Effects of Zinc Finger Mutations on the Nucleic Acid Binding Activities of *Xenopus* Transcription Factor IIIA[†]

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ABSTRACT: Transcription factor IIIA (TFIIIA) is required for the activation of 5S RNA gene transcription as well as the storage of 5S RNA as a 7S ribonucleoprotein particle. Interaction with both nucleic acids is mediated through nine C_2H_2 zinc fingers. In order to determine amino acid regions necessary for nucleic acid interaction, a series of substitution mutants of *Xenopus laevis* TFIIIA have been constructed and expressed as recombinant proteins in *Escherichia coli.* The mutant proteins were purified to homogeneity and analyzed for 5S RNA gene and 5S RNA binding activities using a nitrocellulose filter binding assay. All of the mutant TFIIIA proteins retained full 5S RNA binding activity. Substitution of fingers 2, 3, and 4−6 of TFIIIA with zinc finger sequences from other proteins significantly reduced the interaction of the protein with the 5S RNA gene. In contrast, substitution of finger 1 or finger 7 had little effect on the interaction of TFIIIA with the 5S RNA gene. The results of scanning substitution mutagenesis within the first three zinc fingers of TFIIIA suggested that DNA contacts made by the α-helical regions of finger 2 and particularly of finger 3 provide the majority of the free energy of the TFIIIA−DNA interaction. Basic amino acids found at the same position within the α-helices of fingers 2 and 3 of TFIIIA are required for high-affinity DNA binding activity. The identification of amino acid residues critical for the formation of a TFIIIA−DNA complex contributes to our understanding of zinc finger protein−nucleic acid interactions.

Transcription factor IIIA (TFIIIA) is involved in the developmental regulation of *Xenopus* 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). This second activity of TFIIIA serves in the transport of 5S RNA from the nucleus and storage within the cytoplasm (Guddat et al., 1990; Picard & Wegnez, 1979). TFIIIA is a 38.5 kDa protein which contains nine C_2H_2 zinc fingers tandemly repeated through the N-terminal two-thirds of the protein (Miller et al., 1985, Tso et al., 1986). This 30 kDa N-terminal domain binds in a sequence-specific manner to a 50 base pair internal control region (ICR) of the 5S RNA gene. The zinc fingers of TFIIIA are also the site of interaction with the 5S RNA molecule.

Structural studies by NMR (Carr et al., 1990, Lee et al., 1989a,b; Michael et al., 1992; Párraga et al., 1988) and X-ray crystallography (Fairall et al., 1993; Pavletich & Pabo, 1991, 1993) have revealed that the C_2H_2 zinc fingers contain a two-strand antiparallel β-sheet followed by an α-helix. Each finger adopts a very similar tertiary conformation resulting in a highly compact globular nucleic acid binding domain. However, due to variations of certain key amino acids, each zinc finger recognizes and binds to different DNA (or RNA) sequences (Fairall et al., 1993; Pavletich & Pabo, 1991, 1993).

The interactions of TFIIIA with 5S RNA and the 5S RNA gene have been studied extensively. The majority of energetically important contacts required for the binding of TFIIIA to DNA are formed between the first three fingers of TFIIIA and base pairs in the box C promoter element (Christensen et al., 1991; Clemens et al., 1992; Darby & Joho, 1992; Del Rio et al., 1993; Hayes & Clemens, 1992; Liao et al., 1992; Veldhoen et al., 1994; You et al., 1991). The remaining zinc fingers and the C-terminal domain interact with the intermediate and box A elements within the ICR and are oriented toward the 5′ end of the gene (Vrana et al., 1988). While fingers 1−3 of TFIIIA are required for high-affinity DNA binding, the central zinc fingers 4−7 are sufficient for high-affinity RNA binding (Clemens et al., 1993). The main determinants for high-affinity binding of TFIIIA to 5S RNA are the overall tertiary structure of the RNA and certain regions of the secondary structure that form specific conformations (Baudin & Romaniuk, 1989; Baudin et al., 1991; Romaniuk, 1989; Romaniuk et al., 1987).

To identify amino acid residues within the zinc fingers of TFIIIA critical for binding to the 5S RNA gene, a set of finger swap, scanning, and single amino acid substitution mutants of TFIIIA were generated. The donor finger sequences used in this study originate from the *Xenopus* zinc finger proteins Xfin and p43. Both exhibit RNA binding activity with little or no DNA binding activity, and have amino acid sequences vastly different from the TFIIIA zinc fingers (Andreazzoli et al., 1993; Joho et al., 1990; Ruiz i Altaba et al., 1987; Zang & Romaniuk, 1995). The wild-type and mutant TFIIIAs were expressed as recombinant

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proteins in *E. coli* and purified to homogeneity. The relative association constants for the binding of these mutants to 5S RNA and the 5S RNA gene were determined using a nitrocellulose filter binding assay. The results indicate that certain amino acids in the α-helical regions of zinc fingers 2 and 3 contribute significantly to promoter binding by TFIIIA. Furthermore, the binding data indicate that there is a much more extensive interface formed between the α-helix of finger 3 and DNA than has been observed for other zinc finger−DNA interactions.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Vectors. Plasmid pT7-TF containing the TFIIIA cDNA (Ginsberg et al., 1984) was provided by J. Tso. The *NdeI*/*Bam*HI fragment of pT7-TF was cloned into the same site of pET-11b (Studier et al., 1990) to yield pTF4. Plasmid pKJ66 containing a p43 cDNA insert (Joho et al., 1990) was the generous gift of Dr. D. D. Brown. 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., 1987). All plasmids were maintained in *E. coli* strain JM109, and protein expression was carried out in *E. coli* strain BL21(DE3).

