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Structural elements in the N-terminal half of transcription factor IIIA required for factor binding to the 5S RNA gene internal control region

James F.Smith, Jody Hawkins, Robert E.Leonard and Jay S.Hanas*
Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA

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ABSTRACT

Zinc binding domains and the conserved Thr-Gly-Glu-Lys (TGEK) tetrapeptide in the N-terminal half of transcription factor IIIA (TFIIIA) were subjected to *in vitro* mutagenesis to biochemically assess their role in factor interaction with the 5S gene internal control region (ICR). TFIIIA containing a Leu in place of His33 in the Cys_2His_2 zinc binding site of finger I lost the ability to protect the entire 5S RNA gene ICR (nucleotides +96 to +43) from DNase I digestion. Thus, mutation of one potential zinc ligand in the N-terminal finger inhibited specific DNA binding by the N-terminal as well as downstream fingers. Cooperativity apparently exists among TFIIIA zinc fingers in metal binding/finger folding and DNA binding. Substituting a Ser for Gly69 or a Glu for Lys 71 in the conserved TGEK tetrapeptide in finger II of TFIIIA resulted in the loss of DNA binding. A Gly-dependent bend structure and a terminal positive charge in this tetrapeptide are important for TFIIIA interaction with DNA. Whereas TFIIIA with a Ser substituted for Cys20 in finger I (proposed zinc ligand) did not protect the ICR from DNase I digestion, TFIIIA containing a Ser substituted for Cys35 (not a proposed zinc ligand) retained the ability to bind the ICR. When Cys112 or Cys 164 (proposed zinc ligands in fingers IV and VI) were replaced by Ser, the DNase I footprint patterns afforded by the respective mutant proteins were similar, protection on the ICR from about nucleotides +96 up to +78. A similar pattern was obtained with a TFIIIA mutant in which fingers V,VI,VII, and a portion of VIII were deleted. Maintenance of zinc coordination spheres is necessary for DNA binding by downstream fingers. The six fingers comprising the N-terminal half of TFIIIA appear to act in two groups of three with binding of the second group dependent upon initial binding of the N-terminal group to the +90 to +80 region of the 5S gene ICR.

INTRODUCTION

Elucidation of the structure and function of eukaryotic gene regulatory proteins is necessary for understanding cell differentiation and development. Transcription factor IIIA (TFIIIA) was initially identified in *Xenopus laevis* oocytes as a 39 kDa protein which specifically binds to the internal control region (ICR) of the *Xenopus* 5S ribosomal gene (1,2,3). In concert with TFIIIB and TFIIIC, TFIIIA promotes 5S RNA synthesis by RNA polymerase III (4). TFIIIA protects the ICR from DNase I digestion from about nucleotides +43 to +96 (1) with strong contacts between TFIIIA and the 5S gene residing in the 3′ portion of the ICR (from about +80 to +90, ref. 5). TFIIIA contains zinc and requires the metal for specific DNA binding (6). The zinc in TFIIIA holds the protein in the proper conformation for specific DNA binding (7). The amino acid sequence of *Xenopus laevis* TFIIIA (8) contains nine repetitive domains, each with the potential to coordinate a zinc ion between two cysteines and two histidines (9,10). Examination of species variation in the amino acid sequence of TFIIIA indicates conservation in the proposed Cys_2His_2 zinc coordination sphere in all nine fingers and in a Thr-Gly-Glu-Lys (TGEK, single letter code, ref. 11) tetrapeptide repeat located in the first two fingers (12). This tetrapeptide is present in other transcription factors containing the Cys_2His_2 motif, e.g. the mouse immediate early protein Zif268 and Sp1, the GC box transcription factor (13,14). Previous deletion analyses indicated the two N-terminal zinc fingers of TFIIIA interact with the 3′ portion of the 5S gene ICR (15,16). The conserved tetrapeptide located in these first two fingers could have a role in DNA binding.

