Interaction of Wild-type and Truncated Forms of Transcription Factor IIIA from Saccharomyces cerevisiae with the 5 S RNA Gene*

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Transcription factor (TF) IIIA, which contains nine zinc finger motifs, binds to the internal control region of the 5 S RNA gene as the first step in the assembly of a multifactor complex that promotes accurate initiation of transcription by RNA polymerase III. We have monitored the interaction of wild-type and truncated forms of yeast TFIIIA with the 5 S RNA gene. The DNase I footprints obtained with full-length TFIIIA and a polypeptide containing the amino-terminal five zinc fingers (TF5) were indistinguishable, extending from nucleotides +64 to +99 of the 5 S RNA gene. This suggests that fingers 6 through 9 of yeast TFIIIA are not in tight association with DNA. The DNase I footprint obtained with a polypeptide containing the amino-terminal four zinc fingers (TF4) was 14 base pairs shorter than that of TF5, extending from nucleotides +78 to +99 on the non-transcribed strand and from nucleotides +79 to +98 on the transcribed strand. The binding site of the protein (reviewed by Pieler and Theunissen (1993)). The 30-amino acid zinc finger motif, characterized by pairs of cysteine and histidine residues, consists of an autonomously folding domain in which an anti-parallel β-sheet and an α-helix fold around a zinc ion. The two cysteine and two histidine residues are coordinated to the zinc, and the structure is stabilized by a hydrophobic core (Parra et al., 1988; Lee et al., 1989; Pavletich and Pabo, 1991). X. laevis TFIIIA is an elongated molecule (Bieker and Roeder, 1984) that protects the entire 50-base pair (bp) ICR of the amphibian 5 S RNA gene from degradation by DNase I. The protein is oriented with its amino terminus toward the 3′-end of the ICR and its carboxyl terminus toward the 5′-end of the gene (Miller et al., 1985; Vrana et al., 1988). Early models describing the disposition of the nine zinc fingers of Xenopus TFIIIA over the 50-bp ICR (Fairall et al., 1986; Vrana et al., 1988; Churchill et al., 1990; Berg, 1990) have been modified to take into consideration the crystal structure of the three zinc fingers of Zif268 bound to its 9-bp target site (Pavletich and Pabo, 1991). In these more recent models, based on interpretation of DNase I footprinting patterns, hydroxyl radical footprinting patterns, and missing nucleoside analysis of protein-DNA complexes, the three amino-terminal and three carboxyl-terminal fingers of the molecule are proposed to wrap around the major groove of the DNA helix at each end of the ICR in a manner similar to the Zif268-DNA interaction. The zinc fingers in the middle of the protein are thought to lie on one side of the helix, with finger 5 contacting the major groove and fingers 4 and 6 each crossing the minor groove (Clemens et al., 1992; Hayes and Clemens, 1992; Hayes and Tullius, 1992; Fairall and Rhodes, 1992; Hansen et al., 1993).

The 50-bp ICR of the Xenopus 5 S RNA gene contains three...
elements that contribute to efficient transcription of the gene: the A-box spanning nucleotides +50 to +64, the intermediate element spanning nucleotides +67 to +72, and the C-box spanning nucleotides +80 to +97 (Pieder et al., 1985, 1987; Bogenhagen, 1985). Various studies have indicated that the amino-terminal fingers of Xenopus TFIIIA interact with the C-box element (Vrana et al., 1988; Christensen et al., 1991; Clemens et al., 1992; Hayes and Clemens, 1992; Liao et al., 1992) with a binding energy that is similar to that of the TFIIIA-DNA interaction (Sakonju et al., 1981; Sakonju and Brown, 1982; Fairall et al., 1986; Vrana et al., 1988; You et al., 1991; Darby and J ohn, 1992; Liao et al., 1992; Theunissen et al., 1992; Veldhoen et al., 1994). Indeed, a polypeptide containing only the amino-terminal three zinc fingers of Xenopus TFIIIA was found to have high affinity binding for the 5 S RNA gene, protecting the region from approximately nucleotides +74 to +95 from cleavage by DNase I (Christensen et al., 1991; Liao et al., 1992). The third zinc finger has been implicated as a major contributor to the affinity of Xenopus TFIIIA for DNA (Liao et al., 1992; Darby and J ohn, 1992; Theunissen et al., 1992; Del Rio et al., 1993; Zang et al., 1995). Recent studies confirm that various fingers make differing contributions to the binding energy of complex formation and indicate that the energetic contribution made by a finger can be influenced by neighboring fingers or sets of fingers (Del Rio et al., 1993; Clemens et al., 1994; Zang et al., 1995). The linkers between the amino-terminal fingers have also been shown to have a critical role in DNA binding, although it is unclear whether this contribution is through positioning of the zinc fingers or through contacts with DNA (Choo and Klug, 1993; Clemens et al., 1994; Zang et al., 1995).

