Contribution of Individual Amino Acids to the Nucleic Acid Binding Activities of the *Xenopus* Zinc Finger Proteins TFIIIIA and p43[†]

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ABSTRACT: *Xenopus* transcription factor IIIIA (TFIIIA) binds to both 5S RNA and the 5S RNA gene in immature oocytes, an interaction mediated by nine zinc fingers. To determine the role of the central zinc fingers of the protein in these nucleic acid binding activities, a series of substitution mutants of TFIIIA were constructed and expressed as recombinant proteins in *Escherichia coli*. The mutant proteins were purified to homogeneity and analyzed for DNA and RNA binding activities using a nitrocellulose filter binding assay. Finger 5, but not finger 4, 6, or 7, is involved in sequence-specific binding to the 5S RNA gene. A TWT amino acid motif in finger 6 makes a significant contribution to the binding of TFIIIA to 5S RNA, while mutations in fingers 4, 5, and 7 have little or no effect on RNA binding by TFIIIA. In striking contrast, a TWT motif in finger 6 of p43, another *Xenopus* zinc finger protein that binds to 5S RNA, is not necessary for 5S RNA binding by this protein. Evidence for the presence of inhibitory finger−finger interactions that limit the nucleic acid binding properties of individual zinc fingers within TFIIIA and p43 is discussed.

Transcription factor IIIA (TFIIIA) is involved in the developmental regulation of *Xenopus* 5S RNA gene transcription by RNA polymerase III (*1*, *2*). In immature *Xenopus* oocytes, TFIIIA not only acts as a positive transcription factor but also interacts with approximately 50% of the 5S RNA produced 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 (*3*, *4*). The other 50% of the 5S RNA produced is stored in the cytoplasm in a 42S RNP bound to the p43 protein. 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 (*5*, *6*). This 30 kDa N-terminal domain binds in a sequence-specific manner to a 50 bp 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. The p43 protein also consists of nine zinc fingers; however, this protein does not bind to the 5S RNA gene, and there is little sequence homology between the zinc fingers of p43 and TFIIIA (*7*).

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 (*8*−*15*). 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 (*16*). NMR and X-ray crystallographic studies of zinc finger peptides of TFIIIA bound to DNA sequences from the ICR have confirmed many of these details and provided additional insight into the specific nature of the DNA−protein contacts (*17*, *18*).

Studies with peptides derived from TFIIIA have shown that while fingers 1−3 are required for high-affinity DNA binding, central zinc fingers 4−7 are sufficient for high-affinity RNA binding (*19*). 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 (*20*−*23*). Mutagenesis and phage display studies conducted with zinc fingers 4−7 of TFIIIA have indicated specific roles in 5S RNA binding for a lysine reside in the α-helix of finger 4 and a threonine-tryptophan-threonine motif in the α-helix of finger 6 (*24*−*26*). This TWT motif is also found in finger 6 of p43, another *Xenopus* protein that binds specifically to 5S RNA.

Studies with zinc finger peptides have been important in understanding the interaction of TFIIIA with DNA and RNA ligands. However, it is clear that within the full-length TFIIIA protein, finger−finger interactions can take place that modify the interaction of individual fingers with nucleic acid ligands (*27*, *28*). Therefore, to identify amino acid residues within the zinc fingers of TFIIIA that are critical for binding to the 5S RNA gene and amino acids within both TFIIIA and p43 that are critical for binding to 5S RNA, a set of finger swap, scanning, and single-amino acid substitution mutants were generated. The donor finger sequences used in this study originate from the *Xenopus* zinc finger protein p43 and the human zinc finger protein WT1. The p43 protein exhibits 5S RNA binding activity with little or no DNA binding activity, while WT1 binds to DNA and RNA substrates distinctly different from those of TFIIIA (*7*, *29*, *30*). The wild-type and mutant TFIIIAs were expressed as recombinant

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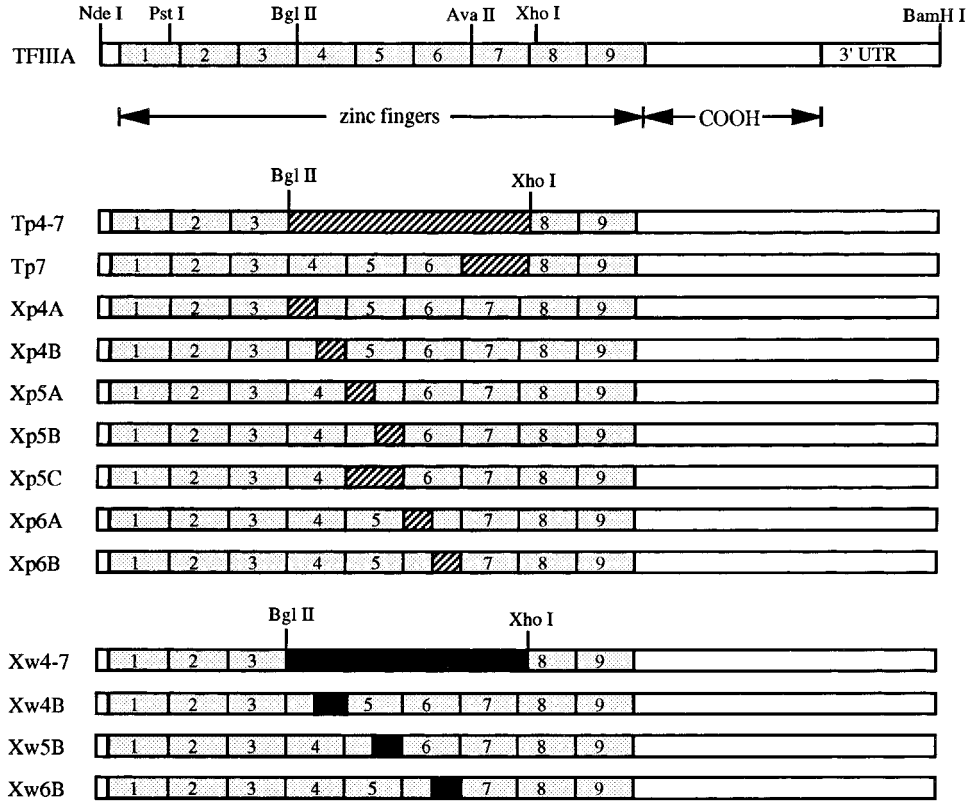

