopus* Transcription Factor IIIA. Use of a 66-Base Pair DNA Fragment Containing the Intragenic Control Region of the 5-S RNA Gene to Study Specific and Nonspecific Interactions." Journal of Biological Chemistry 264 (34): 20394-20402.

Darby, M. K., Andrews, M. T. and Brown, D. D. (1988). "Transcription complexes that program *Xenopus* 5S RNA genes are stable *in vivo*." Proceedings of the National Academy of Sciences of the United States of America 85: 5516-5520.

Darby, M. K. and Joho, K. E. (1992). "Differential Binding of Zinc Fingers from *Xenopus* TFIIIA and p43 to 5S RNA and the 5S RNA Gene." Molecular and Cellular Biology 12 (7): 3155-3164.

Darsillo, P. and Huber, P. W. (1991). "The Use of Chemical Nucleases to Analyze RNA-Protein Interactions - The TFIIIA-5-S rRNA Complex." Journal of Biological Chemistry 266 (31): 21075-21082.

Davanloo, P., Rosenberg, A. H., Dunn, J. J. and Studier, F. W. (1984). "Cloning and expression of the gene for bacteriophage T7 RNA polymerase." Proceedings of the National Academy of Sciences of the United States of America 81: 2035-2039.

de Stevenson, I. L., Romby, P., Baudin, F., Brunel, C., Westhof, E., Ehresmann, C., Ehresmann, B. and Romaniuk, P. J. (1991). "Structural Studies on Site-Directed Mutants of Domain-3 of *Xenopus laevis* Oocyte 5S Ribosomal RNA." Journal of Molecular Biology 219 (2): 243-255.

de Wachter, R., Chen, M. and Vandenberghe, A. (1982). "Conservation of secondary structure in 5S rRNA: a uniform model for eukaryotic, eubacterial, archaebacterial and organelle sequence is energetically favourable." Biochimie 64: 311-329.

de Wachter, R., Chen, M.-W. and Vandenberghe, A. (1984). "Equilibria in 5S ribosomal RNA secondary structure. Bulges and interior loops in 5S RNA secondary structure may serve as articulations for a flexible molecule." European Journal of Biochemistry 143: 175-182.

Del Rio, S. and Setzer, D. R. (1991). "High Yield Purification of Active Transcription Factor IIIA Expressed in *E Coli*." Nucleic Acids Research 19 (22): 6197-6203.

Del Rio, S., Menezes, S. R. and Setzer, D. R. (1993a). "The function of individual zinc fingers in Sequence-Specific DNA recognition by transcription Factor IIIA." Journal of Molecular Biology 233 (4): 567-579.

Del Rio, S. and Setzer, D. R. (1993b). "The role of zinc fingers in transcriptional activation by transcription factor IIIA." Proceedings of the National Academy of Sciences of the United States of America 90 (1): 168-172.

Delihias, N. and Andersen, J. (1982). "Generalized structures of the 5S ribosomal RNAs." Nucleic Acids Research 10: 7323-7344.

Delihias, N., Andersen, J. and Singhal, R. P. (1984). "Structure, function and evolution of 5S ribosomal RNAs." Progress in Nucleic Acid Research and Molecular Biology 31: 161-190.

Denis, H. and Wegnez, M. (1977). "Biochemical research on oogenesis. Oocytes and liver cells of the teleost fish *Tinca tinca* contain different kinds of 5S RNA." Developmental Biology 59: 228-236.

Desjarlais, J. R. and Berg, J. M. (1992). "Toward rules relating zinc finger protein sequences and DNA binding site preferences." Proceedings of the National Academy of Sciences of the United States of America 89 (16): 7345-7349.

Desjarlais, J. R. and Berg, J. M. (1993). "Use of a Zinc-Finger consensus sequence framework and specificity rules to design specific DNA binding proteins." Proceedings of the National Academy of Sciences of the United States of America 90 (6): 2256-2260.

Desjarlais, J. R. and Berg, J. M. (1994). "Length encoded multiplex binding site determination: Application to zinc finger proteins." Proceedings of the National Academy of Sciences of the United States of America 91: 11099-11103.

Diakun, G. P., Fairall, L. and Klug, A. (1986). "EXAFS study of the zinc-binding sites in the protein transcription factor IIIA." Nature 324: 698-699.

Dillon, P. J. and Rosen, C. A. (1990). "A Rapid Method for the Construction of Synthetic Genes Using the Polymerase Chain Reaction." Biotechniques 9 (3): 298.

Eis, P. S. and Lakowicz, J. R. (1993). "Time-Resolved energy transfer measurements of donor acceptor distance distributions and intramolecular flexibility of a CCHH zinc finger peptide." Biochemistry 32 (31): 7981-7993.

Engelke, D. R., Ng, S.-Y., Shastry, B. S. and Roeder, R. G. (1980). "Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes." Cell 19: 717-728.

England, T. E. and Uhlenbeck, O. C. (1978). "Enzymatic Oligoribonucleotide Synthesis with T4 RNA Ligase." Biochemistry 17: 2069-2081.

Erdmann, V. A., Wolters, J., Huysmans, E. and de Wachter, R. (1985). "Collection of published 5S and 4.5S ribosomal sequences." Nucleic Acids Research 13: r105-r153.

Erdmann, V. A., Pieler, T., Wolters, J., Digweed, M., Vogel, D. and Hartmann, R. (1986). Comparative structural and functional studies on small ribosomal RNAs. Structure, function and genetics of ribosomes. New York, Springer-Verlag. 164-183.

Fairall, L., Rhodes, D. and Klug, A. (1986). "Mapping of the sites of protection on a 5S RNA gene by the *Xenopus* transcription factor IIIA. A model for the interaction." Journal of Molecular Biology 192: 577-591.

Fairall, L., Martin, S. and Rhodes, D. (1989). "The DNA binding site of the *Xenopus* transcription factor IIIA has a non-B-form structure." EMBO Journal 8 (6): 1809-1817.

Fairall, L. and Rhodes, D. (1992). "A new approach to the analysis of DNase I footprinting data and its application to the TFIIA/5S DNA complex." Nucleic Acids Research 20 (18): 4727-4731.

Fairall, L., Schwabe, J. W. R., Chapman, L., Finch, J. T. and Rhodes, D. (1993). "The crystal structure of a two Zinc Finger-peptide reveals an extension to the rules for Zinc-Finger DNA recognition." Nature 366 (6454): 483-487.

Fedoroff, N. V. and Brown, D. D. (1977). "The nucleotide sequence of the repeating unit in the oocyte 5S ribosomal DNA of *Xenopus laevis*." Cold Spring Harbor Symposium on Quantitative Biology 42: 1195-1200.

Fedoroff, N. V. and Brown, D. D. (1978). "The nucleotide sequence of oocyte 5S DNA in *Xenopus laevis*. I. The AT-rich spacer." Cell 13: 701-716.