Comparison of the deduced sequences of TFIIIA from S. cerevisiae and from Xenopus laevis indicated that the two proteins are structurally similar in that they both contain nine zinc finger motifs (Ginsberg et al., 1984; Archambault et al., 1992; Woychik and Young, 1992). However, the amino acid sequences of the corresponding fingers of yeast TFIIIA and amphibian TFIIIA as well as the linker sequences between the fingers differ extensively. A distinctive feature of the yeast protein is an 81-amino acid domain interrupting the consecutive zinc finger motifs between fingers 8 and 9. In contrast to the 50-bp DNase I footprints of the Xenopus TFIIIA-DNA complex (Engelke et al., 1980) and the human TFIIIA-DNA complex (Séfart et al., 1989; Moorfield and Roeder, 1994), the DNase I footprint of the yeast TFIIIA-DNA complex is 35 bp, extending from nucleotides +63 to +97 (Braun et al., 1989). Site-specific DNA-protein photocross-linking has revealed, however, that yeast TFIIIA is positioned over a longer region of DNA than that detected by DNase I footprinting (Braun et al., 1992a). The DNase I footprint includes the C-box element of the yeast 5 S RNA gene. This 15-bp sequence, which is positioned at nucleotides +81 to +94, is the only intragenic element that is essential for efficient in vitro transcription of the yeast 5 S RNA gene (Challice and Segall, 1989). An extended ICR has been found to be required for transcription in vivo (Lee et al., 1995).

We have previously shown that a truncated polypeptide containing the amino-terminal three zinc fingers of yeast TFIIIA retains the ability to bind to the yeast 5 S RNA gene (Milne and Segall, 1993). In the present study, we have analyzed in more detail the interaction of full-length and truncated forms of yeast TFIIIA with the 5 S RNA gene. Our data reveal that fingers 6 through 9 of yeast TFIIIA are not in tight association with DNA in the TFIIIA-DNA complex and suggest that the amino-terminal five zinc fingers make major groove contacts from nucleotides +73 to +94. A polypeptide containing the amino-terminal three zinc fingers of TFIIIA has a binding energy that is similar to that of the full-length protein.

### EXPERIMENTAL PROCEDURES

Plasmids for bacterial expression of wild-type and truncated forms of Yeast TFIIIA—A plasmid for bacterial expression of wild-type TFIIIA was obtained by inserting the 2.3-kilobase pair Ncol-BamHI fragment of plj A454 (Archambault et al., 1992) between the corresponding sites of pET-11d (Studier et al., 1990), which places the coding region of TFIIIA under the control of a bacteriophage T7 RNA polymerase promoter. Construction of plasmids for bacterial expression of TFIIIA truncated after the eighth (TF5), seventh (TF6), fourth (TF7), and third (TF3) zinc fingers involved the following restriction endonuclease recognition sites: an EcoRV site present at codon 282 at the end of finger 8, five residues after the second His residue of the zinc finger motif; an XbaI site at codon 265 within finger 8, seven residues after the second Cys residue of the zinc finger motif; a HindIII site at codon 171 within finger 5, three residues after the second Cys residue of the zinc finger motif; and a Bsp2261I site at codon 152 within finger 4, 11 residues after the second Cys residue of the zinc finger motif (see Fig. 1A; also see Fig. 1 of Milne and Segall (1993)). The zinc finger motif is (Tyr-Phe)-Xaa-Cys(Xaa)_5-Phe(Xaa)_5-Phe(Xaa)_5-Leu(Xaa)_5-His(Xaa)_5-His(Xaa)_5 with the pairs of cysteine and histidine residues responsible for coordination of a zinc atom. The code name TFX indicates that the protein contains the first X amino-terminal finger; a suffix Xn indicates that the truncation occurred within finger X + n.

The first step in construction of a plasmid for expression of TFIIIA truncated after the fourth zinc finger motif (TF4°) involved cloning the annealed, self-complementary oligonucleotide 5'-CTAGCTAGCTAG-3' (kindly provided by J. Ingles), which provides stop codons in all reading frames, into the Smal site of plj A453 (Archambault et al., 1992) to generate pJA453-Stsc. pJA453-Stsc, a plasmid containing the TFIIIA-coding region from the initiator ATG codon to the HindIII site at codon 171. The Ncol-BamHI fragment of plj A453-STP was then inserted between the corresponding sites of pET-11d for expression of TF4. The first step in construction of plasmids for expression of TF5, TF7°, and TF3° involved subcloning the 0.85-kilobase pair KpnI-EcoRV fragment, the 0.80-kilobase pair KpnI-XbaI fragment, and the 0.46-kilobase pair KpnI-Bsp2261I fragment, respectively, of plj A454 between the KpnI and EcoRV sites of plj A453-STP. For these constructions, the XbaI end of the KpnI-XbaI fragment and the Bsp2261I end of the KpnI-Bsp2261I fragment were blunted prior to ligation. The Ncol-BamHI fragment of each of the resultant plasmids, containing TFIIIA-coding sequence, was then subcloned between the corresponding sites of the pET-11d expression vector. This cloning procedure introduced the non-TFIIIA, polylinker-encoded amino acids EFLQ-PGDS, NSNCSP, LSINNSCP, and SNNSCP at the carboxy terminus of TF8, TF7°, TF4°, and TF3°, respectively.