FIGURE 1: Construction of TFIIIA finger swap mutants. A schematic representation of the wild-type TFIIIA cDNA and the chimeric cDNAs is shown. Stippled boxes depict regions of the cDNAs which encode wild-type TFIIIA sequences; striped boxes represent regions of the cDNAs which encode donor p43 sequences, and black boxes represent regions of the cDNAs which encode donor WT1 sequences. Restriction enzyme sites used to produce the chimeric cDNAs are shown above the wild-type TFIIIA cDNA.

proteins in *Escherichia 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 equilibrium binding assays. DNA binding results indicate that residues in the α-helix of zinc finger 5 of TFIIIA contribute to the specific binding to the 5S RNA gene. The results from the RNA binding assays indicate that the lysine in the α-helical region of zinc finger 4 does not contribute significantly to 5S RNA binding by full-length TFIIIA. In contrast, amino acid substitution of the TWT motif in finger 6 of full-length TFIIIA reduces 5S RNA binding affinity to an extent similar to that observed for the zinc finger peptide containing fingers 4−7 of the protein. Surprisingly, mutation of this same motif in finger 6 of the p43 protein has no demonstrable effect on the affinity of that protein for 5S RNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Vectors. Plasmid vectors used to express full-length recombinant TFIIIA and p43 proteins in *E. coli* have been described elsewhere (*31*, *32*).

Construction of Mutant TFIIIA and p43 Expression Vectors. Finger swap mutants were constructed using two different methods. First, the polymerase chain reaction (PCR) was employed to produce mutants Tp4−7, Tp5−7, Tp7, and Xw4−7. The segments of p43 cDNA which encode p43 fingers 4−7, 5−7, and 7 were amplified using plasmid pKJ66 as the template. The segment of WT1 encoding fingers 1−4 was amplified from a cDNA construct containing the zinc finger domain of this protein (*29*). PCR primers were designed to amplify regions of the p43 and WT1 cDNAs corresponding to the desired 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 DNA fragments subsequently introduced into pTF4 (Figure 1).

The scanning and single-amino acid substitution mutants within fingers 4−6 of TFIIIA were constructed using site-directed mutagenesis (*33*) (Figure 2). pUC-TF4−7, which contains the *Bgl*II−*Xho*I fragment of the TFIIIA cDNA encoding fingers 4−7 cloned into 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 *Bgl*II and *Xho*I restriction sites.

Expression and Purification of Recombinant Wild-Type and Mutant Proteins. Preparation of wild-type and mutant TFIIIA and p43 proteins was carried out as described previously (*15*, *32*). Protein purity was confirmed by SDS−PAGE analysis, and the concentration of each protein preparation was determined by the method of Bradford (*34*).

Equilibrium Binding of the 5S RNA Gene and 5S RNA to Mutant TFIIIA and p43 Proteins. The apparent association constants for the binding of radiolabeled nucleic acids to wild-type and mutant TFIIIA and p43 proteins were determined using nitrocellulose filter binding assays described elsewhere (*32*, *35*, *36*). 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 or p43 for these nucleic acids were measured in

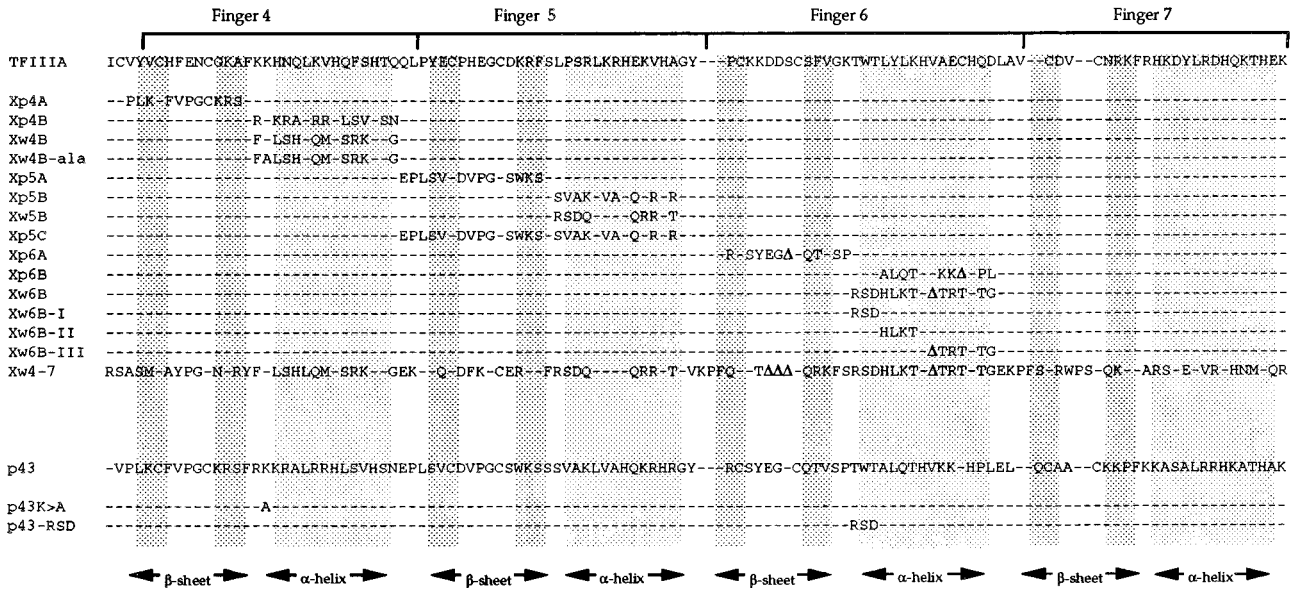

FIGURE 2: Location of scanning and point substitution mutations within fingers 4−7 of TFIIIA (top) and p43 (bottom). For each mutant, a dash indicates that an amino acid at that position is identical with that of wild-type TFIIIA. A Δ indicates that an amino acid has been deleted at that position in the mutant protein. The nomenclature "Xp" refers to mutants containing zinc finger sequences derived from p43. The nomenclature "Xw" refers to mutants containing zinc finger sequences derived from WT1.