Important structural information is available on the Cys_2His_2 type zinc finger first identified in TFIIIA and present in a wide variety of eukaryotic gene regulatory proteins. This type of finger contains about 30 amino acids with the N-terminal Cys_2 side folded into an antiparallel beta sheet and the C-terminal His_2 side folded in an alpha helix (17,18,19). In the DNA binding domain of Zif268, two out of three fingers have the TFIIIA-like TGEK tetrapeptide with the Thr being the last residue of the alpha helix (19). Each finger in Zif268 and Sp1 interacts with 3 bp

* To whom correspondence should be addressed

of DNA (19,20). Although a great deal of structural information is available about the Cys_2His_2-type zinc finger, it is also important to carry out biochemical studies on intact, functional proteins. In the present study, zinc binding domains and the highly conserved TGEK tetrapeptide in the N-terminal half of TFIIIA were subjected to *in vitro* mutagenesis to determine their necessity in binding the 5S RNA gene ICR and to shed light on the overall binding process.

METHODS AND MATERIALS

In vitro mutagenesis of *Xenopus laevis* TFIIIA cDNA

A mutagenesis template was constructed by ligating the cDNA EcoRI-SphI fragment coding for the N-terminal half of *Xenopus laevis* TFIIIA (8) into M13mp18 replicative form digested with EcoRI and SphI. To confirm the presence of the entire EcoRI-SphI fragment, single-stranded phage DNA was purified and sequenced by the chain termination method (21) using a modified form of T7 DNA polymerase (Sequenase, United States Biochemical, Cleveland, OH). Site-directed mutagenesis was performed according to the method of Eckstein and coworkers (22) with reagents provided by Amersham. Briefly, the respective mutant oligonucleotides (Fig. 1 legend, provided by K.Jackson, St. Francis Hospital of Tulsa, Oklahoma) were phosphorylated with bacteriophage T4 polynucleotide kinase and annealed to single-stranded template DNA containing the TFIIIA cDNA which codes for the N-terminal half of the protein. The second DNA strand was synthesized from this template/primer with the Klenow fragment of DNA polymerase I (incorporating dNTPs and αSdCTP) followed by ligation with T4 DNA ligase. After removal of oligonucleotides, the double-stranded circle was nicked with restriction enzyme NciI (nicks only native strand without αSdCMP) followed by digestion with exonuclease III. The second strand was resynthesized using DNA polymerase I and then covalently closed with DNA ligase. The final ligation mixture was used to transform $CaCl_2$-treated *E.coli* TB1 cells. Transformant phage DNAs were screened for the proper mutation by dideoxy sequencing. Once mutated clones were identified, the complete EcoRI-SpHI insert was sequenced to check for unwanted mutations. A TFIIIA deletion mutant was also constructed in which fingers V, VI, VII, and a portion of VIII were removed. This mutant cDNA was constructed by ligating into pT7 vector (digested with EcoRI and PstI) the 436 bp EcoRI-PvuII 5′ fragment and the 529 bp TaqI-PstI 3′ fragment from TFIIIA cDNA (8). The TaqI end of the latter fragment was blunted in a reaction with reverse transcriptase and dNTPs. Conservation of reading frame across the PvuII-TaqI junction was confirmed by double-stranded DNA sequencing.

Cloning and expression of mutant TFIIIA cDNA in *E.coli*

Replicative forms of the mutant phage DNA were purified by ethidium-CsCl equilibrium gradient centrifugation and the TFIIIA EcoRI-SphI insert was isolated by restriction digestion, agarose gel electrophoresis, electroelution, and ethanol precipitation. The mutant EcoRI-SphI fragment coding for the N-terminal half of TFIIIA was subcloned with a SphI-HindIII cDNA fragment (HindIII site derived from the pT7-7 vector) coding for the wild-type, C-terminal half of TFIIIA into plasmid pT7-7 (23) which had been digested with EcoRI and HindIII. The pT7-TFIIIA plasmid (pT7-7 contains a T7 bacteriophage RNA polymerase promoter and ribosome binding site just upstream of the EcoRI site) was sequenced regionally to check the mutation and then transformed into *E.coli* strain K38 (24) harboring plasmid GP1-2. The GP1-2 plasmid contains the T7 RNA polymerase gene under the control of the lambda P_L promoter, the lambda repressor CI857 gene, and a gene for aminoglycoside phosphotransferase (23). Bacterial clones containing both plasmids were selected on nutrient agar plates containing 50 μg/ml ampicillin and kanamycin. For TFIIIA expression and factor isolation, 500 ml cultures of the respective *E.coli* clones were grown overnight with shaking at 40°C, a restrictive temperature for CI857 repressor function. Cell pellets were suspended in 20 mM Tris-HCl, pH 7.6, 320 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM phenylmethyl-sulfonyl fluoride, 0.5 mM DTT and sonicated with a microtip using a Sonics & Materials sonicator. After removal of cell debris by low-speed centrifugation, the supernatant was fractionated by cation exchange chromatography on BIO-REX 70 as described previously (25). TFIIIA in column fractions eluting at 0.50, 0.75, or 1.0 M KCl (in 20 mM Tris-HCl, pH 7.6, 1.5 mM $MgCl_2$, 0.4 mM DTT, and 0.01 mM $ZnCl_2$) was assayed and quantitated by SDS PAGE/immunoblotting with rabbit anti-TFIIIA serum and known amounts of purified *Xenopus*