Construction of Mutant TFIIIA Expression Vectors. Finger swap mutants were constructed using three different methods. First, the polymerase chain reaction (PCR) was employed to produce mutants Tp1−3, Tp4−7, Tp5−7, and Tp7. The segments of p43 cDNA which encode p43 fingers 1−3, 4−7, 5−7, and 7 were amplified using plasmid pKJ66 as the template. PCR primers were designed to amplify regions of the p43 cDNA corresponding to p43 finger sequences and to incorporate unique restriction enzyme sites for introduction into the TFIIIA cDNA of pTF4 (Figure 1). The PCR products were initially cloned by blunt-end ligation into the *Sma*I site of pUC19. The resulting subclones were sequenced and used to generate the desired p43 DNA fragments subsequently introduced into pTF4 (Figure 1).

The second method used to create finger swap mutants employed oligonucleotide overlapping PCR (Dillon & Rosen, 1990). This procedure generated mutants TX2 and Tp3. Four long partially overlapping oligonucleotide primers were used to PCR-amplify DNA fragments encoding fingers 1−3 of TFIIIA that incorporate the designated mutant sequence as well as flanking *Pst*I and *Bam*HI/*Bgl*II sites for insertion into pUC19 and pTF4. The PCR products were initially cloned into the *Pst*I/*Bam*HI site of pUC19 and identified by sequence analysis. The resulting subclones were digested by *Pst*I and *Bgl*II, and the purified DNA fragments were cloned into the same sites in pTF4 (Figure 1).

Finally, 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 the *Pst*I site within finger 1 by shotgun ligation of overlapping oligonucleotides (Romaniuk et al., 1987). The resulting mutant construct (TX1.1) was extended using oligonucleotide-directed mutagenesis to generate the full Xfin finger 31 swap mutant of TFIIIA finger 1 (Nelson & Long, 1989) (Figure 1).

The scanning and single amino acid substitution mutants within fingers 1−3 of TFIIIA were constructed using site-directed mutagenesis (Nelson & Long, 1989) (Figure 2). pUC-TF1−3, which contains the *NdeI*/*Bgl*II fragment of the

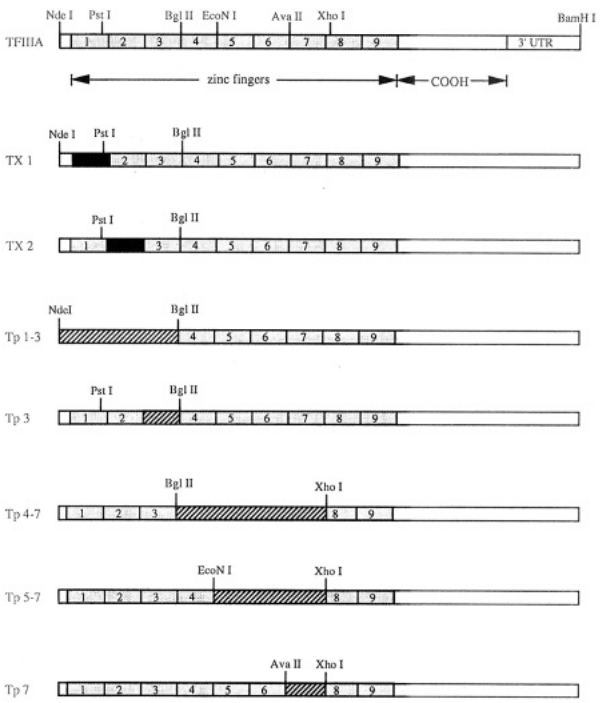

FIGURE 1: Construction of TFIIIA finger swap mutants. A schematic representation of the wild-type TFIIIA cDNA and the chimeric cDNAs is shown. Stippled boxes depict regions of the cDNAs which encode wild-type TFIIIA zinc fingers, solid boxes depict regions of the cDNAS which encode the donor Xfin finger 31 sequence, and striped boxes depict regions of the cDNAs which encode the corresponding zinc fingers from p43. Restriction enzyme sites used to produce the chimeric genes are shown for each construct.

TFIIIA cDNA cloned into the *Kpn*I and *Bam*HI sites of pUC19, was used as the template for mutagenesis. Each mutant DNA was initially subcloned into pUC19 for sequence verification and then introduced back into pTF4 using the unique *Nde*I and *Bgl*II restriction sites.

Expression and Purification of Recombinant Wild-Type and Mutant TFIIIA Proteins. Preparation of wild-type and mutant TFIIIA proteins was carried out as described previously (Veldhoen et al., 1994), except for mutant Tp4−7. In the purification of Tp4−7, 7 M urea was used to extract protein overnight from the cell lysate. Protein purity was confirmed by SDS−PAGE analysis, and the concentration of each protein preparation was determined by the method of Bradford (1976).

Synthesis and Radiolabeling of the 5S RNA Gene and 5S RNA. The 5S RNA gene was released from plasmid pXlo by digestion with the restriction endonucleases *Eco*RI and *Hind*III and end-labeled with [α-^{32}P]dATP (Sambrook et al., 1989). Xlo 5S RNA was produced by *in vitro* transcription and end-labeled at the 3′ terminus with [5′-^{32}P]pCp and T4 RNA ligase (Romaniuk et al., 1987).