parallel as a control. Apparent association constants for the binding 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.

For some mutants of TFIIIA, the affinity of the protein for both DNA and RNA was significantly decreased. In this situation, apparent association constants were determined by Scatchard analysis of mobility shift assays. The protein concentration was held constant at the apparent K_d determined from an initial nitrocellulose filter binding assay, and the 5S RNA concentration was varied from 20 to 80% saturation. Samples were run out on nondenaturing polyacrylamide gels under standard conditions (*37*) and the amounts of free and bound 5S RNA quantified by phosphorimager analysis (*28*). Analysis of mutant p43 proteins by this assay was not possible because the C-terminal domain interferes with the detection of a bimolecular complex in gel electrophoresis assays (*10*).

RESULTS

A series of TFIIIA mutants were created in which either the N-terminal or C-terminal half of the amino acid sequence was replaced with the amino acid sequence from the same zinc finger in p43 (Figure 2). Since both p43 and TFIIIA bind to 5S RNA, but only TFIIIA binds to the 5S RNA gene, we expected that these mutants might selectively reduce the level of binding to DNA rather than that to RNA. Each mutant was expressed as a recombinant protein in *E. coli* and purified to homogeneity. The affinity of each of these mutants for the *Xenopus laevis* oocyte 5S RNA gene or 5S RNA was measured by an equilibrium binding assay and compared to that of wild-type TFIIIA measured in parallel. The results of a typical binding experiment are shown in Figure 3, and the results for all of the mutants are shown in Table 1.

All of the mutants except those in finger 5 bind to the 5S RNA gene with roughly the same affinity as the wild-type TFIIIA. Replacement of either the N-terminal (Xp5A) or C-terminal (Xp5B) part of finger 5 with the corresponding sequences from p43 reduced the affinity of the mutant TFIIIA proteins for the 5S RNA gene by a factor of 3−4. Complete replacement of finger 5 of TFIIIA with the finger 5 sequence from p43 (Xp5C) reduced the affinity for the 5S RNA gene further. This reduction is consistent with the proposed interaction of finger 5 with the intermediate element of the internal promoter of the gene (*15*, *17*).

As the data in Table 1 show, in all cases substitution of the amino acid sequences within fingers 4−7 of TFIIIA with the corresponding p43 sequences has very little effect on the binding of the proteins to 5S RNA. This result was not unexpected, since both proteins bind to 5S RNA with roughly equivalent affinity (*32*, *35*). However, since the precise role of each zinc finger of p43 in RNA binding has not been established, the data in Table 1 suggest that there might be functional equivalency between fingers 4−7 of TFIIIA and p43.

To probe the role of specific amino acids within fingers 4−6 of TFIIIA in 5S RNA binding, we next created a series of substitution mutants using the zinc fingers of the Wilms tumor suppressor protein WT1. This protein has four zinc fingers and binds to both DNA and RNA (*29*, *30*), although to nucleic acid ligands different from those recognized by TFIIIA. Thus, substitution mutations using zinc finger sequences from WT1 should retain the general characteristics necessary for binding to DNA and RNA, but would be expected to lose the specific characteristics necessary to bind to the 5S RNA gene and/or the 5S RNA.

The first substitution mutation we created (Xw4−7) replaced fingers 4−7 of TFIIIA with the four zinc fingers of WT1 (Figure 1). As the data in Figure 4 show, this protein