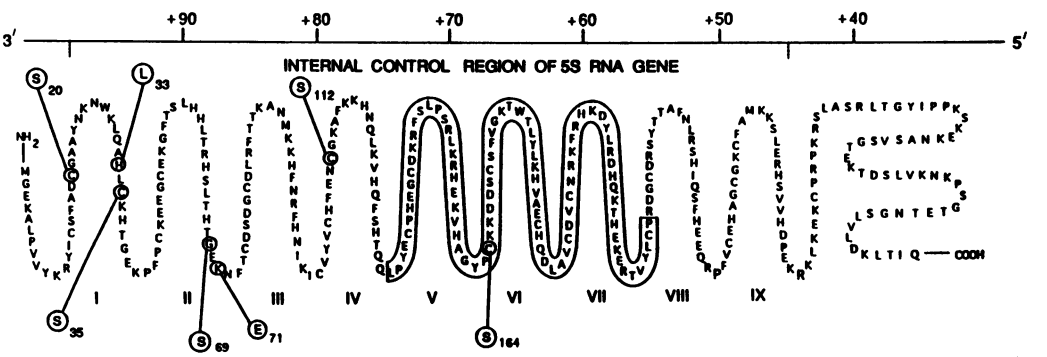

Fig. 1. Amino acids in *Xenopus laevis* TFIIIA targeted for *in vitro* mutagenesis and their replacements. The amino acids in the large circles (numbered) are replacements for the corresponding wild-type amino acids in the small circles in TFIIIA zinc fingers I, II, IV, or VI. Mutagenic oligonucleotides used were as follows (underlined nucleotide is mutation): Cys20 to Ser, GCC GAC TCC GCC GCT; His33 to Leu, CAG GCG CTT CTG TGC; Cys35 to Ser, CG CAT CTG TCC AAA CAC; Lys71 to Glu, GGC GAG GAA AAC TTC; Gly69 to Ser, T CAT ACT AGC GAG AAA; Cys112 to Ser, GAG AAC TCT GGC AAA; Cys164 to Ser, TAT CCC TCC AAA AAG. The boxed region in the structure delineates the TFIIIA finger V–VIII deletion mutation described in Fig. 2B and Fig. 5. The alignment of the TFIIIA amino acid sequence with the 5S gene ICR is according to a previously proposed model (9).

laevis TFIIIA (25). Immunoblot band intensities were quantified with a Molecular Dynamics computing densitometer using Image Quant software.

Isolation of 7S Particles and TFIIIA from *Xenopus laevis* and DNase I footprinting

Immature ovaries, isolated from 4–5 cm *Xenopus laevis* (Nasco, Fort Atkinson, WI), were homogenized in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM phenylmethyl-sulfonyl fluoride. 7S particles, comprised of TFIIIA and oocyte 5S RNA, were isolated by glycerol gradient centrifugation of ovarian homogenates followed by DEAE ion exchange chromatography of the 7S fractions (26). TFIIIA in the 7S particle was liberated from 5S RNA by digesting the particle (100 μg/ml in buffer A, 20 mM Tris-HCl, pH 7.5, 70 mM NH_4CL, 7 mM $MgCl_2$, 0.1% nonidet P-40, and 0.5 mM DTT, or buffer B, 20 mM Tris-HCl, pH 7.5, 320 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) with RNase A (40 μg/ml, Calbiochem) for 30 min at 23°C. Protein amounts were determined by the method of Bradford (27) using bovine serum albumin as the standard.