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 TFIIIAs were determined using a nitrocellulose filter binding assay and the equilibrium binding conditions described previously (Romaniuk, 1985, 1990). The affinities of each mutant protein for RNA and DNA were determined using three or more independent assays in which the affinities of wild-type TFIIIA for these nucleic acids were measured in parallel as a control. Apparent association constants for the binding

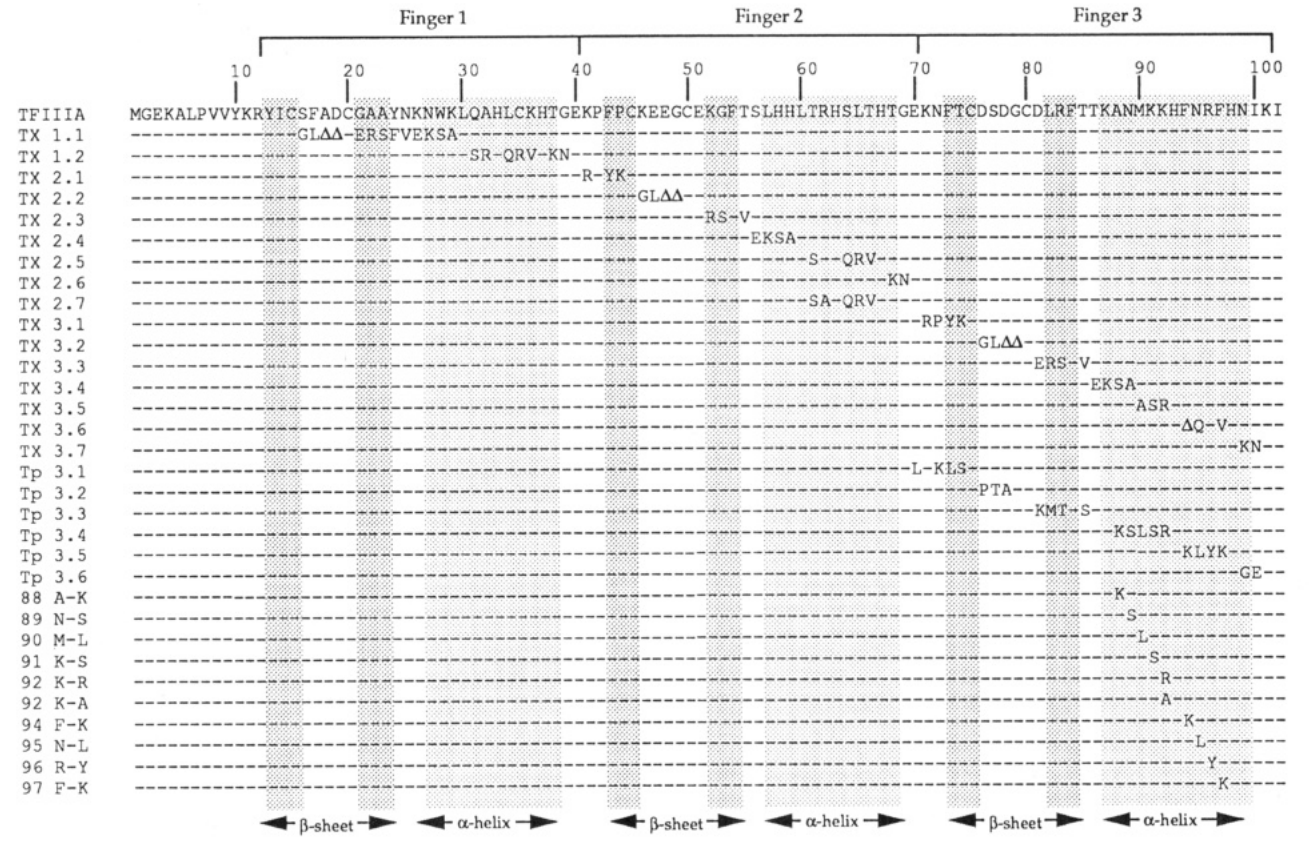

FIGURE 2: Location of scanning and point substitution mutations within the first three zinc fingers of TFIIIA. For each mutant, a dash indicates that an amino acid at that position is identical with that of wild-type TFIIIA. A (Δ) symbol indicates that an amino acid has been deleted at that position in the mutant protein. The nomenclature "TX" refers to mutants containing sequences from finger 31 of the Xfin protein, while "Tp" refers to mutants containing sequences from finger 3 of the p43 protein.

of the mutant and wild-type proteins to DNA and RNA were calculated by fitting the data to a simple bimolecular equilibrium model using the appropriate equation and the general curve-fitting function of Kaleidagraph software (Synergy Software, Reading PA). Relative K_a values for the binding of a mutant protein to DNA and RNA were calculated by dividing the K_a values determined for the mutant protein by the K_a values determined in parallel for the wild-type protein.

RESULTS

To determine the basis of zinc finger−DNA interaction, a series of amino acid substitution mutants of TFIIIA were generated (Figures 1 and 2). There are three types of substitution mutations. The first include mutants of the TX series which were created by substituting amino acid sequences derived from finger 31 of the Xfin protein. The second group are designated Tp mutants and were constructed using sequences from the zinc finger counterparts of p43. Finally, two alanine substitution mutants, TX2.7 and 92 K-A, were created by substituting basic amino acids in TFIIIA with alanine residues.

The 5S RNA gene and 5S RNA binding affinities of the mutant TFIIIA proteins were measured using a nitrocellulose filter binding assay with wild-type TFIIIA included as a control (Figure 3). The apparent association constants of the proteins for these two nucleic acids were determined by fitting the data to the equation for a simple bimolecular equilibrium. By running the wild-type protein in parallel in all assays, it was possible to make an accurate determination of the affinities of each mutant protein for 5S RNA and 5S DNA relative to the wild-type protein. 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 p43 and Xfin zinc finger sequences 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 resulting from amino acid substitutions rather than resulting from drastic conformational changes in zinc finger structure. The results of thermolysin protease digestion analysis of the TFIIIA mutant proteins suggest that none of the substitution mutations introduced major conformational changes from that of wild-type TFIIIA (data not shown). Furthermore, the fact that all of the mutant TFIIIA proteins retained high-affinity 5S RNA binding activities indicates that the mutant zinc fingers fold properly and the purified proteins are fully active in nucleic acid binding.