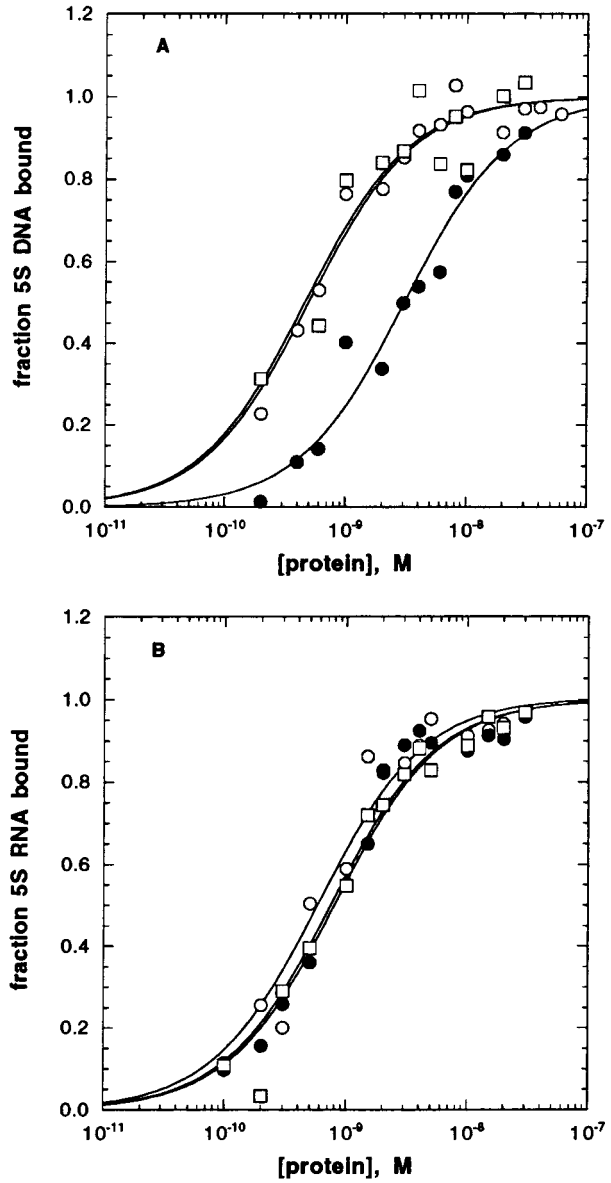

FIGURE 3: Equilibrium binding of wild-type TFIIIA (○), mutant Xp5C (●), and mutant Xp6B (□) to 5S DNA (A) and 5S RNA (B). The data are from a single nitrocellulose filter binding assay. Best-fit curves to a simple bimolecular equilibrium are shown for each protein.

is not capable of binding to either the 5S RNA gene or 5S RNA. However, the Xw4−7 mutant protein has acquired the ability to bind with high affinity to an RNA ligand of WT1 (Figure 4), thus demonstrating that the protein folds correctly and has a specific RNA binding activity. To define the critical residues important for the binding of full-length TFIIIA to 5S RNA, TFIIIA mutants with partial substitution of the amino acids of finger 4 (Xw4B), 5 (Xw5B), or 6 (Xw6B) with the sequence from finger 1, 2, or 3 of WT1 were created (Figure 2). As the data in Table 2 show, the effects of the three mutations on DNA binding are quite similar to the effects observed for the comparable mutant proteins with substituted p43 zinc finger sequences (Table 1). Substitution in mutant Xw5B of the α-helix of finger 5 of TFIIIA with the α-helix of finger 2 of WT1 had the most severe effect on binding to the 5S RNA gene, reducing the affinity by a factor of 12. Substitution of the α-helix of finger 4 or 5 of TFIIIA with WT1 or p43 sequences had small and

Table 1: Affinity of p43 Substitution Mutants of TFIIIA for 5S DNA and 5S RNA[a]

protein	5S DNA	5S RNA
wild type	1.000	1.000
Tp4−7[b]	0.01 ± 0.005	1.01 ± 0.03
Tp7[b]	0.72 ± 0.05	0.93 ± 0.03
Xp4A	0.72 ± 0.14	0.84 ± 0.09
Xp4B	0.59 ± 0.04	0.40 ± 0.10[c]
Xp5A	0.29 ± 0.05	0.50 ± 0.06
Xp5B	0.23 ± 0.02	0.82 ± 0.11
Xp5C	0.09 ± 0.02	0.83 ± 0.10
Xp6A	1.02 ± 0.09	0.88 ± 0.02
Xp6B	1.56 ± 0.09	0.89 ± 0.02

[a] Apparent association constants were determined by the nitrocellulose filter binding assay. Each value represents the mean of three or more independent determinations with the associated standard deviations. Relative affinities were determined by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild-type protein determined in parallel. [b] Data taken from Zang et al. (*31*). [c] Binding affinity determined by Scatchard analysis as described in Materials and Methods.

similar effects on binding to 5S RNA (Tables 1 and 2). However, while substituting the α-helix of finger 6 of TFIIIA with p43 sequence (Xp6B) had no effect on binding to 5S RNA, substitution with the WT1 sequence (Xw6B) did. As the data in Table 2 show, substituting the WT1 sequence into the α-helix of finger 6 of TFIIIA in mutant Xw6B reduced the affinity of the protein for 5S RNA by 100-fold.

Studies on the 5S RNA binding activity of a zinc finger peptide consisting of fingers 4−7 of TFIIIA have identified two key amino acid motifs (*24*, *26*). In finger 4, substitution of the KNQV amino acid sequence found at positions −1, 2, 3, and 6 of the α-helix with alanine residues reduces the affinity of the zinc finger peptide for 5S RNA by 77-fold (*26*). Substitution of just the lysine reside with alanine reduces binding to 5S RNA by 36.5-fold. The two substitution mutants of the finger 4 α-helix sequence constructed in the full-length TFIIIA (Xp4B, Xw4B) reduced the affinity of the protein for 5S RNA by 3−4-fold (Tables 1 and 2). However, in each case only the NQV of the α-helix was substituted, the lysine at position −1 being common to the two donating zinc finger sequences (Figure 2). To establish whether this lysine is critical to the interaction of full-length TFIIIA with 5S RNA, a point mutation in the Xw4B protein was created that substituted the lysine at position −1 of the α-helix with an alanine (Figure 2). As the results in Table 3 show, the Xw4B-Ala mutant protein bound to 5S RNA with the same affinity as the Xw4B protein. Thus, it appears that this lysine in finger 4 of full-length TFIIIA does not contribute significantly to the interaction with 5S RNA.