The *Xenopus borealis* somatic 5S RNA gene was end-labeled on the coding strand by EcoRI digestion of a 5S gene-containing plasmid and filling in the 5′ overhang with [α^{32}P]dATP and reverse transcriptase (25). Unincorporated radioisotope was removed by chromatography on Sephadex G-100 and the DNA peak was ethanol precipitated and redigested with BamHI. The 303 bp EcoRI-BamHI fragment containing the 5S RNA gene was purified by polyacrylamide gel electrophoresis. Specific activity of the 5S gene-containing fragment (cpm/μg) was determined by scintillation counting and absorbance at 260 nm. DNase I protection assays with native TFIIIA isolated from *Xenopus* oocytes, wild-type and mutant TFIIIA expressed in and isolated from *E.coli* were performed in 20 μl reaction volumes with protein and end-labeled DNA amounts as indicated in the Fig. legends. Reaction conditions were the same as those described previously (25).

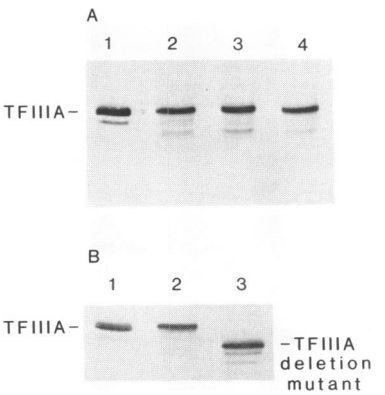

Fig. 2. Immunoblot of native TFIIIA isolated from *Xenopus laevis* and wild-type and mutant TFIIIA expressed in and isolated from *E. coli*. Purification of native TFIIIA, expression and isolation of wild-type and mutant TFIIIA, and SDS PAGE/immunoblotting were all performed as described in the METHODS AND MATERIALS. Samples electrophoresed in panel A: lane 1, 1 μg of native TFIIIA; lanes 2–4, 25 μl of purified wild-type, His33, and Gly69 mutant TFIIIA. Relative amounts of TFIIIA in lanes 2–4 (determined by densitometry of major band relative to lane 1) are 0.8, 0.8, and 0.7 μg. Panel B: native TFIIIA (1 μg, lane 1), 25 μl of wild-type TFIIIA expressed in *E.coli* (lane 2), and 25 μl finger V–VIII deletion mutant TFIIIA (lane 3). Relative amounts of TFIIIA in lanes 2 and 3 (determined by densitometry of major band) are 0.7 and 1.2 μg.

RESULTS

Site-directed mutations in TFIIIA zinc fingers

Previous deletion analyses of TFIIIA zinc fingers indicated the N-terminal fingers interact with the 3′ region of the ICR (15,16). Because large deletions can have adverse effects on overall protein conformation, it is difficult to correlate this loss of function with a well-defined structural alteration. Site-directed mutagenesis can provide more detailed information and understanding of structure-function relationships. Fig. 1 illustrates individual amino acids in TFIIIA targeted in the present study for oligonucleotide-directed mutagenesis and the substituted residues. Cys20 in the N-terminal finger I, a residue proposed to coordinate zinc (9), was replaced by a non-coordinating Ser. Ser was used for the Cys replacement because it best approximates the size and polarity of Cys without the ability to coordinate zinc. His33, also proposed to coordinate zinc in the first finger, was replaced by a Leu. As a control mutation, Cys35, an amino acid in the first finger not proposed to coordinate a metal ion, was replaced by a Ser. Gly69 and Lys71, located in the TGEK tetrapeptide in finger II, were replaced by Ser and Glu respectively. TGEK is also found in finger I and is the largest amino acid repeat in TFIIIA. Cys112 and Cys164, proposed to coordinate zinc ions in TFIIIA fingers IV and VI, were also replaced by Ser. The boxed region in Fig. 1 delineates a finger V–VIII deletion mutation.

Wild-type and mutant TFIIIAs were expressed in *E.coli* and partially purified by ion-exchange chromatography as described previously (25). These TFIIIAs were subjected to SDS PAGE and immunoblotted with rabbit anti-TFIIIA antiserum to determine protein size, integrity, and amount. Fig. 2A shows a SDS PAGE/immunoblot of native, wild-type and two representative mutant TFIIIAs. Lane 1 contains native TFIIIA as isolated from *Xenopus* immature ovarian tissue; this protein has a molecular mass of 39 kDa and is 90% pure (a small amount of a proteolyzed fragment is present in this as well as other lanes). Immunoblotted in lanes 2,3, and 4 are wild-type, His33, and