In many cases, residues other than the "structural" amino acids are found to be conserved between TFIIIA and the donor Xfin or p43 zinc fingers. This conservation of amino acid residues may lead to the retention of critical amino acid−DNA contacts by chimeric TFIIIA mutants. This effect is observed for Xfin and p43 sequence substitutions at positions 62R (mutant TX2.5) and 92K (mutant 92K-R), and it was necessary to construct alanine substitution mutations at these positions to fully investigate the role of these residues in DNA binding by TFIIIA. Thus, the type of substitution mutagenesis that we have employed has the advantage of retaining overall zinc finger structure, but care

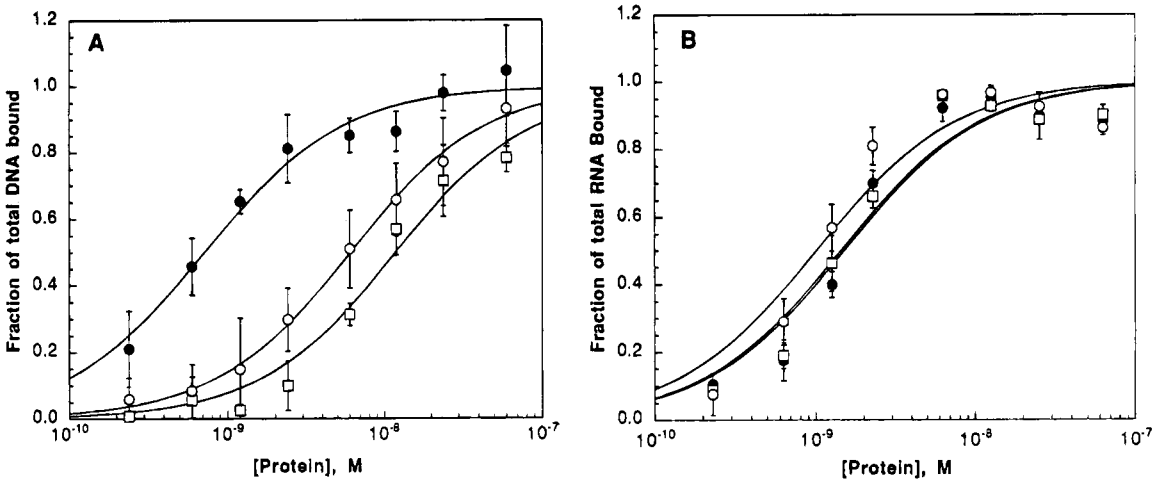

FIGURE 3: Equilibrium binding of wild-type TFIIIA (closed circle) and mutant proteins TX2.4 (open box) and TX3.4 (open circle) to the 5S RNA gene (A) and 5S RNA (B). Each point represents the mean value of three independent determinations. The error bars represent the standard deviations. Best-fit curves to a simple bimolecular equilibrium are shown for each protein.

Table 1: Effects of TFIIIA Finger Substitution Mutations on Binding Activity to the 5S RNA Gene and 5S RNA

mutant[a]	TFIIIA fingers replaced	rel K_a for binding to 5S RNA gene[b]	rel K_a for binding to 5S RNA[b]
TX1.0	1	0.91 ± 0.10	1.17 ± 0.12
TX2.0	2	0.28 ± 0.08	1.15 ± 0.12
Tp1−3	1, 2, 3	0.006 ± 0.001	0.85 ± 0.02
Tp3	3	0.01 ± 0.002	1.13 ± 0.08
Tp4−7	4, 5, 6, 7	0.01 ± 0.005	1.01 ± 0.03
Tp5−7	5, 6, 7	0.04 ± 0.04	0.93 ± 0.07
Tp7	7	0.72 ± 0.05	0.93 ± 0.03

[a] The nomenclature "TX" refers to TFIIIA substitution mutations made using Xfin protein finger 31 as the donor, while "Tp" refers to TFIIIA substitutions made using the corresponding zinc fingers of p43 protein. See Figure 1 for more details. [b] 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. Mean of three or more independent determinations with standard deviations.

must be taken to ensure that potential DNA-contacting amino acids do not evade detection.

As a first step toward the identification of zinc finger domains important for the interaction of TFIIIA with the 5S RNA gene, a series of full finger substitution mutants of TFIIIA were constructed, expressed, and purified (Figure 1). The results of nucleic acid binding analysis indicated that substitutions of fingers 2, 3, 4−7, and 5−7 of TFIIIA reduced 5S RNA gene binding affinity (Table 1). In contrast, the finger 1 and finger 7 substitution mutants retained wild-type DNA binding activity. These results agree with studies performed using truncated forms of TFIIIA (Christensen et al., 1991; Clemens et al., 1994; Hansen et al., 1993; Liao et al., 1992) and "broken finger" mutants (Del Rio et al., 1993) that identified fingers 2, 3, and 4 as important for TFIIIA−DNA complex formation. All of the finger swap substitution mutants exhibited 5S RNA binding activity similar to wild-type TFIIIA. These results are not surprising as it has been demonstrated that fingers 4−7 of TFIIIA are required for high-affinity 5S RNA binding and the donor fingers 4−7 from p43 also function in binding to 5S RNA (Clemens et al., 1993; Darby & Joho, 1992).