For the DNase I and methylation protection footprint experiments, genes encoding proteins with carboxy-terminal truncations that ended after zinc finger sequence of finger 6 (TF6), finger 5 (TF5), finger 4 (TF4), finger 3 (TF3) were generated by polymerase chain reaction primers introduced a stop codon at the end of the linker. TF6, TF5, TF4, and TF3 had a stop codon introduced after codons 223, 193, 163, and 133, respectively. To facilitate subcloning, these primers also contained a BamHI site at their 5'-ends. These primers were used in a polymerase chain reaction, with plj A454 as template and the universal primer as the upstream primer, to generate the truncated TFIIIA-coding sequences. The polymerase chain reaction products were cut with Ncol and BamHI and cloned between the corresponding sites of pET-11d.

For the DNase I experiments, pET-11d-based plasmids were also constructed for bacterial expression of amino-terminal truncated forms of Yeast TFIIIA. Plasmids J A454-3 (Milne, 1992), which encode TFIIIA proteins lacking the sequence for codons 2-43 and 2-69, respectively, were digested with KpnI. After the resultant ends had been blunted by treatment with the Klenow form of DNA polymerase, the DNA was digested with BamHI. The PET-11d expression vector was digested with Ncol, and the resultant ends were treated sequentially with 5' nuclease and the Klenow form of DNA polymerase to generate a new restriction site of the DNA at the KpnI site of BamHI. The J A454-3 plasmids that had been excised from plj A454-2 and plj A454-3 were then subcloned between the Ncol and BamHI sites of pET-11d; this generated plasmids for expression of variant forms of TFIIIA that lack the sequence prior to finger 1 (IFS) and that lack most of the sequence prior to finger 2 (2FS), respectively. The sequence of all polymerase chain reaction-amplified DNA and all subcloning junctions was verified by the chain termination method (Sanger et al., 1977).

Purification of Yeast TFIIIA from Bacteria—The PET-11d-derived plasmids were transformed into the Escherichia coli strain BL21 (DE3),
which contains the T7 RNA polymerase gene under the control of the lacUV5 promoter. One ml of an overnight culture was used to inoculate 100 ml of LB medium (1% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1% NaCl (pH 7.0)) containing 100 μg of ampicillin/ml. When the culture reached an A 660 of 0.5–0.7, ZnSO 4 and isopropyl-β-D-thiogalactopyranosidewere added to 50 μM and 1 μM, respectively. Four h after induction, cells were harvested, washed with 5 ml of buffer A (20 mM HEPES (pH 7.4), 5 mM MgCl 2, 50 μM ZnSO 4, 250 mM NaCl, 10% glycerol, 10 μM β-mercaptoethanol), and resuspended in 4 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride. The cells were broken by sonication, and TFIIIA was then purified from inclusion bodies essentially as described by Del Rio and Setzer (1991). All steps were carried out at 4 °C. The pellet obtained by centrifugation of the sonicated extract at 17,000 g for 10 min was solubilized in 1 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride and 5 μM urea with mixing by inversion for 16 h. The solubilized extract was centrifuged for 20 min at 23,500 × g, and the supernatant was taken. For further purification, the supernatant was brought to 25% saturation with (NH 4 ) 2 SO 4 and mixed by inversion for 1 h. Precipitated protein was removed by centrifugation. The supernatant was brought to 80% saturation with (NH 4 ) 2 SO 4 and mixed by inversion for 2 h, and precipitated protein was recovered by centrifugation. The pellet was dissolved in 10 ml of buffer A containing 5 μM urea and applied to a 2–ml column of Bio-Rex 70 that had been equilibrated with the same buffer. After the column had been washed with buffer A containing 5 μM urea, bound protein was eluted with a 0–1 M gradient of NaCl in buffer A containing 1 mM phenylmethylsulfonyl fluoride. TFIIIA-containing fractions were identified by electrophoresis of an aliquot of each fraction on an SDS-polyacrylamide gel followed by staining with Coomassie Blue. Protein concentration was determined using the Bradford assay (Bradford, 1976), with bovine serum albumin as a standard. Fractions were stored in aliquots at −70 °C and used without dialysis. Active TFIIIA could be obtained by dilution of the urea-containing samples.