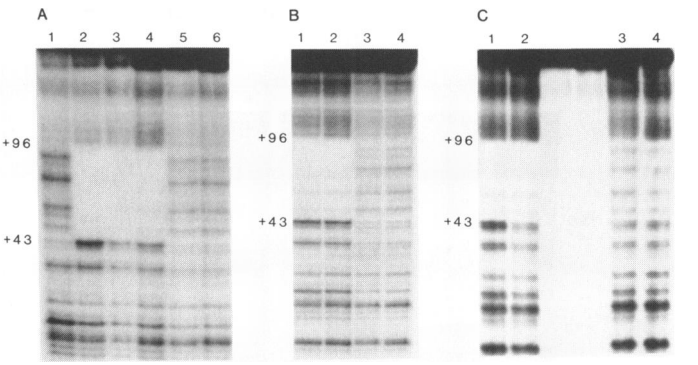

Fig. 3. DNase I protection analyses of histidine and conserved tetrapeptide mutations on TFIIIA-5S gene ICR interactions. Site-directed mutagenesis, isolation of native, wild-type, and mutant TFIIIA, DNA end-labeling, DNase I protection assays, gel electrophoresis, and autoradiography were performed as described in METHODS AND MATERIALS. DNase I protection reactions electrophoresed (panel A): lanes 1 and 2, reaction minus and plus 1×10^{-8} M native TFIIIA (under these conditions, binding saturation occurs at about 3×10^{-9} M TFIIIA, ref. 26); lanes 3 and 4, plus 1.3 and 2.6×10^{-8} M wild-type TFIIIA; lanes 5 and 6, plus 1.3 and 2.6×10^{-8} M mutant TFIIIA (His33 to Leu, finger I). Panels B and C: identical DNase I experiments to that described in panel A, lanes 3–6, except that mutant TFIIIAs used were Lys71 to Glu (B) and Gly69 to Ser (panel C, concentrations of Gly69 mutant were 1 and 2×10^{-8} M). The annotations on the borders mark those nucleotide positions in the 5S RNA gene coding strand (25).

Gly69 mutant TFIIIAs expressed in *E.coli*. The major band in each of these lanes was quantified relative to the native TFIIIA band of similar electrophoretic mobility in lane 1. A small fusion peptide (16 amino acids as deduced from the DNA sequence) is most likely attached to their N-termini because of in-frame translation of the pT7 polylinker and the 5′ region of the TFIIIA cDNA. The presence of these extra amino acids does not impair protein function (16,25). The Cys20, 35, 112, 164, and Lys71 mutant TFIIIAs were immunoblotted in identical fashion (not shown). Fig. 2B is an immunoblot of native TFIIIA from *Xenopus* ovaries (lane 1), wild-type TFIIIA expressed in *E.coli* (lane 2), and the TFIIIA finger V-VIII internal deletion mutant (lane 3). The electrophoretic mobility of the deletion mutant is greater than intact TFIIIA and corresponds to about a 10 kDa reduction in molecular mass in this SDS PAGE system. TFIIIA bands in this blot were quantified in identical fashion as those in panel A.

Requirement for histidine and the conserved tetrapeptide in structure and function of the N-terminal region of TFIIIA

When His33 in TFIIIA finger I was replaced by a non-chelating leucine (a Leu should be compatible with the alpha-helical structure in this region), the mutant protein was unable to protect the 5S gene ICR from DNase I digestion (+96 to +43, lanes 5 and 6, Fig. 3A). The digestion pattern in the presence of this mutant TFIIIA was similar to that observed in the absence of native TFIIIA (lane 1). The DNase protection afforded by wild-type TFIIIA from *E.coli* was the same as native TFIIIA including DNase I hypersensitivity at nucleotide +43 (cf. lanes 3 and 4 with lane 2, Fig. 3A). This DNase I hypersensitivity was missing when no TFIIIA (lane 1) or the His33 mutant (lanes 5 and 6) were included in the reactions (all footprint reactions presented in this study were repeated using a large range of protein concen trations to insure saturation and reproducibility). These results indicate His33 is necessary for TFIIIA structure and function, most likely in zinc coordination. Although only His33 in finger I is mutated, fingers II-IX also fail to interact correctly with the ICR. *Rana catesbeiana* (bullfrog) TFIIIA, which has 49 amino acid changes relative to *Xenopus laevis* TFIIIA in its N-terminal half (none in the proposed zinc coordination spheres, ref. 12), still interacts with the *Xenopus* 5S gene ICR (as assayed by DNase I protection, ref. 28). Therefore, a wide variety of amino acid changes do not deleteriously affect TFIIIA structure and function.