Ford, P. J. and Southern, E. M. (1973). "Different sequences for 5S RNA in kidney cells and ovaries of *Xenopus laevis*." Nature 241: 7-12.

Ford, P. J. and Brown, R. D. (1976). "Sequences of 5S Ribosomal RNA from *Xenopus mulleri* and the Evolution of 5S Gene-Coding Sequences." Cell 8: 485-493.

Fox, G. E. and Woese, C. R. (1975). "5S RNA secondary structure." Nature 256: 505-507.

Frankel, A. D., Berg, J. M. and Pabo, C. O. (1987). "Metal-dependant folding of a single zinc finger from transcription factor IIIA." Proceeding of the National Academy of Sciences of the United States of America 84: 4841-4845.

Gall, J. G. (1968). "Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis." Proceedings of the National Academy of Sciences of the United States of America 60: 553-560.

Gibson, T. J., Postma, J. P. M., Brown, R. S. and Argos, P. (1988). "A model for the tertiary structure of the 28 residue DNA-binding motif ('zinc finger') common to many eukaryotic transcriptional regulatory proteins." Protein Engineering 2 (3): 209-218.

Fiser-Littell, R.M., Duke, A.L., Yanchick, J.S. and Hanas, J.S. (1988). "Deletion of the N-terminal region of *Xenopus* Transcription Factor IIIA inhibits specific binding to the 5S RNA gene." Journal of Biological Chemistry 263 (4): 1607-1610.

Gilbert, D. M. (1986). "Temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells." Proceeding of the National Academy of Sciences of the United States of America 83: 2924-2928.

Ginsberg, A. M., King, B. O. and Roeder, R. G. (1984). "*Xenopus* 5S Gene Transcription Factor, TFIIA: Characterization of a cDNA Clone and Measurement of RNA Levels throughout Development." Cell 39: 479-489.

Gottesfeld, J. and Bloomer, L. S. (1982). "Assembly of transcriptionally active 5S RNA gene chromatin *in vitro*." Cell 28: 781-791.

Gottesfeld, J. M., Blanco, J. and Tennant, L. L. (1987). "The 5S gene internal control region is B-form both free in solution and in a complex with TFIIA." Nature 329: 460-462.

Gottesfeld, J. M., Wolf, V. J., Dang, T., Forbes, D. J. and Hart, P. (1994). "Mitotic repression of RNA polymerase III transcription *in vitro* mediated by phosphorylation of a TFIIB component." Science 263: 81-84.

Gottlieb, E. and Steitz, J. A. (1989a). "Function of the mammalian La protein: evidence for its action in transcription termination by RNA polymerase III." EMBO Journal 8 (3): 851-861.

Gottlieb, E. and Steitz, J. A. (1989b). "The RNA binding protein La influences both the accuracy and the efficiency of RNA polymerase transcription *in vitro*." EMBO Journal 8 (3): 841-850.

Green, L. M. and Berg, J. M. (1989). "A retroviral Cys-Xaa<sub>2</sub>-Cys-Xaa<sub>4</sub>-His-Xaa<sub>4</sub>-Cys peptide binds metal ions: spectroscopic studies and a proposed three dimensional structure." Proceeding of the National Academy of Sciences of the United States of America 86 (6): 4047-4051.

Guddat, U., Bakker A. H. and Pieter, T. (1990). "Protein-Mediated Nuclear Export of RNA: 5S RNA Containing Small RNPs in *Xenopus* Oocytes." Cell 60 (4): 619-628.

Guinta, D. R., Tso, J. Y., Narayanswami, S., Hamkalo, B. A. and Korn, L. J. (1986). "Early replication and expression of oocyte-type 5S RNA genes in a *Xenopus* somatic cell line carrying a translocation." Proceeding of the National Academy of Sciences of the United States of America 83: 5150-5154.

Hall, R. K. and Taylor, W. L. (1989). "Transcription Factor IIIA Gene Expression in *Xenopus* Oocytes Utilizes a Transcription Factor Similar to the Major Late Transcription Factor." Molecular and Cellular Biology 9 (11): 5003-5011.

Han, M. K., Cyran, F. P., Fisher, M. T., Kim, S. H. and Ginsburg, A. (1990). "*Xenopus* Transcription Factor IIIA. Evidence for Heterogeneity of  $Zn^{2+}$  Binding Affinities and Specific Labeling of Cysteine-287." Journal of Biological Chemistry 265 (23): 13792-13799.

Hanas, J. S., Hazuda, D. J., Bogenhagen, D. F., Wu, F. Y.-H. and Wu, C.-W. (1983a). "*Xenopus* transcription factor A requires Zinc for binding to the 5S RNA gene." Journal of Biological Chemistry 258: 14120-14125.

Hanas, J. S., Bogenhagen, D. F. and Wu, C.-W. (1983b). "Cooperative model for the binding of *Xenopus* transcription factor A to the 5S RNA gene." Proceeding of the National Academy of Sciences of the United States of America 80: 2142-2145.

Hanas, J. S., Bogenhagen, D. F. and Wu, C.-W. (1984). "Binding of *Xenopus* transcription factor A to 5S RNA and to single stranded DNA." Nucleic Acids Research 12: 2745-2758.

Hanas, J. S., Duke, A. L. and Gaskins, C. J. (1989). "Conformational states of *Xenopus* transcription factor IIIA." Biochemistry 28: 4083-4088.

Hanas, J. S. and Smith, J. F. (1990). "Identification of a TFIIIA binding site on the 5' flanking region of the TFIIIA gene." Nucleic Acids Research 18 (10): 2923-2928.

Hancock, J. and Wagner, R. (1982). "A structural model of 5S RNA from *E. coli* based on intramolecular crosslinking evidence." Nucleic Acids Research 10: 1257-1269.

Hansen, J. C. and Wolffe, A. P. (1992). "Influence of chromatin folding on transcription initiation and elongation by RNA polymerase III." Biochemistry 31: 7977-7988.

Hansen, P. K., Christensen, J. H., Nyborg, J., Lillelund, O. and Thogersen, H. C. (1993). "Dissection of the DNA-Binding domain of *Xenopus laevis* TFIIA - quantitative DNase-I footprinting analysis of specific complexes between a 5S RNA gene fragment and N-Terminal fragments of TFIIA containing 3-Zinc Finger, 4-Zinc Finger or 5-Zinc Finger domains." Journal of Molecular Biology 233 (2): 191-202.

Harland, R. and Misher, L. (1988). "Stability of RNA in developing *Xenopus* embryos and identification of a destabilizing sequence in TFIIA messenger RNA." Development 102: 837-852.

Harper, M. E., Price, J. and Korn, L. J. (1983). "Chromosomal mapping of *Xenopus* 5S genes: somatic-type versus oocyte-type." Nucleic Acids Research 11 (8): 2313-2323.