Gel Retardation and in Vitro Transcription Assays—Gel retardation assays were performed as described previously (Challice and Segall, 1989). The standard 20-μl reaction contained 10 μl of HEPES (pH 7.9), 110 mM KCl, 11 mM MgCl 2, 50 μM ZnSO 4, 10% glycerol, 0.65 mM dithiothreitol, 100 μg/ml bovine serum albumin, 2 μg/ml of uniquely end-labeled 5 S DNA (see above), 200 μg/ml of TFIIIA, and 2.5 ng of uniquely end-labeled 5 S DNA (see above). The reaction mixture was incubated for 5 min at room temperature with occasional mixing and then loaded onto a 4% nondenaturing polyacrylamide gel. The positions of protein-DNA complexes were separated from free DNA by electrophoresis on a nondenaturing gel to separate protein-DNA complexes from free DNA. Control experiments indicated that equilibrium was reached very quickly: the amount of protein-DNA complex present after a 1-min incubation was the same as the amount present after a 10-min incubation. The ratio of bound DNA to free DNA in each lane was determined using ImageQuant software (Molecular Dynamics, Inc.) to analyze phosphorimages of dried gels obtained with a Molecular Dynamics PhosphorImager. These ratios were then used in conjunction with the known concentration of input DNA to determine the concentrations of TFIIIA-DNA complex and free DNA. Apparent dissociation constants were determined by nonlinear regression analysis using Res Geom software (Scientific Publications Inc.) and Kaleidograph (Abelbeck Software) to fit the data to the following equation derived from the law of mass action: [P–D] = ([P] 0 ([D]/[K d + [D]]), where [P–D], [P], and [D] refer to TFIIIA-DNA complex, total active TFIIIA, and free DNA, respectively. [P–D] and [D] were the input variables to the nonlinear regression analysis, and K d and [P] 0 were the unconstrained output parameters. The calculation assumes that TFIIIA binds to DNA with 1:1 stoichiometry and does not require that the fraction of protein that is active be known. The standard errors for the individual K d determinations varied from 14 to 35% for the standard errors for [P] 0 varied from 5 to 25%. We emphasize that our measurements reflect apparent K d values since we cannot exclude the possibility that the equilibrium is perturbed during electrophoresis.

DNaSe I Footprint Analysis—DNA fragments of 270 bp containing the 5 S RNA gene, referred to as 5 S DNA, uniquely labeled at the 5′-end of the transcribed strand or the nontranscribed strand were prepared by digestion of p18-SS and p19-SS, respectively, with EcoRI and XbaI (Challice and Segall, 1989). After end filling of the digested DNA with the Klenow form of DNA polymerase I in the presence of [γ-32P]ATP, the fragment containing the 5 S RNA gene was purified by gel electrophoresis and recovered by electroelution. An 80-μl gel retardation reaction contained 1.5 ng of DNA and an amount of protein that was sufficient to bind the DNA. For these experiments, the protein solubilized from inclusion bodies (see above) was used without further purification. The reaction was incubated for 5 min at room temperature prior to the addition of 8 μl of DNase I (4 μg/ml). After an additional 3 min of incubation, 4 μl of 0.45 M EDTA was added to stop the reaction. The reaction was extracted with phenol/chloroform (50:50, ν/ν). The DNA was precipitated in the presence of 70% ethanol, 0.3 M NaOAc (pH 7.0) using oyster glycogen as carrier. The pellet was rinsed with 70% ethanol, dried in vacuo, and resuspended in loading buffer (95% formamide, 20 μM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF). The samples were analyzed on a 7 μl urea, 8% polyacrylamide sequencing gel.

Methylation Protection Analysis—Standard 20-μl gel retardation reactions containing 2.5 ng of uniquely end-labeled 5 S DNA (see above) were set up in triplicate and incubated with protein for 5 min at room temperature. Protein solubilized from inclusion bodies (see above) was used without further purification. Two μl of a 3% dimethyl sulfate solution in 0.5 M sodium cacodylate (pH 8.0) was then added, and the samples were incubated for 5 min at room temperature prior to being loaded onto a 4% nondenaturing polyacrylamide gel. The positions of free DNA and protein-DNA complexes were determined by autoradiography of the wet gel. DNA was recovered from gel slices by electroelution and precipitated with ethanol in the presence of 0.3 M NaOAc (pH 7.0) using oyster glycogen as carrier. The DNA was resuspended in H 2 O, extracted with phenol/chloroform (50:50, ν/ν), and precipitated in the presence of 0.3 M NaOAc (pH 7.0). The purified DNA was cleaved by treatment with piperidine (Maxam and Gilbert, 1980) and run on a 7 μl urea, 8% polyacrylamide sequencing gel. Quantification of relative intensities of bands was carried out using IPLab Gel software (Signal Analytics Corp.) to analyze phosphorimages of dried gels obtained with a Molecular Dynamics PhosphorImager.