In the *Rana* frog genus, the Lys present in the conserved TGEK tetrapeptide is replaced by the similarily charged Arg (12). To examine whether a positive charge at this position may be required for proper TFIIIA structure and function, Lys71 in finger II was changed to a Glu (Fig. 1) and the resultant protein tested for its ability to protect the 5S gene ICR from DNase I digestion (Fig. 3B). When compared with wild-type TFIIIA footprint pattern (lanes 1 and 2), this mutant TFIIIA did not adequately protect the control region from DNase I digestion (lanes 3 and 4). In addition, the Lys71 mutant TFIIIA did not induce DNase I hypersensitivity at +43, also indicative of a lack of proper TFIIIA binding. Point mutations in either fingers I or II have the identical result: complete loss of DNA binding by all nine fingers.

Because of its small size, Gly is often found in bends in proteins (29). To examine whether Gly69 has a possible role in bend formation between adjacent zinc fingers in TFIIIA, this residue was replaced with a larger and more polar amino acid, Ser (Fig. 1) and the effect of this change in protein structure was assayed by DNase I protection. Fig. 3C shows the DNase I digestion patterns in the presence of wild-type or Gly69 mutant TFIIIA. Lanes 3 and 4 exhibit the patterns on the 5S gene ICR in the presence of increasing concentrations of Gly69 mutant TFIIIA. Incomplete protection over the entire ICR (nucleotides +96 to +43) is observed in these lanes in contrast to strong protection observed in the presence of wild-type protein (cf. lanes 3 and 4 with lanes 1 and 2, the TFIIIA control). Quantification by densitometry of the +96 to +43 regions indicated a three-fold decrease in protection of the Gly69 mutant than wild-type

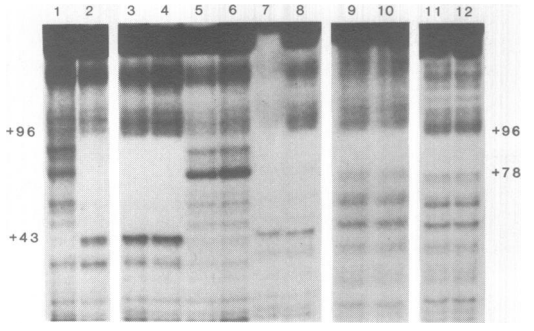

Fig. 4. Influence of cysteine residue changes in TFIIIA on factor interaction with the 5S gene ICR. Experiments were performed in identical fashion to that described in Fig. 3 legend. In all DNase I protection assays, the *Xenopus* 5S RNA gene-containing fragment was end-labeled on the coding strand at a final concentration of 1×10^{-9} M. DNase I protection reactions electrophoresed in lanes 1 and 2, minus and plus 1×10^{-8} M native TFIIIA; lanes 3 and 4, plus 1.3 and 2.6×10^{-8} M wild-type TFIIIA; lanes 5 and 6, 1.3 and $2.6x^{-8}$ M mutant TFIIIA (Cys20 to Ser, finger I); lanes 7 and 8, 1 and 2×10^{-8} M mutant TFIIIA (Cys35 to Ser, finger I); lanes 9 and 10, 1 and 2×10^{-8} M mutant TFIIIA (Cys112 to Ser, finger IV); lanes 11 and 12, 1.3 and 2.6^{-8} M mutant TFIIIA (Cys164 to Ser, finger VI).