Hayes, J., Tullius, T. D. and Wolffe, A. P. (1989). "A protein-protein interaction is essential for stable complex formation on a 5S RNA gene." Journal of Biological Chemistry 264 (11): 6009-6012.

Hayes, J. J. and Clemens, K. R. (1992). "Locations of contacts between individual zinc fingers of *Xenopus laevis* transcription Factor IIIa and the internal control region of a 5S RNA gene." Biochemistry 31 (46): 11600-11605.

Hayes, J. J. and Tullius, T. D. (1992). "Structure of the TFIIA-5S DNA complex." Journal of Molecular Biology 227 (2): 407-417.

Hayes, J. J. and Wolffe, A. P. (1992). "Histones H2A/H2B Inhibit the Interaction of Transcription Factor IIIA with the *Xenopus borealis* Somatic 5S RNA Gene in a Nucleosome." Proceedings of the National Academy of Sciences of the United States of America 89 (4): 1229-1233.

Holmberg, L., Melander, Y. and Nygård, O. (1992). "Ribosome-bound eukaryotic elongation factor 2 protects 5S rRNA from modification." Journal of Biological Chemistry 267 (30): 21906-21910.

Honda, B. M. and Roeder, R. G. (1980). "Association of a 5S gene transcription factor with 5S RNA and altered levels of the factor during cell differentiation." Cell 22: 119-126.

Hori, H. and Osawa, S. (1986). "Evolutionary change in 5S rRNA secondary structure and a phylogenetic tree of 352 5S rRNA species." BioSystems 19: 163-172.

Hori, H. and Osawa, S. (1987). "Origin and evolution of organisms as deduced from 5S ribosomal RNA sequences." Molecular Biology and Evolution 4 (5): 445-472.

Huber, P. W. and Wool, I. G. (1984). "Nuclease protection analysis of ribonucleoprotein complexes: use of the cytotoxic ribonuclease  $\alpha$ -sarcin to determine the binding sites for *Escherichia coli* ribosomal proteins L5, L18, and L25 on 5S rRNA." Proceeding of the National Academy of Sciences of the United States of America 81: 322-326.

Huber, P. W. and Wool, I. G. (1986). "Identification of the binding site on 5S rRNA for the transcription factor IIIA: Proposed structure of a common binding site on 5S rRNA and on the gene." Proceeding of the National Academy of Sciences of the United States of America 83: 1593-1597.

Huber, P. W., Blobel, G. C. and Hartmann, K. M. (1991a). "Conformational Studies of the Nucleic Acid Binding Sites for *Xenopus* Transcription Factor IIIA." Journal of Biological Chemistry 266 (5): 3278-3286.

Huber, P. W., Morii, T., Mei, H. Y. and Barton, J. K. (1991b). "Structural Polymorphism in the Major Groove of a 5S RNA Gene Complements the Zinc Finger Domains of Transcription Factor IIIA." Proceedings of the National Academy of Sciences of the United States of America 88 (23): 10801-10805.

Jackson, S. P. and Tjian, R. (1988). "O-glycosylation of eukaryotic transcription factors: Implications for mechanisms of transcriptional regulation." Cell 55: 125-133.

Jacobs, G. H. (1992). "Determination of the base recognition positions of zinc fingers from sequence analysis." EMBO Journal 11 (12): 4507-4517.

Jamieson, A. C., Kim, S. H. and Wells, J. A. (1994). "In vitro selection of zinc fingers with altered DNA-binding specificity." Biochemistry 33 (19): 5689-5695.

Jasanoff, A. and Weiss, M. A. (1993). "Aromatic-Histidine interactions in the zinc finger motif - structural inequivalence of phenylalanine and tyrosine in the hydrophobic core." Biochemistry 32 (6): 1423-1432.

Kassavetis, G. A., Braun, B. R., Nguyen, L. H. and Geiduschek, E. P. (1990). "S. *Cerevisiae* TFIIB Is the Transcription Initiation Factor Proper of RNA Polymerase III, While TFIIA and TFIIC Are Assembly Factors." Cell 60 (2): 235-245.

Kaulen, H., Pognonec, P., Gregor, P. D. and Roeder, R. G. (1991). "The *Xenopus* B1 Factor Is Closely Related to the Mammalian Activator USF and Is Implicated in the Developmental Regulation of TFIIA Gene Expression." Molecular and Cellular Biology 11 (1): 412-424.

Keller, H. J., You, Q., Romaniuk, P. J. and Gottesfeld, J. M. (1990). "Additional Intragenic Promoter Elements of the *Xenopus* 5S RNA Genes Upstream from the TFIIA Binding Site." Molecular and Cellular Biology 10 (10): 5166-5176.

Keller, H. J., Romaniuk, P. J. and Gottesfeld, J. M. (1992). "Interaction of *Xenopus* TFIIC with the TFIIA-5S RNA gene complex." Journal of Biological Chemistry 267 (25): 18190-18198.

Kim, S. H., Darby, M. K., Joho, K. E. and Brown, D. D. (1990). "The Characterization of the TFIIA Synthesized in Somatic Cells of *Xenopus laevis*." Gene & Development 4 (9): 1602-1610.

Kinzler, K. W., Ruppert, J. M., Bigner, S. H. and Vogelstein, B. (1988) "The *GLI* gene is a member of the *Kruppel* family of zinc finger proteins." Nature 332:371-374.

Kjems, J., Olesen, S. O. and Garrett, R. A. (1985). "Comparison of Eubacterial and Eukaryotic 5S RNA Structures: A chemical modification study." Biochemistry 24: 241-250.

Klevit, R. E. (1991). "Recognition of DNA by Cys2, His2 Zinc Fingers." Science 253 (5026): 1367.

Kochoyan, M., Keutmann, H. T. and Weiss, M. A. (1991a). "Alternating Zinc Fingers in the Human Male Associated Protein ZFY - Refinement of the NMR Structure of an Even Finger by Selective Deuterium Labeling and Implications for DNA Recognition." Biochemistry 30 (29): 7063-7072.

Kochoyan, M., Keutmann, H. T. and Weiss, M. A. (1991b). "Architectural rules of the zinc finger motif: Comparative two-dimensional NMR studies of native and "aromatic-swap" domains define a "weakly polar switch"." Proceedings of the National Academy of Sciences of the United States of America 88: 8455-8459.

Kochoyan, M., Keutmann, H. T. and Weiss, M. A. (1991c). "Alternating Zinc Fingers in the Human Male-Associated Protein ZFY - HX3H and HX4H Motifs Encode a Local Structural Switch." Biochemistry 30 (39): 9396-9402.

Kochoyan, M., Havel, T. F., Nguyen, D. T., Dahl, C. E., Keutmann, H. T. and Weiss, M. A. (1991d). "Alternating Zinc Fingers in the Human Male Associated Protein ZFY - 2D NMR Structure of an Even Finger and Implications for "Jumping Linker" DNA Recognition." Biochemistry 30 (14): 3371-3386.