RESULTS

Determination of Apparent Dissociation Constants (K d)—As a first step in further characterization of yeast TFIIIA, we determined the affinity of the protein for the 5 S RNA gene. Wild-type TFIIIA, expressed in E. coli under the control of a phage T7 RNA polymerase promoter, was purified by subjecting the protein solubilized from inclusion bodies to gel retardation on Bio-Rex 70 (Fig. 1B; see "Experimental Procedures"). This purified TFIIIA was active in binding to the 5 S RNA gene as monitored by a gel retardation assay (Fig. 1C). As a more stringent assay of the activity of the bacterially produced transcription factor, we tested its ability to support in vitro transcription of the yeast 5 S RNA gene in the presence of a yeast-derived fraction containing TFIIIB, TFIIIC, and RNA polymerase III. This fraction directs accurate transcription of a tRNA gene, but requires the addition of TFIIIA for transcription on the 5 S RNA gene (Fig. 1D, lanes 1–3). Bacterially produced wild-type TFIIIA was able to substitute for partially purified yeast TFIIIA in promoting transcription of the yeast 5 S RNA gene (Fig. 1D, lanes 3 and 4).

We next measured the affinity of TFIIIA for the 5 S RNA gene by incubating a constant amount of protein with increasing amounts of a radiolabeled 5 S DNA fragment. Protein-DNA complexes were separated from free DNA by electrophoresis on a nondenaturing gel (Fig. 2A). The relative amounts of free DNA and bound DNA were determined at each input DNA concentration, and the apparent dissociation constant (K d) was derived by nonlinear regression analysis of the data (Fig. 2A; see "Experimental Procedures"). We found that the K d of the
We determined the binding affinities of carboxyl-terminal truncated forms of yeast TFIIIA. Versions of TFIIIA that contained the first three zinc fingers (TF3*), the first four zinc fingers (TF4*), and the first seven zinc fingers (TF7*) were purified from bacteria. These truncated forms of TFIIIA contained up to 25 amino acids after the last intact zinc finger; this additional sequence (denoted by the asterisk) included a portion of the next zinc finger and six to eight amino acids introduced from vector sequence (see “Experimental Procedures”). We considered that this sequence would be relatively unstructured and would be unlikely to have a significant effect on the affinity measurements. Each form of truncated TFIIIA eluted from Bio-Rex 70 at a unique salt concentration, ran as a single band of the expected molecular mass on an SDS-polyacrylamide gel (Fig. 1B), and was active in binding to the 5 S RNA gene (Fig. 1C). Consistent with the reduction in size of the carboxyl-terminal truncated proteins, the protein-DNA complexes formed with the truncated proteins (Fig. 1C, lanes 3–5) had increased mobilities relative to the complex formed with wild-type protein (lane 2). The protein-DNA interactions visualized in the gel shift assay were shown to be specific by monitoring the effects of addition of competitor DNAs (data not shown). As expected from the observation that the 81-amino acid domain located between fingers 8 and 9 of yeast TFIIIA is essential for the transcriptional activity of the protein (Milne and Segall, 1993), bacterially purified TF7*, TF4*, and TF3* were unable to support in vitro transcription of the 5 S RNA gene (Fig. 1D, lanes 5–7).

Removal of zinc fingers 9 and 8 from yeast TFIIIA did not have a significant effect on the apparent affinity of the protein-DNA interaction (Fig. 2B and Table I). Removal of zinc fingers 9 through 5 led to an ~2-fold increase in the apparent \( K_D \) (Fig. 2C and Table I), and removal of zinc finger 4 led to an additional 3.5-fold increase in the apparent \( K_D \) (Fig. 2D and Table I). Using the measured \( K_D \) values, we calculated the \( \Delta G^\circ \) values for the protein-DNA interactions (Table I). This representation of the data emphasizes that a polypeptide containing the amino-terminal three zinc fingers of yeast TFIIIA has a high binding energy; the binding energy of TF3 was 90% that of the full-length protein. In previous studies using protein synthesized in vitro, we were unable to detect a TFIIIA-DNA interaction with a truncated molecule containing only the amino-terminal two zinc fingers (Milne and Segall, 1993) or with a truncated molecule containing only fingers 3 through 9.2 This suggests that if these proteins can bind to the 5 S RNA gene, they do so with a relatively high \( K_D \). It should be noted that our analysis using truncated proteins would not necessarily detect the effects that finger-DNA interactions in one portion of the molecule might exert on the energetics of interactions occurring elsewhere in the complex (for example, see Del Rio et al. (1993), Clemens et al. (1994), and Zang et al. (1995)).