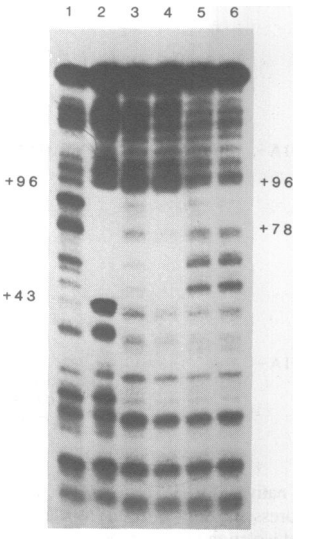

Fig. 5. Effect of deletion of TFIIIA fingers V-VIII on factor interaction with the 5S gene ICR. Native, wild-type, and mutant TFIIIAs were isolated as described in the Fig. 3 legend. DNase I protection reactions were identical to those described in the Fig. 3 legend. Protection reactions electrophoresed: lanes 1 and 2, minus and plus 1×10^{-8} M native TFIIIA; lanes 3 and 4, plus 1 and 2×10^{-8} M wild-type TFIIIA; lanes 5 and 6, plus 2 and 4×10^{-8} M finger V−VIII deletion mutant TFIIIA. Annotations on the left and right borders were described in ref. 25.

protein. This result indicates the Gly69 to serine mutation has significantly decreased the ability of TFIIIA to interact with the 5S gene ICR.

DNase I protection patterns afforded by TFIIIAs containing cysteine or deletion mutations

Specific Cys residues in the nine TFIIIA zinc fingers are also proposed to coordinate zinc ions which are required for TFIIIA function (9,6). Fig. 4 illustrates the DNase I digestion patterns of the end-labeled 5S RNA gene (coding strand) obtained in the presence of TFIIIAs containing various Cys mutations. Lanes 3 and 4 exhibit the DNAse I digestion products in the presence of increasing amounts of wild-type TFIIIA expressed in and isolated from *E.coli*; normal protection patterns from +43 to +96 were observed including the presence of TFIIIA-induced DNase I hypersensitivity at +43 (compare with lane 2, the native TFIIIA control). When increasing concentrations of mutant TFIIIA containing a Ser substituted for Cys20 (proposed to coordinate zinc in finger I) were included in the DNase I footprint assay, protection was no longer observed between nucleotides +43 to +96 and hypersensitivity was not observed at +43 (lanes 5 and 6). Slight DNase I hypersensitivity was observed at about +78 which is not readily observed in the minus TFIIIA control (lane 1). A TFIIIA mutant with a Ser substituted for Cys35 (residue in finger I *not* proposed to coordinate zinc) was capable of protecting the 5S gene ICR from DNase I digestion as well as inducing DNase I hypersensitivity at nucleotide +43 (lanes 7,8, Fig. 4).

To determine the effect of Cys mutations in the more downstream fingers on the overall ICR DNase I protection, Cys112 in finger IV or Cys164 in finger VI were substituted with serines (Fig. 1). When TFIIIA containing the finger IV mutation was assayed for its ability to protect the 5S gene ICR from DNase I digestion (Fig. 4), strong protection was only observed in the 3′ region of the ICR from nucleotides +96 up to but not including +78 (lanes 9 and 10). An identical pattern was previously obtained with a finger IV deletion mutant whereas a finger VII/VIII deletion mutant resulted in DNase I protection from +96, past +78, and up to but not including +63 (25). TFIIIA containing the Cys164 to serine substitution in finger VI gave strong protection in this same 3′ region (+96 up to but not including +78, lanes 11 and 12). Thus, replacement of Cys112 or Cys164 with non-chelating serines disrupted the structure and function of their respective fingers resulting in apparent normal binding by the N-terminal three fingers but not by the downstream fingers. In addition, disruption of either finger IV or finger VI (mutations 52 residues apart) yielded an identical DNase I digestion pattern which suggests that fingers IV, V, and VI act as a binding unit. Changes in this unit would lead to default binding by the N-terminal region comprising fingers I-III.

To further examine this possibility, a TFIIIA mutant was constructed in which finger V was deleted and also, because of convenient restriction sites, fingers VI, VII, and a portion of VIII (amino acids 133 to 224, Fig. 1, Fig. 2B). The ability of this mutant TFIIIA to protect the end-labeled coding strand of the 5S RNA gene ICR from DNase I digestion is exhibited in Fig. 5. Lanes 3 and 4 or lanes 5 and 6 exhibit the DNase I protection patterns in the presence of increasing concentrations of wild-type or deletion mutant TFIIIA respectively. Whereas wild-type TFIIIA protects the ICR from +96 to +43 (lane 4) like native TFIIIA (lane 2), the deletion mutant TFIIIA affords DNase I protection from +96 up to but not including +78 (lane 6). This protection pattern is the same as that observed with the Cys mutations in fingers IV and VI (Fig. 4).