In the peptide consisting of fingers 4−7 of TFIIIA, substitution of the TTLK sequence at positions −1, 2, 3, and 6 of the α-helix of finger 6 with alanines reduces the affinity of the peptide for 5S RNA by 43-fold (*26*). In a peptide consisting of fingers 1−7 of TFIIIA, substitution of the threonine at position −1 with isoleucine reduced the affinity for 5S RNA by 30-fold (*24*). It was noted that amino acid sequence TWT found in positions −1 to 2 of the α-helix of finger 6 in TFIIIA is also found in finger 6 of p43, suggesting that this motif is critical for the binding of both proteins to 5S RNA. Mutation of either threonine had only a modest effect on the binding of the peptide to 5S RNA (*24*). In the case of full-length TFIIIA, the data for mutant

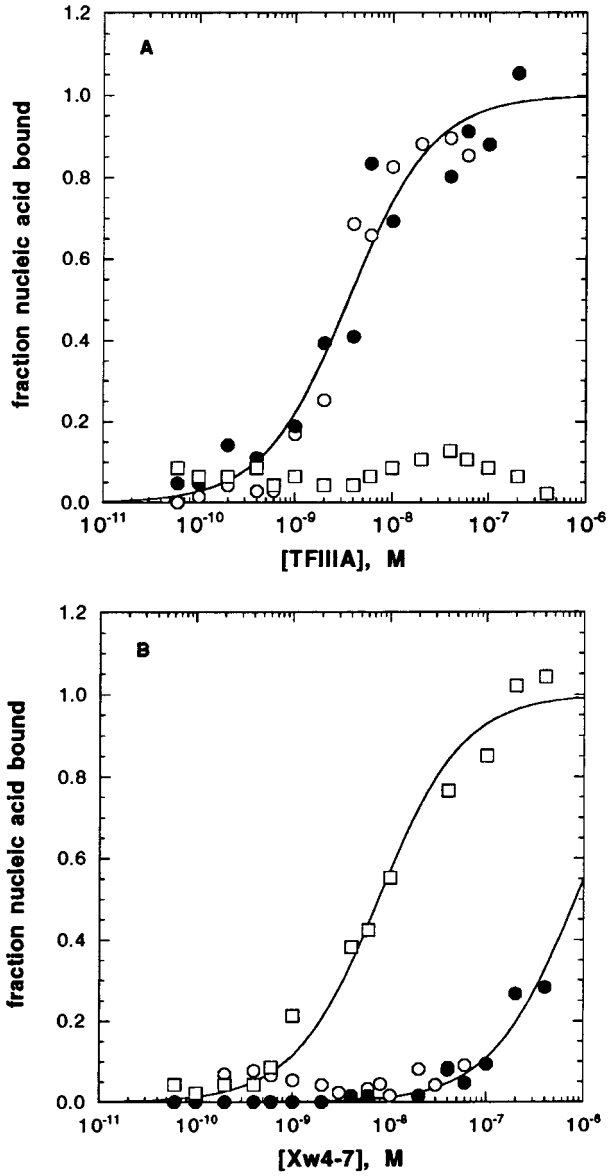

FIGURE 4: Equilibrium binding of wild-type TFIIIA (A) and mutant Xw4−7 (B) to 5S DNA (○), 5S RNA (●), and RNA22 (□), a WT1-specific RNA aptamer (*30*). The data are from a single nitrocellulose filter binding assay. Best-fit curves to a simple bimolecular equilibrium are shown for nucleic acid ligands that display binding above background levels.

Table 2: Affinity of WT1 Substitution Mutants of TFIIIA for 5S DNA and 5S RNA[a]

protein	5S DNA	5S RNA
wild type	1.000	1.000
Xw4−7	<0.01[b]	<0.01[b]
Xw4B	0.34 ± 0.04	0.28 ± 0.07[c]
Xw5B	0.08 ± 0.01	0.35 ± 0.02[c]
Xw6B	0.55 ± 0.04	<0.01[b]

[a] Apparent association constants were determined by the nitrocellulose filter binding assay. Each value represents the mean of three or more independent determinations with the associated standard deviations. Relative affinities were determined by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild-type protein determined in parallel. [b] Binding affinity below the limits of detection in the assay. [c] Binding affinity determined by Scatchard analysis as described in Materials and Methods.

Xw6B (Table 2) demonstrate that the α-helix of finger 6 is critical to 5S RNA binding. We created three scanning mutations that subdivided the substitution of residues in this α-helix with the corresponding residues from the α-helix of finger 3 of WT1 (Figure 2). Only the substitution of the TWT motif in finger 6 of TFIIIA with the RSD motif from WT1 in mutant Xw6B-I reduced the affinity of the protein for 5S RNA, substitutions further down the helix in mutants Xw6B-II and Xw6B-III having little effect. The effect on 5S RNA binding that this substitution has in full-length TFIIIA is slightly smaller than the effect observed for substitution mutations in the zinc finger peptides, but is within the same order of magnitude.

Table 3: Affinity of Scanning and Point Mutants of TFIIIA and p43 for 5S RNA[a]

protein	5S RNA	protein	5S RNA
wild type	1.000	Xw6B-III	0.85 ± 0.12[b]
Xw4B-Ala	0.38 ± 0.09	p43 K>A	0.28 ± 0.07
Xw6B-I	0.09 ± 0.04[b]	p43 TWT>RSD	0.90 ± 0.07
Xw6B-II	0.43 ± 0.12[b]		

[a] Apparent association constants were determined by the nitrocellulose filter binding assay. Each value represents the mean of three or more independent determinations with the associated standard deviations. Relative affinities were determined by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild-type protein determined in parallel. [b] Binding affinity determined by Scatchard analysis as described in Materials and Methods.

The key residues involved in the binding to 5S RNA of zinc finger peptides derived from TFIIIA are conserved in the same positions of the p43 protein. To test for the potential role of these residues in 5S RNA binding by p43, two targeted mutations were created (Figure 2). As the results in Table 3 show, substitution with alanine of the lysine at position −1 of the α-helix of finger 4 of p43 decreased the 5S RNA binding affinity by 3.5-fold. On the other hand, substitution of the TWT motif in finger 6 of p43 with RSD had no effect on 5S RNA binding. Apparently, this motif does not play the same role in the binding of p43 to 5S RNA that it does in the binding of TFIIIA to 5S RNA.

DISCUSSION

Zinc finger proteins play a variety of key biological roles, mainly mediated by their interaction with DNA or RNA. Our understanding of the interaction of zinc fingers with DNA has been greatly aided by the structural analysis of a number of protein−DNA complexes by NMR spectroscopy and X-ray crystallography. In comparison, we know relatively little about the binding of zinc finger proteins to RNA.