Several groups have found that fingers 1−3 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 (Christensen et al., 1991; Clemens et al., 1994; Del Rio et al., 1993; Liao et al., 1992; Vrana et al., 1988). 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 2). The effects of these scanning substitution mutations of TFIIIA on nucleic acid binding activity are shown in Table 2. The "finger tip" region of finger 2 and the α-helical regions of both fingers 2 and 3 of TFIIIA are clearly involved in DNA binding. These α-helical regions contain a number of basic amino acid residues that are in positions suitable for contacting the 5S RNA gene (Figure 4). An extensive region throughout the α-helix of finger 3 is required to maintain high-affinity DNA binding (Table 2, Figure 2). The use of α-helical residues within a zinc finger to form direct

Table 2: Effects of TFIIIA Scanning Substitution Mutations on Binding Activity to the 5S RNA Gene and 5S RNA

mutant[a]	rel K_a for binding to 5S RNA gene[b]	rel K_a for binding to 5S RNA[b]
TX1.1	0.94 ± 0.06	0.97 ± 0.11
TX1.2	0.84 ± 0.06	1.23 ± 0.05
TX2.1	0.88 ± 0.12	1.00 ± 0.02
TX2.2	0.77 ± 0.05	1.12 ± 0.22
TX2.3	0.89 ± 0.10	1.16 ± 0.12
TX2.4	0.34 ± 0.04	1.07 ± 0.10
TX2.5	0.75 ± 0.17	0.87 ± 0.08
TX2.6	0.72 ± 0.19	0.96 ● 0.01
TX2.7	0.22 ± 0.07	0.92 ± 0.07
TX3.1	1.25 ± 0.23	0.97 ± 0.02
TX3.2	0.51 ± 0.17	0.96 ± 0.06
TX3.3	0.65 ± 0.18	1.01 ± 0.13
TX3.4	0.16 ± 0.03	1.07 ± 0.15
TX3.5	1.23 ± 0.21	1.10 ± 0.07
TX3.6	0.33 ± 0.02	1.22 ± 0.17
TX3.7	0.67 ± 0.10	1.32 ± 0.10
Tp3.1	0.69 ± 0.13	0.91 ± 0.07
Tp3.2	0.95 ± 0.06	1.05 ± 0.12
Tp3.3	0.74 ± 0.12	0.96 ± 0.23
Tp3.4	0.24 ± 0.05	0.94 ± 0.19
Tp3.5	0.14 ± 0.04	0.72 ± 0.17
Tp3.6	0.77 ± 0.14	0.87 ± 0.22

[a] The nomenclature "TX" refers to TFIIIA substitution mutations made using Xfin protein finger 31 sequence as the donor, while "Tp" refers to TFIIIA substitutions made using the corresponding zinc finger sequence of p43 protein. See Figure 2 for more details. [b] 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. Mean of three or more independent determinations with standard deviations.

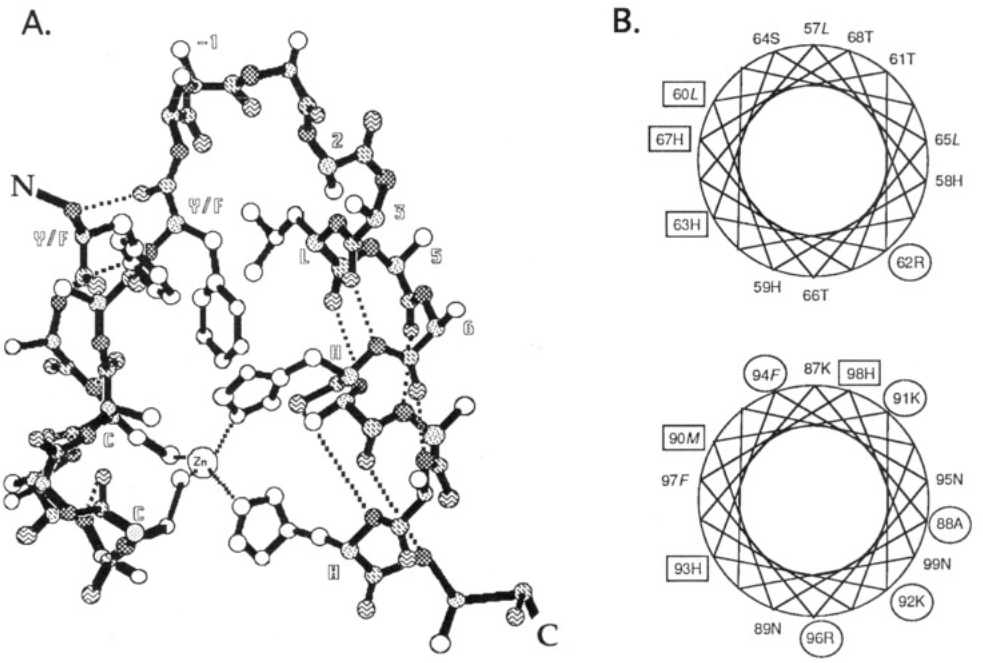

FIGURE 4: Structural represention of a C_2H_2 zinc finger. (A) The zinc finger structure is based on a model proposed by Berg (1988) and shows the polypeptide backbone conformation stabilized by zinc ion binding and a hydrophobic core. (B) Helical wheel diagrams of TFIIIA zinc fingers 2 and 3 are shown. Amino acids involved in stabilization of the finger structure are boxed, hydrophobic residues are shown in italics, and amino acid positions that reduce DNA binding activity 3-fold or greater when substituted are circled.