Korn, L. J. (1982). "Transcription of *Xenopus* 5S ribosomal RNA genes." Nature 295: 101-105.

Korn, L. J. and Brown, D. D. (1978). "Nucleotide sequence of *Xenopus borealis* oocyte 5S DNA: Comparison of sequences that flank several related eucaryotic genes." Cell 15: 1145-1156.

Kriwacki, R. W., Schultz, S. C., Steitz, T. A. and Caradonna, J. P. (1992). "Sequence-Specific recognition of DNA by Zinc Finger peptides derived from the transcription factor Sp1." Proceedings of the National Academy of Sciences of the United States of America 89 (20): 9759-9763.

Krizek, B. A., Amann, B. T., Kilfoil, V. J., Merkle, D. L. and Berg, J. M. (1991). "A Consensus Zinc Finger Peptide - Design, High-Affinity Metal Binding, a pH-Dependent Structure, and a His to Cys Sequence Variant." Journal of the American Chemical Society 113 (12): 4518-4523.

Küntzel, H., Piechulla, B. and Hahn, U. (1983). "Consensus structure and evolution of 5S rRNA." Nucleic Acids Research 11 (3): 893-900.

Kuwahara, J. and Coleman, J. E. (1990). "Role of the Zinc(II) Ions in the Structure of the three-finger DNA Binding Domain of the Sp1 Transcription Factor." Biochemistry 29 (37): 8627-8631.

Lassar, A. B., Martin, P. L. and Roeder, R. G. (1983). "Transcription of class III genes: formation of preinitiation complexes." Science 222: 740-748.

Lee, M. S., Gippert, G. P., Soman, K. V., Case, D. A. and Wright, P. E. (1989a). "Three-Dimensional Solution Structure of a Single Zinc Finger DNA-Binding Domain." Science 245 (4918): 635-637.

Lee, M. S., Cavanagh, J. and Wright, P. E. (1989b). "Complete Assignment of the H-1 NMR Spectrum of a Synthetic Zinc Finger from Xfin- Sequential Resonance Assignments and Secondary Structure." FEBS Letters 254 (1-2): 159-164.

Lee, M. S., Gottesfeld, J. M. and Wright, P. E. (1991a). "Zinc Is Required for Folding and Binding of a Single Zinc Finger to DNA." FEBS Letters 279 (2): 289-294.

Lee, D. K., Evans, R. K., Blanco, J., Gottesfeld, J. and Johnson, J. D. (1991b). "Contacts Between 5' DNA and *Xenopus* TFIIIA Identified Using 5-azido-2'-Deoxyuridine-Substituted DNA." Journal of Biological Chemistry 266 (25): 16478-16484.

Liao, X. B., Clemens, K. R., Tennant, L., Wright, P. E. and Gottesfeld, J. M. (1992). "Specific Interaction of the First-Three Zinc Fingers of TFIIIA with the Internal Control Region of the *Xenopus* 5S RNA Gene." Journal of Molecular Biology 223 (4): 857-871.

Liao, X. B., Clemens, K., Cavanagh, J., Tennant, L. and Wright, P. E. (1994). "H-1, N-15 and C-13 resonance assignments for the first three zinc fingers of transcription factor IIIA." Journal of Biomolecular NMR 4 (3): 433-454.

Lorch, Y., LaPointe, J. W. and Kornberg, R. D. (1987). "Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones." Cell 49: 203-210.

Losa, R. and Brown, D. D. (1987). "A bacteriophage RNA polymerase transcribes *in vitro* through a nucleosome core without displacing it." Cell 50: 801-808.

Majowski, K., Mentzel, H. and Pieler, T. (1987). "A split binding site for TFIIC on the *Xenopus* 5S gene." EMBO Journal 6: 3057-3063.

Mannella, C. A., Frank, J. and Delihias, N. (1987). "Interrelatedness of 5S RNA sequences investigated by correspondence analysis." Journal of Molecular Evolution 24: 228-235.

Mao, X. Z. and Darby, M. K. (1993). "A Position-Dependent Transcription-Activating domain in TFIIA." Molecular Cellular Biology 13 (12): 7496-7506.

Martinez, E., Lagna, G. and Roeder, R. G. (1994). "Overlapping transcription by RNA polymerase II and III of the *Xenopus* TFIIA gene in somatic cells." Journal of Biological Chemistry 269: 25692-25698.

Matsumoto, Y. and Korn, L. J. (1988). "Upstream sequences required for transcription of the TFIIA gene in *Xenopus* oocytes." Nucleic Acids Research 16: 3801-3814.

McBryant, S.J., Veldhoen, N., Gedulin, B., Leresche, A., Foster, M., Wright, P.E., Romaniuk, P.J. and Gottesfeld, J.M. (1995) "Interaction of the RNA binding fingers of *Xenopus* transcription factor IIIA with specific regions of 5S ribosomal RNA." Journal of Molecular Biology 248: 44-57.

McCall, M., Brown, T., Hunter, W. N. and Kennard, O. (1986). "The crystal structure of d(GGATGGGAG):d(CTCCCATCC) forms an essential part of the binding site for transcription factor IIIA." Nature 322: 661-664.

McConkey, G. A. and Bogenhagen, D. F. (1987). "Transition mutations within the *Xenopus borealis* somatic 5S RNA gene can have independent effects on transcription and TFIIA binding." Molecular and Cellular Biology 7: 486-494.

McConkey, G. A. and Bogenhagen, D. F. (1988). "TFIIA binds with equal affinity to somatic and major oocyte 5S RNA genes." Genes & Development 2: 205-214.

Michael, S. F., Kilfoil, V. J., Schmidt, M. H., Amann, B. T. and Berg, J. M. (1992). "Metal Binding and Folding Properties of a Minimalist Cys<sup>2</sup>His<sup>2</sup> Zinc Finger Peptide." Proceedings of the National Academy of Sciences of the United States of America 89 (11): 4796-4800.

Miller, J. R., Cartwright, E. M., Brownlee, G. G., Fedoroff, N. V. and Brown, D. D. (1978). "The nucleotide sequence of oocyte 5S DNA in *Xenopus laevis*. II. The GC-rich region." Cell 13: 717-725.

Miller, J., McLachlan, A. D. and Klug, A. (1985). "Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus oocytes*." EMBO Journal 4: 1609-1614.

Miller, J., Fairall, L. and Rhodes, D. (1989). "A Novel Method for the Purification of the *Xenopus* Transcription Factor IIIA." Nucleic Acids Research 17 (22): 9185-9192.

Millstein, L., Eversole-Cire, P., Blanco, J. and Gottesfeld, J. M. (1987). "Differential transcription of *Xenopus* oocyte and somatic-type 5S genes in a *Xenopus* oocyte extract." Journal of Biological Chemistry 262: 17100-17110.