TFIIIA has the ability to bind to specific sequences within the 5S RNA gene, and to the resulting 5S RNA transcript. Binding to DNA is mediated by sequence-specific contacts formed between amino acids on the protein and base pairs in the DNA (*17*, *18*). Disruption of one or more of these contacts by mutation of the DNA ligand or zinc fingers of the protein results in a significant loss of free energy of binding (*13*−*15*, *20*, *25*, *27*, *31*). In comparison, binding of TFIIIA to 5S RNA appears to be mediated primarily by the three-dimensional structure of the RNA rather than by the formation of bonds to specific bases on the nucleic acid (*14*, *20*−*23*, *38*−*40*).

The ability of TFIIIA to bind to both DNA and RNA is explained in part by a partitioning of the two activities with different sets of zinc fingers within the protein (*19*, *24*). Much

of the DNA binding activity can be retained by a zinc finger peptide that consists of fingers 1−3 of the protein. Much of the RNA binding activity can be retained by zinc finger peptides that consist of fingers 4−6 or 4−7 of the protein (*10*, *19*, *25*, *26*, *37*, *40*, *41*). While these zinc finger peptides have proven to be useful in structural studies of the interaction of TFIIIA with DNA and RNA, other studies have demonstrated that they may not accurately model the interaction of the full-length protein with nucleic acids (*13*, *27*, *28*). In particular, the interactions between zinc fingers within the full-length protein may provide structural stability that modifies the nucleic acid binding properties of individual zinc fingers.

We have been studying the role of individual zinc fingers of TFIIIA in DNA and RNA binding by creating finger swap and substitution mutations within the full-length protein (*31*). This approach led to the identification of key amino acids within zinc finger 3 that are involved in sequence-specific DNA binding. NMR and X-ray crystallographic analysis of zinc finger peptide−DNA complexes confirmed that these residues were involved in DNA binding (*17*, *18*). In the report presented here, we have extended our analysis to the central zinc fingers of TFIIIA.

Analysis of the Role of Zinc Fingers 4−7 in the Binding of 5S DNA by TFIIIA. Only substitution of the α-helix of finger 5 with sequences from either p43 or WT1 significantly reduced the level of interaction of TFIIIA with 5S DNA, but not 5S RNA. This result is consistent with the crystal structure of a complex of fingers 1−6 of TFIIIA with a fragment of the 5S RNA gene (*17*). In the crystal structure, sequence-specific protein−DNA contacts are formed between the side chains of amino acids L148, S150, R151, and R154 in finger 5 and functional groups facing the major groove on base pairs 70, 71, 73, and 74. Mutation of a zinc-coordinating histidine in finger 5 results in a loss of the DNase I footprint in the region of base pairs 70−73 (*13*). Base substitutions within the intermediate element of the promoter significantly reduce the binding affinity for wild-type TFIIIA, with the largest effect observed for substitutions of the G·C base pair at position 70 of the gene (*15*). In contrast, substitution of base pair 71 had a relatively minor effect on TFIIIA binding and simultaneous substitution of base pairs 73 and 74 had no effect on TFIIIA binding affinity. These data suggest that the interactions of R151 with base pair 71 and R154 with base pair 70 are likely to be the main contributors from finger 5 to the overall free energy of binding of TFIIIA to 5S DNA. The data we have measured for scanning mutants of this region of finger 5 are consistent with this conclusion. In mutant Xp5B, R151 is replaced with a lysine and R154 is replaced with an alanine. This mutant binds to the 5S DNA with a 4-fold lower affinity than wild-type TFIIIA, an effect similar to that of introducing a substitution of base pair 70 in the DNA (*15*). The lysine that replaces arginine 151 might continue to contact base pair 71. In mutant Xw5B, R151 is substituted with an asparagine, while R154 is not replaced. This mutant binds the 5S DNA with a 10-fold lower affinity than the wild-type TFIIIA, confirming the importance of the contact with base pair 71 to the overall interaction of TFIIIA with DNA.

Analysis of the Role of Zinc Fingers 4−7 in the Binding of 5S RNA by TFIIIA. Comparison of the effects that specific nucleotide substitutions in the 5S RNA gene versus the 5S RNA have on TFIIIA has established that DNA binding is sequence-specific while RNA binding is structure-specific (*14*). A series of experiments with deletion and truncation mutants of TFIIIA have implicated central zinc fingers 4−6 or 4−7 in high-affinity binding to 5S RNA (*19*, *25*). These zinc fingers are resistant to proteolysis of a TFIIIA−5S RNA complex (*42*). Comparison of the ribonuclease footprints of full-length TFIIIA and zinc finger peptides on 5S RNA established that fingers 4−7 of TFIIIA interact with the central structure of the 5S RNA (*37*, *41*). In general, mutations of the 5S RNA have similar effects on the binding of full-length TFIIIA and the zf4−7 peptide, although the magnitudes of the effects on the interaction of the peptide were often greater (*37*). This result suggested that a 5S RNA−protein complex formed with the zf4−7 peptide is more easily perturbed by disruption of one part of the protein−RNA interface than a complex formed with the full-length TFIIIA.

We have evaluated the role of individual amino acids within fingers 4−6 of TFIIIA in 5S RNA binding by scanning and point mutagenesis. Studies on the binding of TFIIIA zinc finger peptides to 5S RNA have implicated lysine 118 and glutamine 121 within finger 4 as being critical for the interaction (*19*, *26*). Replacement of the lysine with an alanine in a peptide containing zinc fingers 4−7 decreases the affinity of the peptide for the RNA by almost 37-fold (*26*). In comparison, the RNA binding data for mutants Xw4B and Xw4B-Ala suggest that lysine 118 in finger 4 is not as critical for binding of full-length TFIIIA to 5S RNA. The small effect observed in mutant Xw4B could result from a conformational change in the α-helix of finger 4 which disrupts the lysine−RNA interaction. In this case, replacement of the lysine with alanine in the Xw4B-Ala mutant would not be expected to reduce the RNA binding affinity any further.