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**DNease I Footprinting—To approximate the position of various fingers of yeast TFIIIA on the 5 S RNA gene, we compared the DNease I cleavage patterns of the gene complexed with wild-type and truncated forms of TFIIIA (Fig. 3). Because we wished to avoid any unpredictable effects that imprecise truncations might have on determination of the boundaries of the protein-DNA interactions, we prepared versions of TF8, TF6, TF5, TF4, and TF3 with carboxyl-terminal truncations that terminated at the end of the linker sequence (see “Experimental Procedures”). The series of truncation mutants analyzed also included TF7* and amino-terminal truncated forms of TFIIIA that began before the first finger and before the second finger, referred to as IFS and 2FS, respectively (see “Experimental Procedures”). As seen previously with TFIIIA purified**

**protein-DNA complex was 0.11 nm (Table I). For comparison, the \( K_D \) values measured for the Xenopus TFIIIA-DNA interaction range from 0.42 to 2.2 nm (Hanas et al., 1983; Romaniuk, 1990; Del Rio and Setzer, 1991; Liao et al., 1992; Del Rio et al., 1993).**

We also determined the binding affinities of carboxyl-termi-
from yeast (Braun et al., 1989), we found that wild-type TFIIIA expressed in bacteria protected a 35-bp region of the 5 S RNA gene from DNase I cleavage; this region extended from nucleotides +64 to +98 on the transcribed strand (Fig. 3A, fourth lane) and from nucleotides +66 to +99 on the nontranscribed strand (Fig. 3B, fourth lane). Within this region, nucleotides +70 and +71 on the transcribed strand and nucleotides +74 and +75 on the nontranscribed strand remained accessible to DNase I cleavage. The DNase I cleavage patterns of the TFIIIA-DNA complexes (Fig. 3, A and B, fifth to eighth lanes) were identical to that of the wild-type TFIIIA-DNA complex with the exception that the TF7*-DNA complex gave rise to DNase I-hypersensitive sites at nucleotides +51 and +52 on the transcribed strand, 12 bp upstream of the protected region. These data indicate that fingers 6 through 9 and the 81-amino acid domain of yeast TFIIIA either are not in close proximity to DNA in the TFIIIA-DNA complex or have such weak affinity for DNA that they are readily displaced by DNase I. It is possible that the hypersensitivity present at nucleotides +51 and +52 in the DNase I footprint of TF7* is generated by the 17-amino acid carboxyl-terminal extension present in this protein; if this is the case, it is possible that the carboxyl-terminal fingers of yeast TFIIIA are in closer proximity to DNA than is implied by the DNase I protection patterns. Indeed, the observation of Braun et al. (1992a) that yeast TFIIIA can be cross-linked to templates containing photoactive nucleotides at residues +48 to +59 of the 5 S RNA gene is consistent with the possibility that a portion of TFIIIA carboxyl-terminal to finger 5 is weakly bound to DNA in the TFIIIA-DNA complex.

TFIIIA containing four intact zinc fingers generated a smaller DNase I footprint on the 5 S RNA gene than did TF5. On the transcribed strand, TF4 appeared to protect a 20-bp region, extending from nucleotides +79 to +98 (Fig. 3A, ninth lane). Comparison of the relative intensities of the bands at nucleotide +76 suggested that the upstream boundary of this footprint might extend to nucleotide +76. On the nontranscribed strand, the protected region extended from nucleotides +78 to +99 (Fig. 3B, ninth lane). Comparison of the footprints obtained with TF4 and TF3 showed that deletion of the fourth zinc finger led to a change in the DNase I footprint only on the nontranscribed strand (Fig. 3, A and B, tenth lanes). Whereas TF4 provided protection from nucleotides +78 to +99, TF3 did not protect residue +78 from DNase I digestion. In summary, our data confirmed, as previously suggested (Milne and Segall,
The sequence of the 5 S RNA gene is given from nucleotides 58 to 112. The ICR is boxed. The extent of DNase I protection on the transcribed strand (closed rectangles) and transcribed strand (open rectangles) in complexes with wild-type TFIIIA, TF8, TF5*, TF6, TF5, TF4, TF3, and TF5 is shown. The shaded portions of the solid rectangles indicate uncertainty in positioning the boundary of the protected region. The solid arrowheads denote DNase I-sensitive sites within the region protected by wild-type TFIIIA, TF6, TF7*, and TF8.

1993), that yeast TFIIIA, like its amphibian counterpart, binds to the 5 S RNA gene with its carboxyl terminus toward the 5' end of the gene. Comparison of the DNase I protection patterns obtained with the carboxyl-terminal truncated proteins TF5, TF4, and TF3 indicated that the amino-terminal three zinc fingers span the ICR, which maps approximately from nucleotides +81 to +94 (Challice and Segall, 1989); the presence of finger 4 leads to only a modest change in the protection pattern at the 5'-end of the ICR; and the presence of finger 5 leads to protection of an additional 12 bp upstream of the ICR.

Inspection of the DNase I footprints generated by the two amino-terminal truncated proteins indicated that TFIIIA lacking the non-zinc finger amino-terminal extension (IF5) and TFIIIA lacking the first zinc finger (2FS) did not protect nucleotide +98 on the transcribed strand (Fig. 3A, sixth lane). Additionally, protection of nucleotides +96 and +94 on the transcribed strand (Fig. 3A, twelfth lane) and nucleotide +97 on the nontranscribed strand (Fig. 3B, twelfth lane) was reduced in the 2FS-DNA complex (Fig. 3A, twelfth lane). These data position finger 1 at the 3'-end of the ICR.