Table 3: Effects of TFIIIA Single Amino Acid Substitution Mutations within Finger 3 on Binding Activity to the 5S RNA Gene and 5S RNA

mutant[a]	rel K_a for binding to 5S RNA gene[b]	rel K_a for binding to 5S RNA[b]
88A-K	0.20 ± 0.02	0.95 ± 0.15
89N-S	0.66 ± 0.20	1.14 ± 0.16
90M-L	0.84 ± 0.19	0.92 ± 0.16
91K-S	0.30 ± 0.12	0.86 ± 0.08
92K-R	0.96 ± 0.10	1.17 ± 0.12
92K-A	0.13 ± 0.04	0.73 ± 0.18
94F-K	0.28 ± 0.15	0.71 ± 0.19
95N-L	0.40 ± 0.08	0.83 ± 0.39
96R-Y	0.25 ± 0.08	0.67 ± 0.22
97F-K	0.72 ± 0.06	1.05 ± 0.15

[a] The nomenclature refers to the amino acid position mutated within TFIIIA followed by the wild-type residue and mutant amino acid substitution (Figure 2). [b] 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. Mean of three or more independent determinations with standard deviations.

contacts to bases within the major groove of the DNA has been demonstrated by X-ray crystallographic studies of DNA−zinc finger peptide complexes derived from the proteins *zif*268 (Pavletich & Pabo, 1991), GLI (Pavletich & Pabo, 1993), and Tramtrack (*TTK*) (Fairall et al., 1993).

A number of single amino acid substitution mutations were constructed to further identify individual amino acids within the α-helical region of finger 3 of TFIIIA required for high-affinity binding to the 5S RNA gene (Figure 2). In addition, a double mutant designated TX2.7, containing an alanine substitution within the sequence context of the scanning mutant TX2.5, was constructed. The influence of these mutations on the nucleic acid binding activities of TFIIIA is shown in Table 3. The data for mutant TX2.7, when compared with the data for mutant TX2.5, suggest that the arginine residue at position 62 within finger 2 is essential for specific recognition of the 5S RNA gene promoter (Table 2). Substitution mutations within finger 3 of TFIIIA at amino acid positions 88A, 92K, 94F, and 96R reduce DNA binding affinity 4−8-fold (Table 3). Less severe reductions of 1−3-fold are also observed for mutations at positions 91K and 95N. The remaining finger 3 single residue mutants 89N-S, 90M-L, and 97F-K did not significantly affect DNA binding activity. It is interesting to note that an arginine substitution at position 92 can functionally replace the wild-type lysine residue and maintain energetically important sequence-specific contacts with the 5S RNA gene (Table 3).

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 for the binding of these mutants to 5S RNA and the 5S RNA gene were measured using a nitrocellulose filter binding assay. In general, the effects of substitution mutagenesis on the nucleic acid binding activities of TFIIIA are independent of the donor sequence (Xfin or p43) present at a given position. All of the mutants retained wild-type levels of 5S RNA binding activity. 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 activities of individual TFIIIA mutants reflects either the loss of specific protein−DNA contacts or a localized change in finger conformation that may affect docking of the recognition α-helix in the major groove of the DNA.

The N-terminal fingers of TFIIIA form specific contacts with the box C promoter element of the 5S rRNA gene, while the C-terminal zinc fingers interact with the box A promoter element (Del Rio et al., 1993; Liao et al., 1992; Vrana et al., 1988). The locations of contacts between individual zinc fingers of TFIIIA and the ICR of the 5S rRNA gene were elucidated using nuclease and chemical protection analyses of truncated and broken finger mutants of TFIIIA (Christensen et al., 1991; Clemens et al., 1992, 1994; Del Rio et al., 1993; Hansen et al., 1993; Hayes & Clemens, 1992). These studies provide a model for the interaction of TFIIIA with the 5S RNA gene promoter in which fingers 1−3, finger 5, and fingers 7−9 contact the box C, intermediate, and box A elements, respectively.

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 (Clemens et al., 1994; Liao et al., 1992; Vrana et al., 1988). Association of TFIIIA with the 5S RNA gene may be initiated by these N-terminal fingers, as the remaining C-terminal six zinc fingers cannot bind to DNA independently. DNA binding analysis of "broken finger" TFIIIA mutants indicated that zinc fingers 3 and 4 provide energetically critical promoter contacts, while fingers 1 and 2 are dispensable for high-affinity binding of TFIIIA to DNA (Del Rio et al., 1993). This apparent variability in determining the energetic contributions made by individual zinc fingers may arise from differences in the conformations of the types of TFIIIA mutants studied and the methods of analysis used to study the DNA binding activities of these mutants. For example, the use of *in vitro* translated TFIIIA restricts the quantity of protein available for analysis (Vrana et al., 1988), making it impossible to accurately measure association constants for DNA binding of mutants with less than 25% of wild-type binding activity. In addition, the use of truncated or "broken finger" mutants of TFIIIA may result in long-range effects on the protein−DNA interaction. The local disruption of finger conformation resulting from the "broken finger" mutations could lead to either a misalignment of downstream fingers within the DNA major groove of the 5S RNA gene or a alteration in finger−finger interactions within TFIIIA. Thus, the effect of this local mutation on the DNA binding energy may be amplified by an overall alteration in the position of TFIIIA on the 5S gene promoter. The use of finger swapping and substitution mutants of TFIIIA expressed as recombinant proteins in *E. coli* in this study overcomes many of these limitations.