In a study with a peptide containing zinc fingers 1−7 of TFIIIA, replacement of the glutamine at position 121 within finger 4 with an arginine reduced the affinity of the peptide for the RNA by 4.5-fold (*19*). In the scanning mutant Xp4B, this glutamine is replaced with an alanine, while in scanning mutant Xw4B, the glutamine is replaced with a histidine. The effects of these scanning mutants on 5S RNA binding affinity are quite small. Thus, the most straightforward explanation of our data with scanning mutants would be that finger 4 makes a relatively small contribution to the overall free energy of binding of full-length TFIIIA to 5S RNA.

A threonine-tryptophan-threonine (TWT) motif beginning at amino acid 176 in zinc finger 6 has also been demonstrated to be important for binding of TFIIIA peptides to 5S RNA (*19*, *26*, *40*). In mutant Xw6B-I, replacing the TWT motif of finger 6 in full-length TFIIIA with the sequence RSD from a WT1 zinc finger has a significant effect on RNA binding activity, reducing it by approximately 11-fold. Clearly, this motif in finger 6 is critical for the interaction of full-length TFIIIA or zinc finger peptides with 5S RNA.

Analysis of the Role of Zinc Fingers 4 and 6 in the Binding of 5S RNA by p43. Both TFIIIA and p43 have nine zinc fingers, and both bind specifically to 5S RNA. Given the weak sequence similarity between the two proteins, it is striking that both proteins have the TWT motif in finger 6. It is therefore surprising that substitution of this motif in p43 does not reduce the affinity of the protein for 5S RNA,

while substitution of the lysine in finger 4 does slightly decrease the level of 5S RNA binding in p43 but apparently not in TFIIIA. This result is consistent with the fact that equilibrium binding of TFIIIA and the binding of p43 to 5S RNA have distinctly different characteristics (*32*, *35*). In addition, while both proteins rely upon the three-dimensional shape of the 5S RNA for binding specificity, the effects of specific mutations in the 5S RNA differ for the two proteins. The data on TFIIIA and p43 mutations and their effects on binding of the proteins to 5S RNA simply underscore these differences. The fact that the TWT motif of zinc finger 6 of p43 can be substituted without significantly reducing the 5S RNA binding affinity is also consistent with the observation that a peptide containing zinc fingers 5−9 of p43 does not bind to 5S RNA (*10*). A peptide containing zinc fingers 1−4 of p43 does bind with high affinity to 5S RNA, and the results obtained with the p43 K>A mutant suggest that the lysine in finger 4 may contribute to this binding.

Comparison to Previous Studies with Zinc Finger Peptides or Broken Finger Mutants of TFIIIA. Much of what is known about the role of specific zinc fingers and amino acid residues in the binding of TFIIIA to DNA and RNA has come from studies with either zinc finger peptides (*10*, *19*, *25*, *26*, *37*, *40*, *41*) or finger disruption mutants of full-length TFIIIA (*13*, *27*, *28*). One interpretation of the results obtained with TFIIIA peptides has been that the two nucleic acid binding activities of TFIIIA are partitioned between different sets of zinc fingers. However, results obtained with finger disruption mutations in full-length TFIIIA suggest that finger−finger interactions may either stabilize or restrict the interaction of individual zinc fingers with DNA or RNA in ways that do not apply to the peptide−nucleic acid interactions.

Arriving at an understanding of how full-length TFIIIA interacts with two distinct nucleic acid ligands will ultimately require additional information about the role of finger−finger interactions as well as finger−nucleic acid interactions. A comparison of the effects that the residue swapping mutations we have introduced into full-length TFIIIA have with the results of these previous studies using peptides or finger disruption mutants leads to some intriguing observations that can generally be organized into three categories of differential effects.

The first category of differential effects consists of substitution mutations in full-length TFIIIA that result in smaller effects on DNA or RNA binding than do mutations that disrupt the structure of specific zinc fingers. For example, the p43 and WT1 substitutions in fingers 4, 6, and 7 all have significantly smaller effects on the binding of TFIIIA to 5S DNA than do the corresponding "broken finger" mutations of those same zinc fingers (*13*, *27*). Indeed, the substitution mutations in these three zinc fingers either have no effect on the affinity of TFIIIA for 5S DNA or reduce it by less than 2-fold. In comparison, the three broken finger mutants all reduce the level of binding of TFIIIA to 5S DNA, the effects being 9.3-fold for finger 4, 3.4-fold for finger 6, and 5.8-fold for finger 7. One possible interpretation of this difference could be that nearest-neighbor finger−finger interactions establish spatial constraints that are required for the interaction of each finger with DNA. Such finger−finger interactions would be disrupted in the broken finger mutants of TFIIIA, but would not necessarily be affected in the substitution mutations, which maintain structural integrity of the zinc finger.

Substitution mutations in fingers 4 and 5 and disruption of these fingers have effects of similar magnitude on the binding of TFIIIA to 5S RNA. In comparison, substitution of residues in finger 6 (e.g., mutant Xw6B) has a much larger deleterious effect on 5S RNA binding than does disrupting zinc coordination of this finger (*28*). Thus, the trend for RNA binding is different than that discussed above for DNA binding. One potential explanation for this difference lies in the observation that productive interaction of fingers 1−3 with 5S RNA is somehow inhibited in wild-type TFIIIA, but not in the broken finger 6 mutant (*28*). The potential disruption of specific amino acid−nucleic acid contacts in this broken finger mutant may be offset by the loss of inhibitory finger−finger interactions that normally prevent the interaction of fingers 1−3 with the RNA. In the Xw6B mutant protein, the amino acid substitutions that are introduced may disrupt specific interactions between the protein and RNA without relieving the inhibitory finger−finger interactions that prevent fingers 1−3 from binding to the 5S RNA.