Nagai, K., Nakaseko, Y., Nasmyth, K. and Rhodes, D. (1988). "Zinc-finger motifs expressed in *E. coli* and folded *in vitro* direct specific binding to DNA." Nature 332: 284-286.

Nakaseko, Y., Neuhaus, D., Klug, A. and Rhodes, D. (1992). "Adjacent Zinc-Finger motifs in multiple Zinc-Finger peptides from C<sup>1</sup>WI5 form structurally independent, flexibly linked domains." Journal of Molecular Biology 228 (2): 619-636.

Nardelli, J., Gibson, T. J., Vesque, C. and Charnay, P. (1991). "Base Sequence Discrimination by Zinc-Finger DNA-Binding Domains." Nature 349 (6305): 175-178.

Nazar, R. N. and Wildeman, A. G. (1983). "Three helical domains form a protein binding site in the 5S RNA-protein complex from eukaryotic ribosomes." Nucleic Acids Research 11: 3155-3168.

Nekludova, L. and Pabo, C. O. (1994). "Distinctive DNA conformation with enlarged major groove is found in zinc finger-DNA and other protein-DNA complexes." Proceedings of the National Academy of Sciences of the United States of America 91 (15): 6948-6952.

Nelson, R. M. and Long, G. L. (1989). "A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction." Analytical Biochemistry 180: 147-151.

Nendza, R., Digweed, M., Meyer, H. E., Erdmann, V. A. and Mayr, G. W. (1987). "5S rRNA-containing ribonucleoproteins from rabbit muscle and liver. Complex and partial primary structures." European Journal of Biochemistry 109: 85-95.

Neuhaus, D., Nakaseko, Y., Schwabe, J. W. R. and Klug, A. (1992). "Solution structures of two Zinc-Finger domains from SWI5 obtained using 2-Dimensional H-1 nuclear magnetic resonance spectroscopy - a Zinc Finger structure with a 3rd strand of  $\beta$ -Sheet." Journal of Molecular Biology 228 (2): 637-651.

Ng, S. Y., Parker, C. S. and Roeder, R. G. (1979). "Transcription of cloned *Xenopus* 5S RNA genes by *X. laevis* RNA polymerase III in reconstituted systems." Proceedings of the National Academy of Sciences of the United States of America 76 (1): 136-140.

Nickol, J. and Rau, D. C. (1992). "Zinc induces a bend within the transcription factor IIIA Binding region of the 5-S RNA gene." Journal of Molecular Biology 228 (4): 1115-1123.

Nishikawa, K. and Takemura, S. (1974). "Nucleotide sequence of 5S RNA from *Torulopsis utilis*." FEBS Letters 40: 106-109.

Nygård, O. and Nilsson, L. (1987). "The ribosomal binding site for eukaryotic elongation factor EF-2 contains 5S ribosomal RNA." Biochimica et Biophysica Acta 908: 46-53.

Oei, S.-L. and Pieler, T. (1990). "A transcription stimulatory factor binds to the upstream region of *Xenopus* 5S RNA and tRNA genes." Journal of Biological Chemistry 265 (13): 7485-7491.

Omichinski, J. G., Clore, G. M., Appella, E., Sakaguchi, K. and Gronenborn, A. M. (1990). "High-Resolution 3-Dimensional Structure of a Single Zinc Finger from a Human Enhancer Binding Protein in Solution." Biochemistry 29 (40): 9324-9334.

Omichinski, J. G., Clore, G. M., Robien, M., Sakaguchi, K., Appella, E. and Gronenborn, A. M. (1992). "High-Resolution Solution Structure of the Double Cys2His2 Zinc Finger from the Human Enhancer Binding Protein MBP-1." Biochemistry 31 (16): 3907-3917.

Palmer, A. G., Rance, M. and Wright, P. E. (1991). "Intramolecular Motions of a Zinc Finger DNA-Binding Domain from Xfin Characterized by Proton-Detected Natural Abundance C-12 Heteronuclear NMR Spectroscopy." Journal of the American Chemical Society 113 (12): 4371-4380.

Pardue, M. L., Brown, D. D. and Birnstiel, M. L. (1973). "Location of the genes for 5S Ribosomal RNA in *Xenopus laevis*." Chromosoma 42: 191-203.

Parker, C. S. and Roeder, R. G. (1977). "Selective and accurate transcription of the *Xenopus laevis* 5S RNA genes in isolated chromatin by purified RNA polymerase III." Proceedings of the National Academy of Sciences of the United States of America 74 (1): 44-48.

Párraga, G., Horvath, S. J., Eisen, A., Taylor, W. E., Hood, L., Young, E. T. and Kleit, R. E. (1988). "Zinc-dependent structure of a single finger domain of yeast ADR1." Science 241: 1489-1492.

Parsons, M. C. and Weil, P. A. (1990). "Purification and characterization of *Saccharomyces cerevisiae* transcription factor TFIIC." Journal of Biological Chemistry 265 (9): 5095-5103.

Pavletich, N. P. and Pabo, C. O. (1991). "Zinc Finger-DNA Recognition - Crystal Structure of a Zif268-DNA Complex at 2.1Å." Science 252 (5007): 809-817.

Pavletich, N. P. and Pabo, C. O. (1993). "Crystal structure of a 5-finger GIL-DNA complex - new perspectives on zinc fingers." Science 261 (5129): 1701-1707.

Peattie, D. A., Douthwaite, S., Garrett, R. A. and Noller, H. F. (1981). "A bulged double helix in a RNA-protein contact site." Proceeding of the National Academy of Sciences of the United States of America 78: 7331-7335.

Pelham, H. R. B. and Brown, D. D. (1980). "A specific transcription factor that can bind either the 5S RNA gene or 5S RNA." Proceeding of the National Academy of Sciences of the United States of America 77: 4170-4174.

Pelham, H. R. B., Wormington, W. M. and Brown, D. D. (1981). "Related 5S RNA transcription factors in *Xenopus* oocytes and somatic cells." Proceeding of the National Academy of Sciences of the United States of America 78: 1760-1764.

Pellegrino, G. R. and Berg, J. M. (1991). "Identification and Characterization of Zinc Finger Domains by the Polymerase Chain Reaction." Proceedings of the National Academy of Sciences of the United States of America 88 (2): 671-675.

Peterson, R. C., Doering, J. L. and Brown, D. D. (1980). "Characterization of two *Xenopus* somatic 5S DNAs and one minor oocyte-specific 5S DNA." Cell 20: 131-141.

Pfaff, S. L., Hall, R. K., Hart, G. C. and Taylor, W. L. (1991). "Regulation of the *Xenopus laevis* Transcription Factor IIIA Gene During Oogenesis and Early Embryogenesis - Negative Elements Repress the O-TFIIIA Promoter in Embryonic Cells." Developmental Biology 145 (2): 241-254.