The lack of energetically significant contacts between the 5S RNA gene and finger 1 of TFIIIA is supported by the present results using Xfin finger 31 substitution mutagenesis. In contrast, a reduction in DNA binding activity of 4-fold is observed when finger 2 of TFIIIA is substituted with the amino acid sequence from Xfin. This decrease in the association constant may represent the loss of a small number of sequence-specific contacts within the box C promoter element (Liao et al., 1992; Veldhoen et al., 1994; Vrana et al., 1988). The importance of finger 3 in the association of TFIIIA with the 5S RNA gene is supported by the severe reductions in DNA binding activity for both Xfin finger 31 and p43 finger 3 substitution mutations. The critical role finger 3 plays in the TFIIIA−DNA interaction has been observed in previous TFIIIA mutagenesis studies (Clemens et al., 1994; Del Rio et al., 1993). Substitution of TFIIIA fingers 4−7 with the corresponding p43 fingers reduces DNA binding activity 100-fold. This reduction in DNA binding has been observed previously with "broken finger" mutations of the central zinc fingers of TFIIIA (Del Rio et al., 1993). These results suggest either that the central fingers of TFIIIA form multiple sequence-specific contacts to the 5S RNA gene or that their conformation is important for the spatial alignment of N- and C-terminal fingers along the ICR. Several lines of evidence indicate that fingers 4 and 6 may not directly contact base pairs within the ICR but provide specific linkers that connect the zinc fingers that interact with bases in the major groove of the box C, intermediate, and box A promoter elements (Clemens et al., 1992; Hayes & Clemens, 1992; Hayes & Tullius, 1992). The severe reduction in DNA binding activity observed for mutations in the central fingers is not found for the N-terminal three-finger peptide of TFIIIA, where these fingers have been omitted (Clemens et al., 1994; Liao et al., 1992). This truncated TFIIIA peptide may retain the majority of energetically important sequence contacts but lack the negative positioning constraints imposed by the central zinc fingers.

The first three fingers of TFIIIA are connected by the linker sequence, TGEKP/N, which is highly conserved in many zinc finger proteins. Amino acid sequences within these linker segments contribute to high-affinity DNA binding by the zf1−3 peptide of TFIIIA (Choo & Klug, 1993; Clemens et al., 1994). Substitution of these sequences with heterologous linkers from *Xenopus* p43 protein or other linker regions of TFIIIA, or substituting with nonconserved amino acids, reduces the affinity of zf1−3 for DNA by as much as 24-fold. This result suggests that individual linker sequences are functionally distinct in their contributions to the formation of a stable zf1−3−DNA complex. In the present study, 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 2, Figure 2). Partial substitution of the third linker sequence (NIKI to KNKI or GEKI) results in a modest reduction of less than 2-fold (Table 2, Figure 2). Thus, in the context of the full-length TFIIIA protein, the contribution made to DNA binding by the wild-type N-terminal linker sequences is minimal. Possible changes in finger orientation resulting from the linker substitutions that could alter the interaction of TFIIIA with the 5S RNA gene may be compensated for by favorable DNA−finger interactions toward the C-terminus of the full-length protein. Thus, 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.

Each zinc finger consists of an N-terminal two-stranded β-sheet and a C-terminal α-helix stabilized by four zinc binding residues and a hydrophobic core (Berg, 1988; Gibson et al., 1988; Lee et al., 1989a,b; Liao et al., 1994; Párraga et al., 1988). Determination of the X-ray crystallographic structures of zinc finger peptides derived from *zif*268 (Pavletich & Pabo, 1991), GLI (Pavletich & Pabo, 1993), and TTK (Fairall et al., 1993) complexed with their respective DNA binding sites indicate that amino acids within the α-helices of the zinc fingers provide the majority of contacts to the DNA bases (Figure 5). The results of scanning mutagenesis of TFIIIA suggest that the α-helical regions of both fingers 2 and 3 are required for high-affinity interaction with the 5S RNA gene. However, the contributions made

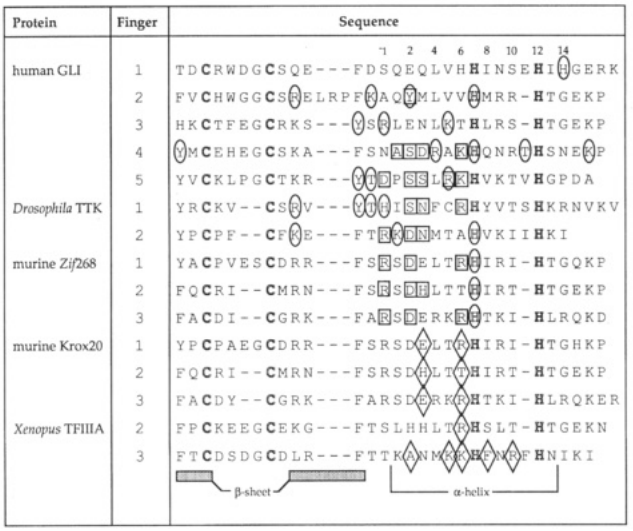

FIGURE 5: Alignment of zinc finger sequences indicating the location of amino acid residues that contact DNA. Protein−DNA contacts were identified by X-ray crystallography for GLI (Pavletich & Pabo, 1993), TTK (Fairall et al., 1993), and Zif 268 (Pavletich & Pabo, 1991) proteins. Residues that interact with the phosphate backbone are enclosed in ovals, while those that contact base pairs are enclosed in boxes. Amino acid residues within Krox20 (Nardelli et al., 1991) and TFIIIA that contribute to high-affinity DNA binding are indicated with diamonds. The amino acid positions within the α-helix are numbered above the alignment, where 1 denotes the first α-helical residue.

to the TFIIIA−DNA interaction are not equivalent between the N-terminal zinc fingers. Replacement of the finger 3 sequence of TFIIIA reduces DNA binding activity 100-fold, while finger 2 substitution of TFIIIA results in a 4-fold reduction in DNA binding. Differences in the contribution to the free energy of DNA binding by individual zinc fingers are also observed for "broken finger" and truncated mutants of TFIIIA (Clemens et al., 1994; Del Rio et al., 1993; Vrana et al., 1988).