DISCUSSION

Single amino acid substitutions in proposed zinc ligands (His33 or Cys20) in the N-terminal finger of TFIIIA inhibit interaction of the entire protein with the 5S gene ICR as measured by DNase I protection (Figs. 3 and 4). Possible causes of this phenomenon include disruption of zinc binding in the N-terminal as well as downstream fingers and the resulting negative effect on DNA binding. This conclusion is consistent with previous observations which showed that the three zinc fingers of transcription factor Sp1 unfold and lose most of their zinc and DNA binding ability when one zinc binding site is disrupted (30). Sp1 also loses its DNA binding ability when only one finger is mutated in the DNA binding site (20). These results indicate that zinc binding, finger folding, and DNA binding are highly cooperative processes in proteins containing multiple fingers. In TFIIIA this cooperativity appears to be directional (N to C-terminal) since mutations in downstream fingers do not affect DNA binding by N-terminal fingers (see discussion below). TFIIIA undergoes a significant conformational change upon DNA binding (7). This change may be propagated down the polypeptide chain in the N to to C-terminal direction as DNA binding progresses.

Replacement of Gly or Lys in the conserved tetrapeptide TGEK in N-terminal region of TFIIIA (Gly69, Lys71) inhibited factor interaction with the entire 5S gene ICR (Fig. 3). X-ray crystallographic data on the Zif268 finger region-DNA interaction indicated that the Gly is at the C-terminal border of the DNA binding helix and is located in a slight bend linking adjacent fingers (19). Replacing Gly69 with Ser probably distorts the alpha helix and/or spacer region leading to DNA binding inhibition by the N-terminal and downstream fingers. This conclusion based on biochemical evidence supports conclusions obtained from X-ray crystallographic data (19). The N-terminal region of TFIIIA from the bullfrog contains a Gly21 to Ser replacement (outside the TGEK) relative to *Xenopus laevis* TFIIIA and footprints normally to the 3′ portion of the *Xenopus* 5S gene ICR (28). Thus, not all glycines in N-terminal region of TFIIIA are required.

Although X-ray crystallographic data did not assign a structure/function role for Lys in the TGEK tetrapeptide in the mouse protein Zif268 (19), the strict conservation of a positive charge in this position (either Lys or Arg) in the Cys_2His_2-type zinc finger would suggest an important role. The observation from the present study that replacement of this positive charge inhibits TFIIIA interaction with the 5S gene ICR (Fig. 3) lends biochemical support for a role in DNA binding. This role in DNA binding would be non-specific since this tetrapeptide is present in a wide variety of zinc finger proteins that bind to disparate sequences. The basic amino acid in the tetrapeptide is always four residues on the N-terminal side of the first zinc coordinating cysteine of the next finger. X-ray data did identify a positive charge in a similar position (although not in TGEK) 4 residues upstream of the first coordinating Cys in the first zinc finger in Zif268 binding to the DNA backbone (19). The Lys in TGEK could be performing a similar function. The positive charge might also be required for proper folding of this spacer region.

Replacement of Cys residues proposed to coordinate zinc ions in fingers IV or VI (Cys112 or Cys164) resulted in DNA binding by the N-terminal fingers but not by fingers IV−IX (fig. 4). This

result is consistent with zinc coordination being required for proper folding and DNA binding by downstream fingers as well as N-terminal fingers. The Cys112 and Cys164 mutant TFIIIAs as well as a finger V−VIII deletion mutant all produce the same DNase I protection pattern on the 5S RNA gene ICR, from about nucleotides +96 up to, but not including +78; this region is likely being protected by fingers I−III. Previous studies demonstrated that a TFIIIA mutant containing a finger VII−VIII deletion protected from about +96, past +78, and up to but not including +63 (25). These results taken together would suggest that the six fingers comprising the N-terminal half of TFIIIA are interacting in two units of three with a region in the 3′ portion of the ICR resulting in DNase I protection from about +96 up to but not including +63. The strong contacts between TFIIIA and the 5S RNA gene ICR have been mapped to a 10 bp region, about +80 to +90 (GGATGGGAGA, ref. 5). X-ray cystallographic or solution data have demonstrated that three zinc fingers are capable of strongly interacting as a unit with about a full turn of duplex DNA (e.g. Zif268 and Sp1 binding domains, refs. 19,20). These observations suggest that fingers I−III of TFIIIA interact with the +80 to +90 region in the 3′ portion of the ICR.

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