Methylation Protection Analysis—We also used dimethyl sulfate to probe the protein-DNA complexes formed with wild-type and carboxyl-terminal truncated forms of TFIIIA (Fig. 4). In the TFIIIA-DNA complexes formed with wild-type TFIIIA, guanines -73, +74, +93, and +94 on the transcribed strand and guanines +82, +85, +87, +88, +89, and +91 on the nontranscribed strand were protected from modification by dimethyl sulfate (Fig. 4, A and B, third lanes) (Challie and Segall, 1989). Similarly, modification of these residues interferes with binding of TFIIIA (Wang and Weil, 1989). As noted in Fig. 4C, 8 of the 10 G residues that are in close contact with TFIIIA are within the ICR. The methylation protection patterns obtained with TF6 and TF5 were identical to that obtained with wild-type TFIIIA (Fig. 4, A and B, third to fifth lanes). In the TF4-DNA complex, the G residues at positions +73 and +74 on the transcribed strand, which map upstream of the ICR, were no longer protected (Fig. 4A, sixth lane). These data place finger 5 in close contact with G+73 and G+74 on the transcribed strand. G+82 on the nontranscribed strand was partially protected in the TF4-DNA complex and not protected in the
TF3-DNA complex (Fig. 4B, sixth and seventh lanes; data not shown). Quantification of the analysis shown in Fig. 4 (see “Experimental Procedures”) indicated that modification of G\textsuperscript{32} relative to modification of the unprotected residue G\textsuperscript{99} in wild-type TFIII\textalpha-DNA, TF6-DNA, TF5-DNA, TF4-DNA, and TF3-DNA complexes was 0.09, 0.12, 0.13, 0.42, and 0.75, respectively, and 0.83 in free DNA. Our interpretation of these results is that finger 4, or the finger 4-finger 3 linker, contacts G\textsuperscript{32} and that this interaction occurs more efficiently when finger 5 is docked onto DNA (see “Discussion”).