Pfaff, S. L. and Taylor, W. L. (1992). "Characterization of a *Xenopus* Oocyte Factor That Binds to a Developmentally Regulated cis-Element in the TFIIIA Gene." Developmental Biology 151 (1): 306-316.

Picard, B. and Wegnez, M. (1979). "Isolation of a 7S particle from *Xenopus laevis* oocytes: a 5S RNA-protein complex." Proceeding of the National Academy of Sciences of the United States of America 76: 241-245.

Picard, B., le Maire, M., Wegnez, M. and Denis, H. (1980). "Biochemical research on oogenesis: composition of the 42S storage particles of *Xenopus laevis* oocytes." European Journal of Biochemistry 109: 359-368.

Pieler, T. and Erdmann, V. A. (1982). "Three-dimensional structural model of eubacterial 5S RNA that has functional implications." Proceeding of the National Academy of Sciences of the United States of America 79: 4599-4603.

Pieler, T. and Erdmann, V. A. (1983). "Isolation and characterization of a 7S RNP particle from mature *Xenopus laevis* oocytes." FEBS Letters 157: 283-287.

Pieler, T., Schreiber, A. and Erdmann, V. A. (1984a). "Comparative structural analysis of eubacterial 5S rRNA by oxidation of adenines in the N-1 position." Nucleic Acids Research 12: 3115-3126.

Pieler, T., Erdmann, V. A. and Appel, B. (1984b). "Structural requirements for the interaction of 5S rRNA with the eukaryotic transcription factor IIIA." Nucleic Acids Research 12: 8393-8406.

Pieler, T., Appel, B., Oei, S. L., Mentzel, H. and Erdmann, V. A. (1985a). "Point mutational analysis of the *Xenopus laevis* 5S gene promoter." EMBO Journal 4 (7): 1847-1853.

Pieler, T., Oei, S.-L., Hamm, J., Engelke, U. and Erdmann, V. A. (1985b). "Functional domains of the *Xenopus laevis* 5S gene promoter." EMBO Journal 4: 3751-3756.

Pieler, T., Guddat, U., Oei, S. L. and Erdmann, V. A. (1986). "Analysis of the RNA structural elements involved in the binding of the transcription factor IIIA from *Xenopus laevis*." Nucleic Acids Research 14: 6313-6327.

Pieler, T., Hamm, J. and Roeder, R. G. (1987). "The 5S gene internal control region is composed of three distinct sequence elements, organized as two functional domains with variable spacing." Cell 48: 91-100.

Pierandrei-Amaldi, P. and Campioni, N. (1982). "Expression of ribosomal protein genes in *Xenopus laevis* development." Cell 30: 163-171.

Rebar, E. J. and Pabo, C. O. (1994). "Zinc finger phage - affinity selection of fingers with new DNA-Binding specificities." Science 263 (5147): 671-673.

Regan, L. and Clarke, N. D. (1990). "A tetrahedral Zinc(II) binding site introduced into a designed protein." Biochemistry 29: 10878-10883.

Reynolds, W. F. and Gottesfeld, J. M. (1983). "5S rRNA gene transcription factor IIIA alters the helical configuration of DNA." Proceeding of the National Academy of Sciences of the United States of America 80: 1862-1866.

Rhodes, D. (1985). "Structural analysis of a triple complex between the histone octamer, a *Xenopus* gene for 5S RNA and transcription factor IIIA." EMBO Journal 4: 3473-3482.

Rhodes, D. and Klug, A. (1986). "An underlying repeat in some transcriptional control sequences corresponding to half a double helical turn of DNA." Cell 46: 123-132.

Rollins, M. B., Delrio, S., Galey, A. L., Setzer, D. R. and Andrews, M. T. (1993). "Role of TFIIIA zinc fingers in vivo - analysis of Single-Finger function in developing *Xenopus* embryos." Molecular and Cellular Biology 13 (8): 4776-4783.

Romaniuk, P. J. (1985). "Characterization of the RNA binding properties of transcription factor IIIA of *Xenopus laevis* oocytes." Nucleic Acids Research 13 (14): 5369-5387.

Romaniuk, P. J., de Stevenson, I. L. and Wong, H.-H. A. (1987a). "Defining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric 5S RNA molecules." Nucleic Acids Research 15 (6): 2737-2755.

Romaniuk, P. J., Lowary, P., Wu, H.-N., Stormo, G. and Uhlenbeck, O. C. (1987b). "RNA binding site of R17 coat protein." Biochemistry 26: 1563-1568.

Romaniuk, P. J., de Stevenson, I. L., Ehresmann, C., Romby, P. and Ehresmann, B. (1988). "A comparison of the solution structures and conformational properties of the somatic and oocyte 5S rRNAs of *Xenopus laevis*." Nucleic Acids Research 16 (5): 2295-2312.

Romaniuk, P. J. (1989). "The role of highly conserved single-stranded nucleotides of *Xenopus* 5S RNA in the binding of transcription factor IIIA." Biochemistry 28: 1388-1395.

Romaniuk, P. J., de Stevenson, I. L. and You, Q., Ed. T. R. Cech (1989). The specificity of the RNA binding activity of *Xenopus* transcription factor IIIA. Molecular Biology of RNA. New York, Alan R. Liss, Inc.

Romaniuk, P. J. (1990). "Characterization of the Equilibrium Binding of *Xenopus* Transcription Factor IIIA to the 5S RNA Gene." Journal of Biological Chemistry 265 (29): 17593-17600.

Romby, P., Westhof, E., Toukifimpa, R., Mache, R., Ebel, J.-P., Ehresmann, C. and Ehresmann, B. (1988). "Higher order structure of chloroplastic 5S ribosomal RNA from spinach." Biochemistry 27: 4721-4730.

Ruiz i Altaba, A., Perry-O'Keefe, H. and D.A., M. (1987). "*Xfin*: an embryonic gene encoding a multifingered protein in *Xenopus*." EMBO Journal 6: 3065-3070.

Sakonju, S., Bogenhagen, D. F. and Brown, D. D. (1980). "A control region in the center of the 5S RNA gene directs specific initiation of transcription: I. The 5' border of the region." Cell 19: 13-25.

Sakonju, S., Brown, D. D., Engelke, D., Ng, S.-Y., Shastry, B. S. and Roeder, R. G. (1981). "The binding of a transcription factor to deletion mutants of a 5S rRNA gene." Cell 23: 665-669.

Sakonju, S. and Brown, D. D. (1982). "Contact points between a positive transcription factor and the *Xenopus* 5S RNA gene." Cell 31: 395-405.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Sands, M. S. and Bogenhagen, D. F. (1987). "TFIIIA binds to different domains of 5S RNA and the *Xenopus borealis* 5S RNA gene." Molecular and Cellular Biology 7: 3985-3993.