Comparison of the role of amino acid residues in the α-helices of a number of zinc finger proteins indicates a unique property of the α-helix of finger 3 of TFIIIA (Figure 5). For the other zinc finger proteins studied so far, it appears that the α-helix of each finger is situated in the major groove of the DNA so that only the amino acids from the start of the α-helix up to the first zinc-coordinating histidine residue are positioned to form contacts with the DNA. In contrast, the results of our mutagenesis study indicate that the α-helix of finger 3 of TFIIIA is positioned in the major groove of the DNA in such a way that residues along the entire α-helix, including those between the two zinc-coordinating histidine residues, are involved in making direct contacts to the DNA bases.

The packing of the α-helix in the zinc finger domain and its role in DNA binding suggest that two functionally distinct groups of amino acid residues 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 4A). Substitution of either residue type may lead to loss of DNA contacts and a reduction in complex stability. Amino acid residues along the solvent-exposed face of the α-helix may form nonspecific interactions with the DNA backbone or provide direct contacts to DNA bases that contribute to DNA binding affinity and specificity.

Amino acids 57−68 within finger 2 and 87−99 within finger 3 of TFIIIA are predicted to form α-helices (Figure 4B). Helical wheel representations of these zinc finger regions indicate that hydrophilic residues are located on one side of the α-helix and hydrophobic residues are positioned on the opposite side facing toward the internal core of the zinc finger domain (Figure 4B). All single amino acid substitution mutants within the α-helix of finger 3 that reduce DNA binding activity, with the exception of 94F-K, are located on the side facing away from the core of the zinc finger (Figure 4B). Amino acids positioned on this "external" face of the α-helix are expected to contribute to high-affinity interaction with DNA by forming contacts to the bases in the major groove (Jacobs, 1992; Párraga et al., 1988). Replacement of the alanine at position 88 with a lysine results in a 5-fold reduction in DNA binding affinity (Table 3). This alanine may act as an auxiliary amino acid, enhancing or modulating the specificity of base recognition by a neighboring amino acid (Choo & Klug, 1994a,b). Alternatively, alanine 88 might form a hydrophobic interaction with a thymine base located within the box C element of the ICR. Candidates for this interaction include base pairs TA79 or TA80 that are essential for high-affinity binding by TFIIIA (Veldhoen et al., 1994). Alternatively, replacement of alanine with the more bulky lysine at position 88 could affect docking of the α-helix of finger 3 within the major groove, resulting in the loss of additional amino acid−base contacts. The importance of the "finger tip" region in high-affinity DNA binding is also seen for finger 2. Substitution of this region with the corresponding Xfin finger 31 sequence reduces DNA binding activity 3-fold (Table 2). Such a reduction could result from loss of contacts between the box C element and residues 56S, 58H, or 59H. Substitution of phenylalanine at position 94 with lysine reduces DNA binding activity 4-fold. This may result from a destabilization of the hydrophobic core of finger 3. The positioning of this α-helical residue toward the interior of the zinc finger suggests a critical role for 94F in maintaining finger 3 structural integrity (Figure 4B).

Many of the GC base pairs within the 5S DNA promoter and the neighboring phosphates are involved in TFIIIA binding (Churchill et al., 1990; Lee et al., 1991; Sakonju & Brown, 1982; Veldhoen et al., 1994). The 3−8-fold reduction in DNA binding activity observed for the TFIIIA mutants 91K-S, 92K-A, and 96R-Y suggests that these basic amino acid residues provide direct contacts with the critical GC base pairs within the ICR. Substitution of lysine 92 with arginine does not affect TFIIIA DNA binding activity, indicating that these basic residues are functionally equivalent at this position. Arginine is required at the same α-helical position (62R) in finger 2 of TFIIIA for high-affinity DNA binding (Figure 5). It is possible that 62R in finger 2 and 92K in finger 3 of TFIIIA provide direct contacts with guanine bases on the noncoding strand of the ICR.

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). Hydroxyl radical footprinting and binding site selection analysis identify a smaller region within box C from $^{+}$79 to $^{+}$91 as critical for high affinity binding of TFIIIA to the 5S RNA gene (Clemens et al., 1992; Hayes & Clemens, 1992; Veldhoen et al., 1994). Mutations of three GC base pairs within this region ($^{+}$81, $^{+}$85, and $^{+}$89) result in the greatest reduction in the affinity of TFIIIA for the 5S

RNA gene (Veldhoen et al., 1994). The protein sequence linking fingers 2 and 3 is adjacent to base pair TA84, as determined using photoreactive cross-linking (Lee et al., 1991). Thus, it is possible that finger 3 interacts with a region of the ICR that includes GC81, while finger 2 may contact a DNA subsite that includes GC85.

Our data have demonstrated that zinc fingers 2 and 3 of TFIIIA are required for high-affinity DNA binding. The α-helices of both fingers are involved in forming direct contacts to the box C element within the ICR of the 5S RNA gene. Many of the amino acids implicated in DNA binding are clustered along one face of the α helix of finger 3. The interaction of this α-helix with DNA is more extensive than other zinc finger−DNA interactions described previously, which might account for the importance of this finger to the free energy of DNA binding by TFIIIA. Two basic amino acids that function in DNA binding are found at a conserved position within zinc fingers 2 and 3 of TFIIIA and may form contacts with guanine bases on the noncoding strand or the backbone of the 5S RNA gene.

SUPPORTING INFORMATION AVAILABLE

Supporting information is available containing three tables listing the sequences of the oligonucleotides used to generate the TFIIIA mutant proteins listed in Tables 1−3 of the main text (3 pages). Ordering information is given on any current masthead page.

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