**DISCUSSION**

We expressed yeast TFII\textalpha in E. coli and found, as observed by others (Ottonello et al., 1994), that bacterially produced yeast TFII\textalpha supports accurate in vitro transcription of the 5 S RNA gene. Using wild-type and truncated forms of TFII\textalpha that had been purified from bacteria, we studied the interaction of the protein with the 5 S RNA gene using DNase I and dimethyl sulfate as probes. We note that DNase I allows only approximate mapping of the boundaries of a protein-DNA complex. First, not all phosphodiester bonds are cleaved efficiently by this enzyme in naked DNA. Second, the boundary of a footprint could be influenced by a weak transient interaction of a finger with DNA, displacement of a weakly bound finger by DNase I, or steric hindrance between the enzyme and DNA-bound protein. Despite these limitations, inspection of the protection patterns allowed us to position various portions of yeast TFII\textalpha along the 5 S RNA gene. Although yeast TFII\textalpha and Xenopus TFII\textalpha both contain nine zinc fingers, the 40-kDa amphibian protein generates a 50-bp DNase I footprint (Engelke et al., 1980), whereas the 50-kDa yeast protein generates a 35-bp footprint (Braun et al., 1989; this study). We found that the smaller footprint of yeast TFII\textalpha can be accounted for by the absence of an intimate interaction of yeast fingers 6–9 with DNA. Studies of the Xenopus TFII\textalpha-DNA interaction (Clemens et al., 1992; Fairall and Rhodes, 1992; Hayes and Clemens, 1992; Hayes and Tullius, 1992; Del Rio et al., 1993) suggest that the carboxyl-terminal three fingers (fingers 7–9) of Xenopus TFII\textalpha wrap around the major groove of the DNA helix at the 5′-end of the amphibian ICR and immediately upstream of this element. The three zinc fingers in the middle of the protein are thought to lie on one side of the helix, with finger 5 contacting the major groove and fingers 4 and 6 each crossing the minor groove. The amino-terminal three zinc fingers are proposed to wrap around the major groove of the DNA helix, contacting a 13-bp region of the C-box (Christensen et al., 1991; Hayes and Clemens, 1992; Clemens et al., 1992; Liao et al., 1992; Bogenhagen, 1993; Hansen et al., 1993; Veldhoen et al., 1994) with an affinity that is similar to that of the entire protein (Liao et al., 1992; Choo and Klug, 1993; Zang et al., 1995). Our methylation protection analysis indicated that finger 5 of yeast TFII\textalpha is in close contact with G\textsuperscript{73} and G\textsuperscript{74}, placing it in the major groove, as is finger 5 in the Xenopus TFII\textalpha-DNA complex. Because the DNase I protection analysis indicated that finger 5 provides protection to nucleotide +64, the finger 5-DNA interaction presumably extends upstream of nucleotides +74 and +75. The methylation protection analysis also indicated that G\textsuperscript{52} was not protected from modification in the yeast TF4-DNA complex, was partially protected in the TF5-DNA complex, and was completely protected in the TF6-DNA complex. This suggests that yeast finger 4 makes a major groove contact with G\textsuperscript{52} and that this interaction occurs more efficiently when finger 5 is docked at G\textsuperscript{73}/G\textsuperscript{74}. If this is the case, it is possible that the 12-bp difference between the DNase I footprints of TF5 and TF4 represents loss of protection not only by finger 5, but also by finger 4, i.e. DNase I might partially displace finger 4 from the TF4-DNA complex. This could explain why the only difference between the DNase I protection patterns of the yeast TF4-DNA and TF3-DNA complexes was protection of nucleotide +78 in the TF4-DNA complex. The interactions suggested above, which position the major groove contacts made by fingers 4 and 5 10 bp apart on the same side of the helix, require that the finger 4-finger 5 linker, which is only five amino acids, cross the minor groove (e.g. see Kochoyan et al. (1991)). TFII\textalpha-induced bending of DNA (Braun et al., 1992b) might facilitate this proposed spacing. Alternatively, it is possible that the finger 4-finger 3 linker is responsible for protection of G\textsuperscript{52} and that most of the finger lies over the minor groove, extending from approximately nucleotides +82 to +75, leaving this region accessible to DNase I. Finally, it is possible that finger 4 approaches DNA in a novel manner. In the above model, irrespective of how finger 4 interacts with DNA, fingers 3, 2, and 1 would then be responsible for the major groove contacts represented by protection of G residues from positions +85 to +94. Although this differs from the model proposed for the Xenopus TFII\textalpha-DNA complex, in which the amino-terminal three zinc fingers contact 13 bp of the C-box element (Clemens et al., 1992; Liao et al., 1992) and finger 4 lies over the minor groove, the fact that G\textsuperscript{52} is not protected from methylation in the yeast TF3-DNA complex argues against G\textsuperscript{52} being contacted by finger 3. If finger 3 is in contact with G\textsuperscript{52}, then our data indicate that this contact occurs only in the presence of finger 4. In this case, TF3, which binds to DNA with an affinity that is similar to that of the full-length protein, would have only two fingers in contact with DNA. We note that using protein synthesized in vitro, we did not detect an interaction between the 5 S RNA gene and a polypeptide containing the first two fingers of yeast TFII\textalpha (Milne and Segall, 1993).

Further studies are required to allow detailed comparisons between the finger-DNA interactions in the yeast and Xenopus TFII\textalpha-DNA complexes. Exact positioning of individual fingers awaits deduction of the three-dimensional structure of the protein-DNA complexes. As discussed above, however, our preliminary study suggests that some differences may exist. This is perhaps not unexpected. Yeast TFII\textalpha does not contain the conserved linker motif TGXEK found between fingers 1 and 2 and between fingers 2 and 3 of Xenopus TFII\textalpha. The His-His spacings, which could affect the structure of the DNA-binding α-helix, also differ between fingers 1, 3, and 5 of the TFII\textalpha proteins from the two organisms (Ginsberg et al., 1984; Archambault et al., 1992; Woychik and Young, 1992). These differences, in addition to differences in potential DNA-contacting amino acids, could lead to differences in the DNA binding properties of the polypeptides. Deduction of the structures of protein-DNA crystals of Zif268 (Pavletich and Pabo, 1991), GL1 (Pavletich and Pabo, 1993), and Tramtrack (Fairall et al., 1993) has revealed that zinc fingers can dock into the major groove of DNA with variations in base and phosphate contacts and in the spacing of adjacent binding sites. Linkers have been shown to have a major influence on the binding affinity of zinc fingers (Choo and Klug, 1993; Clemens et al., 1994; Zang et al., 1995). Furthermore, it is interesting to note that the amino-terminal three fingers of yeast TFII\textalpha serve not only in DNA binding, but also to recruit TFII\textbeta to the TFII\textalpha-DNA complex (Milne and Segall, 1993). The observations that putative human TFII\textalpha (Arakawa et al., 1995; Drew et al., 1995) and Xenopus TFII\textalpha share more extensive identity than do Xenopus TFII\textalpha and yeast TFII\textalpha (Archambault et al., 1992; Woychik and Young, 1992) and that no similarity has yet been found between the deduced amino acid sequences of subunits of human TFII\textalpha and yeast TFII\textalpha (L’Etoile et al., 1994; Lagna et al., 1994; Sinn et al., 1995) are consistent with the notion that a
yeast-specific TFIIIA-DNA interaction is established to direct assembly of yeast TFIIIC into the transcription complex.

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