Sands, M. S. and Bogenhagen, D. F. (1991). "The Carboxyterminal Zinc Fingers of TFIIIA Interact with the Tip of Helix V of 5S RNA in the 7S Ribonucleoprotein Particle." Nucleic Acids Research 19 (8): 1791-1796.

Sarge, K. D. and Maxwell, E. S. (1991). "Intermolecular hybridization of 5S rRNA with 18S rRNA: Identification of a 5'-terminally-located nucleotide sequence in mouse 5S rRNA which base-pairs with two specific complementary sequences in 18S rRNA." Biochimica et Biophysica Acta **1088**: 57-70.

Sawadogo, M. and Roeder, R. G. (1985). "Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region." Cell **43**: 439-448.

Schlissel, M. S. and Brown, D. D. (1984). "The Transcriptional Regulation of *Xenopus* 5S RNA Genes in Chromatin: The Roles of Active Stable Transcription Complexes and Histone H1." Cell **37**: 903-913.

Schneider, H. R., Waldschmidt, R. and Seifart, K. H. (1990). "Human Transcription Factor III C Contains a Polypeptide of 55 kDa Specifically Binding to Pol III Genes." Nucleic Acids Research **18** (16): 4743-4750.

Schroth, G. P., Cook, G. R., Bradbury, E. M. and Gottesfeld, J. M. (1989). "Transcription Factor III A Induced Bending of the *Xenopus* Somatic 5S Gene Promoter." Nature **340** (6233): 487-488.

Schroth, G. P., Gottesfeld, J. M. and Bradbury, E. M. (1991). "TFIIIA Induced DNA Bending - Effect of Low Ionic Strength Electrophoresis Buffer Conditions." Nucleic Acids Research **19** (3): 511-516.

Scotto, K. W., Kaulen, H. and Roeder, R. G. (1989). "Positive and negative regulation of the gene for transcription factor III A in *Xenopus laevis* oocytes." Genes & Development **3**: 651-662.

Segall, J., Matsui, T. and Roeder, R. G. (1980). "Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III." Journal of Biological Chemistry **255** (24): 11986-11991.

Sekiguchi, J. A. M., Cole, A. D., Gardner, K. H. and Kmiec, E. B. (1990). "Transcription Factor TFIIIA Stimulates DNA Supercoiling Promoted by a Fractionated Cell-Free Extract from *Xenopus laevis*." European Journal of Biochemistry **192** (2): 311-320.

Setzer, D. R. and Brown, D. D. (1985). "Formation and stability of the 5S RNA transcription complex." Journal of Biological Chemistry 260 (4): 2483-2492.

Shang, Z., Liao, Y.-D., Wu, F. Y.-H. and Wu, C.-W. (1989). "Zinc release from *Xenopus* transcription factor IIIA induced by chemical modifications." Biochemistry 28: 9790-9795.

Shastry, B. S., Ng, S.-Y. and Roeder, R. G. (1982). "Multiple factors involved in the transcription of class III genes in *Xenopus laevis*." Journal of Biological Chemistry 257: 12979-12986.

Shastry, B. S., Honda, B. M. and Roeder, R. G. (1984). "Altered levels of a 5S gene-specific transcription factor (TFIIIA) during oogenesis and embryonic development of *Xenopus laevis*." Journal of Biological Chemistry 259: 11373-11382.

Smith, D. R., Jackson, I. J. and Brown, D. D. (1984). "Domains of the positive transcription factor specific for the *Xenopus* 5S RNA gene." Cell 37: 645-652.

Smith, J. F., Hawkins, J., Leonard, R. E. and Hanas, J. S. (1991). "Structural Elements in the N-Terminal Half of Transcription Factor IIIA Required for Factor Binding to the 5S RNA Gene Internal Control Region." Nucleic Acids Research 19 (24): 6871-6876.

Stahl, D. A., Luehrsen, K. R., Woese, C. R. and Pace, N. R. (1981). "An unusual 5S rRNA, from *Sulfolobus acidocaldarius*, and its implications for a general 5S rRNA structure." Nucleic Acids Research 9: 6129-6137.

Steitz, J. A., Berg, C., Hendrick, J. P., La Branche-Chabot, H., Metspalu, A., Rinke, J. and Yario, T. (1988). "A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells." Journal of Cell Biology 106: 545-556.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990). "Gene Expression Technology. Use of T7 RNA polymerase to direct expression of cloned genes." Methods of Enzymology 185: 60-89.

Tang, B. Z. and Nazar, R. N. (1991). "Structure of the Yeast Ribosomal 5S RNA Binding Protein YL3." Journal of Biological Chemistry 266 (10): 6120-6123.

Taylor, W., Jackson, I. J., Siegel, N., Kumar, A. and Brown, D. D. (1986). "The developmental expression of the gene for TFIIIA in *Xenopus laevis*." Nucleic Acids Research 14: 6185-6195.

Theunissen, O., Rudt, F., Guddat, U., Mentzel, H. and Pieler, T. (1992). "RNA and DNA binding zinc fingers in *Xenopus* TFIIIA." Cell 71 (4): 679-690.

Timmins, P. A., Langowski, J. and Brown, R. S. (1988). "An elongated model of the *Xenopus laevis* transcription factor IIIA-5S ribosomal RNA complex derived from neutron scattering and hydrodynamic measurements." Nucleic Acids Research 16: 8633-8644.

Toots, I., Misselwitz, R., Böhm, S., Welfle, H., VILLEMS, R. and Saarma, M. (1982). "Two distinct conformations of rat liver ribosomal 5S RNA." Nucleic Acids Research 10: 3381-3389.

Tremethick, D., Zucker, K. and Worcel, A. (1990). "The Transcription Complex of the 5S RNA Gene, But Not Transcription Factor IIIA Alone, Prevents Nucleosomal Repression of Transcription." Journal of Biological Chemistry 265 (9): 5014-5023.

Tso, J. Y., Van Der Berg, D. J. and Korn, L. J. (1986). "Structure of the gene for *Xenopus* transcription factor TFIIIA." Nucleic Acids Research 14: 2187-2200.

Veldhoen, N., You, Q. M., Setzer, D. R. and Romaniuk, P. J. (1994). "Contribution of individual base pairs to the interaction of TFIIIA with the *Xenopus* 5S RNA gene." Biochemistry 33 (24): 7568-7575.

Vogel, D. W., Hartmann, R. K., Bartsch, M., Subramanian, A. R., Kleinow, W., O'Brien, T. W., Pieler, T. and Erdmann, V. A. (1984). "Reconstitution of 50S ribosomal subunits from *Bacillus stearothermophilus* with 5S RNA from spinach chloroplast and low-Mr RNA from mitochondria of *Locusta migratoria* and bovine liver." FEBS Letters 169: 67-72.

Vrana, K. E., Churchill, M. E. A., Tullius, T. D. and Brown, D. D. (1988). "Mapping functional regions of transcription factor TFIIIA." Molecular and Cellular Biology 8: 1684-1696.