The second category of differential effects consists of substitution mutations in full-length TFIIIA that result in smaller effects on DNA or RNA binding than do substitution mutations in zinc finger peptides of TFIIIA. Comparison of these effects on a one-to-one basis can be complicated by the fact that different amino acid substitutions in the α-helix region of a zinc finger can alter the helical conformation in ways that change the orientation of amino acid side chains toward the nucleic acid. Therefore, caution is required in attempting to ascribe too much significance to such differences. However, we do observe clear differences in the effects that substitution mutations in fingers 4 and 6 in full-length TFIIIA (Xw4B and Xw6B mutants) have on both DNA and RNA binding compared to the effects that substitution mutations in the same zinc fingers of truncated TFIIIA molecules have on the binding of those proteins to DNA and RNA.

A point mutation (Q121R) within finger 4 significantly reduces the 5S DNA binding affinity of a finger 1−7 peptide of TFIIIA, as does a point mutation (T176I) within finger 6 (*24*). Finger swap mutations that target these two residues in full-length TFIIIA (e.g., Xw4B and Xw6B) have little or no effect on 5S DNA binding affinity. Comparison of the effects on 5S RNA binding affinity of substitution mutations in fingers 4 and 6 of full-length TFIIIA and zinc finger peptides has already been discussed above. Once again, the mutations in the full-length protein have smaller effects on the binding to 5S RNA than do the mutations in the zinc finger peptides. One potential explanation of this phenomenon may be the "compensatory" binding model proposed on the basis of the results obtained with the broken finger mutations (*27*, *28*). For example, in the full-length protein, finger−finger interactions may occur that destabilize the interaction of finger 4 with 5S RNA. If such a destabilizing interaction were absent in the finger 4−7 peptide of TFIIIA, it would explain why amino acid substitutions in finger 4 of the peptide have a more deleterious effect on RNA binding than do substitution mutations of the same finger in full-length TFIIIA. Further resolution of this question will require careful studies of matched substitution mutations in full-

length TFIIIA and the relevant zinc finger peptide, because of the limitations inherent in comparing different types of substitution mutations.

The third category of differential effects consists of substitution mutations in full-length TFIIIA that result in larger effects on DNA or RNA binding than does complete elimination of the relevant zinc finger via construction of truncated peptide fragments of TFIIIA. Such a difference suggests that these zinc fingers in the full-length protein may interfere with potential modes of nucleic acid recognition observable only in the minimal zinc finger peptides. For example, a peptide containing only fingers 1−3 of TFIIIA has a binding affinity for 5S DNA that is close to that of the full-length protein (*24*). However, replacing zinc fingers 4−7 with the corresponding fingers from p43 in mutant Tp4−7 reduces the affinity of TFIIIA for DNA by 100-fold, even though no mutations have occurred in zinc fingers 1−3. Indeed, point mutations in fingers 4 and 6 of a zf1−7 peptide have a greater effect on DNA binding affinity than simply deleting fingers 4−7 (*24*). Thus, it appears that the ability of zinc fingers 1−3 alone to direct high-specific activity binding of TFIIIA to 5S DNA is only possible in a truncated peptide, and not in the full-length protein.

Investigation of the nucleic acid binding properties of the Xw4−7 mutant provided additional novel insights into the interference properties of zinc fingers. Although this mutant has five wild-type TFIIIA zinc fingers, it is unable to bind to either 5S RNA or 5S DNA. In addition, the WT1 zinc fingers within this mutant are unable to bind to a WT1 consensus DNA binding site (data not shown). However, the WT1 zinc fingers within this mutant are capable of binding to a WT1-specific RNA aptamer with an affinity equivalent to that of a zinc finger peptide containing only the four WT1 zinc fingers. These observations suggest the possibility of additional zinc finger interference within TFIIIA that restricts the ability of zinc fingers within the finger 4−6 or 4−7 positions to bind to DNA molecules.

Role of Finger−Finger Interactions in the Modes of Binding to 5S RNA by p43. The results of this study raise some fascinating questions about the interaction of TFIIIA and p43 with nucleic acid ligands, and the functional relationships between these two proteins. It is intriguing that fingers 4−7 of p43 can functionally replace fingers 4−7 of TFIIIA in 5S RNA binding activity, even though a peptide that includes these p43 fingers is unable to bind to the RNA. It is also intriguing that the TWT motif is present in both proteins but only required for 5S RNA binding by TFIIIA. These observations raise the question of what additional functions fingers 4−7, and particularly the TWT motif, might provide to p43 and TFIIIA. Another mystery surrounds the negative effects that finger−finger interactions may play in TFIIIA, and whether such interactions occur in p43. Even though a peptide containing fingers 1−3 of TFIIIA can bind to DNA and RNA with high specificity and affinity (*28*, *43*), mutations introduced downstream of these fingers in the full-length protein prevent binding to either nucleic acid ligand. In a similar way, zinc fingers 4−7 of p43 can functionally substitute for the corresponding fingers in TFIIIA in 5S RNA binding activity, even though mutational evidence suggests a small contribution by finger 4 in RNA binding by p43 and no role for finger 6. This observation raises the question of whether a strong interaction by the other zinc fingers of p43 with 5S RNA prevents the interaction of fingers 4−7 with the RNA. This model of p43−RNA interaction is similar to that proposed for TFIIIA, except that in the case of TFIIIA fingers 1−3 are prevented from strongly interacting with the RNA (*28*). Development of a genetic assay for the 5S RNA binding activities of TFIIIA and p43 may allow for screening of mutations and second site revertants that could shed light on how these interfering finger−finger interactions occur.

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