Weil, P., Segall, J., Harris, B., Ng, S.-Y. and Roeder, R. G. (1979). "Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates." Journal of Biological Chemistry 254 (13): 6163-6173.

Westhof, E., Romby, P., Romaniuk, P. J., Ebel, J.-P., Ehresmann, C. and Ehresmann, B. (1989). "Computer modeling from solution data of spinach chloroplast and of *Xenopus laevis* somatic and oocyte 5S rRNAs." Journal of Molecular Biology 207: 417-431.

Wimberly, B., Varani, G. and Tinoco, I. (1993). "The conformation of Loop-E of eukaryotic 5S Ribosomal RNA." Biochemistry 32 (4): 1078-1087.

Wolffe, A. P., Jordan, E. and Brown, D. D. (1986). "A Bacteriophage RNA Polymerase Transcribes through a *Xenopus* 5S RNA Gene Transcription Complex without Disrupting it." Cell 44: 381-389.

Wolffe, A. P. and Brown, D. D. (1986). "DNA replication in vitro erases a *Xenopus* 5S RNA gene transcription complex." Cell 47: 217-227.

Wolffe, A. P. and Brown, D. D. (1987). "Differential 5S RNA gene expression *in vitro*." Cell 51: 733-740.

Wolffe, A. P. (1988). "Transcription factor TFIIC can regulate differential *Xenopus* 5S RNA gene transcription *in vitro*." EMBO Journal 7: 1071-1079.

Wolffe, A. P. and Drew, H. R. (1989). "Initiation of transcription on nucleosomal templates." Proceeding of the National Academy of Sciences of the United States of America 86: 9817-9821.

Wolffe, A. P. and Morse, R. H. (1990). "The transcription complex of the *Xenopus* somatic 5S RNA gene: A functional analysis of protein-DNA interactions outside the internal control region." Journal of Biological Chemistry 265 (8): 4592-4599.

Wolters, J. and Erdmann, V. A. (1988). "Compilation of 5S rRNA and 5S rRNA gene sequences." Nucleic Acids Research 16 (supplement): r1-r9.

Wormington, W. M., Bogenhagen, D. F., Jordan, E. and Brown, D. D. (1981). "A quantitative assay for *Xenopus* 5S RNA gene transcription *in vitro*." Cell 24: 809-817.

Wormington, W. M. and Brown, D. D. (1983). "Onset of 5S RNA gene regulation during *Xenopus* embryogenesis." Developmental Biology 99: 248-257.

Wormington, W. M. (1989). "Developmental Expression and 5S rRNA-Binding Activity of *Xenopus laevis* Ribosomal Protein L5." Molecular and Cellular Biology 9 (12): 5281-5288.

Wu, H.-N. and Uhlenbeck, O. C. (1987). "Role of a bulged A residue in a specific RNA-protein interaction." Biochemistry 26: 8221-8227.

Xing, Y. Y. and Worcel, A. (1989a). "The C-terminal domain of transcription factor IIIA interacts differently with different 5S RNA genes." Molecular and Cellular Biology 9: 499-514.

Xing, Y. Y. and Worcel, A. (1989b). "A 3' Exonuclease Activity Degrades the Pseudogene 5S RNA Transcript and Processes the Major Oocyte 5S RNA Transcript in *Xenopus* Oocytes." Genes & Development 3 (7): 1008-1018.

Xu, R. X., Horvath, S. J. and Klevit, R. E. (1991). "ADR1a, a Zinc Finger Peptide, Exists in Two Folded Conformations." Biochemistry 30 (14): 3365-3371.

Yamashita, M. M., Wesson, L., Eisenman, G. and Eisenberg, D. (1990). "Where metal ions bind in proteins." Proceedings of the National Academy of Sciences of the United States of America 87: 5648-5652.

You, Q. and Romaniuk, P. J. (1990). "The Effects of Disrupting 5S RNA Helical Structures on the Binding of *Xenopus* Transcription Factor IIIA." Nucleic Acids Research 18 (17): 5055-5062.

You, Q., Veldhoen, N., Baudin, F. and Romaniuk, P. J. (1991). "Mutations in 5S DNA and 5S RNA Have Different Effects on the Binding of *Xenopus* Transcription Factor IIIA." Biochemistry 30 (9): 2495-2500.

Zang, W-Q., Veldhoen, N. and Romaniuk, P.J. (1995). "The effect of substitution mutations of transcription factor IIIA on nucleic acid binding activity." in preparation.

Zhang, P. and Moore, P. B. (1989). "An NMR study of the helix V-loop E region of the 5S RNA from *Escherichia coli*." Biochemistry 28: 4607-4615.

Zwieb, C. and Brown, R. S. (1990). "Absence of substantial bending in *Xenopus laevis* transcription factor IIIA-DNA complexes." Nucleic Acids Research 18 (3): 583-587.

## VITA

Surname: Veldhoen

Given Names: Nicholas Jan

Place of Birth: Nairobi, Kenya

### Educational Institutions Attended:

University of Victoria

1984 to 1995

### Degrees Awarded:

B. Sc.

University of Victoria

1989

### Publications:

Zang, W-Q., Veldhoen, N. and Romaniuk, P.J. (1995) "The effect of substitution mutations of transcription factor IIIA on nucleic acid binding activity." in preparation.

McBryant, S.J., Veldhoen, N., Gedulin, B., Leresche, A., Foster, M., Wright, P.E., Romaniuk, P.J. and Gottesfeld, J.M. (1995) "Interaction of the RNA binding fingers of *Xenopus* transcription factor IIIA with specific regions of 5S ribosomal RNA." Journal of Molecular Biology. Vol. 248, No. 1. pp. 44-57.

Veldhoen, N., You, Q., Setzer, D.R. and Romaniuk, P.J. (1994) "Contributions of individual base pairs to the interaction of TFIIIA with the *Xenopus* 5S RNA gene." Biochemistry Vol. 33, No. 24. pp. 7568-7575.

You, Q., Veldhoen, N., Baudin, F. and Romaniuk, P.J. (1991) "Mutations in 5S DNA and 5S RNA have different effects on the binding of *Xenopus* transcription factor IIIA." Biochemistry Vol. 30, No. 9. pp. 2495-2500.

PARTIAL COPYRIGHT LICENSE

I hereby grant the right to lend my dissertation to users of the University of Victoria library, and to make single copies only for such users or in response to a request from the library of any other University, or similar institution, on behalf or for one of its users. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by me or a member of the University designated by me. It is understood that copying or publication of this dissertation for financial gain shall not be allowed without my written permission.

Title of Dissertation:

Studies on the Nucleic Acid Interactions of *Xenopus* Transcription Factor IIIA

Author \_\_\_\_\_

Nicholas J. Veldhoen

May 10, 1995