TFIIIA Binds to Different Domains of 5S RNA and the *Xenopus borealis* 5S RNA Gene

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We have established the conditions for the reassociation of 5S RNA and TFIIIA to form 7S particles. We tested the ability of altered 5S RNAs to bind TFIIIA, taking advantage of the slower mobility of 7S particles compared with free 5S RNA in native polyacrylamide gels. Linker substitution mutants were constructed encompassing the entire gene, including the intragenic control region. In vitro transcripts of the linker substitution mutants were tested for their ability to bind TFIIIA to form 7S ribonucleoprotein particles. Altered 5S RNAs with base changes in or around helices IV and V, which would interfere with the normal base pairing of that region, showed decreased ability to bind TFIIIA. The transcripts of some mutant genes that were efficiently transcribed (>50% of wild-type efficiency) failed to bind TFIIIA in this gel assay. In contrast, the RNA synthesized from a poorly transcribed mutant, LS 86/97, in which residues 87 to 96 of the RNA were replaced in the single-stranded loop at the base of helix V, bound TFIIIA well. The data indicate that TFIIIA binds to different domains in the 5S RNA gene and 5S RNA.

The binding of a protein, designated transcription factor IIIA (TFIIIA), to an intragenic control region in the center of the *Xenopus borealis* 5S RNA gene is an early event in gene activation (6, 9, 32). Although at least two other factors are required for assembly of the 5S RNA gene into a stable active complex (4, 19, 35, 36), the interaction of TFIIIA with the gene has been studied in greatest detail. TFIIIA is the only one of these factors that has been purified to homogeneity.

TFIIIA is available in large quantities in immature oocytes bound to 5S RNA within a 7S ribonucleoprotein particle (RNP) (16, 24). This dual function of TFIIIA has led to the suggestion that the accumulation of 5S RNA may feedback-regulate 5S RNA synthesis (24). Accumulation of 5S RNA may be sufficient to sequester TFIIIA, depleting the pool available for transcription. Any rigorous test of this hypothesis will require a detailed understanding of the interactions between TFIIIA and 5S RNA.

Interactions between TFIIIA and 5S RNA have been studied by a variety of approaches. 5S RNA has been shown to inhibit specific transcription in vitro (24) and to compete for TFIIIA in 5S DNA-binding reactions (14). These footprint competition experiments suggest that 5S RNA associates with TFIIIA with a dissociation constant (K_d) of approximately 10 nM. More recently, Romaniuk (30) used a filter binding assay to characterize the binding of TFIIIA to 5S RNA and reported an apparent K_d of 1 nM. Two experimental approaches have been used to begin to define the 5S RNA sequences involved in binding TFIIIA. Pieler and Erdman (28) tested the ability of chemically modified 5S RNAs or nuclease-generated fragments to inhibit 5S RNA gene transcription, presumably by binding TFIIIA. These experiments suggested that TFIIIA contacts the helix IV/V region of 5S RNA. Studies performed with substantially deleted RNAs must be interpreted cautiously, since these RNAs may possess secondary structures bearing little resemblance to intact 5S RNA. Nuclease probes have also been used in an attempt to identify specific bases interacting with or pro-

tested by TFIIIA. The results of digestions of end-labeled 5S RNA within 7S RNPs with α -sarcin or structure-specific nucleases are also consistent with binding of TFIIIA to the 3' portion of 5S RNA (3, 17).

In the present study we used nondenaturing polyacrylamide gel electrophoresis (PAGE) to study the RNA sequences required to reconstitute 7S RNPs from free 5S RNA and TFIIIA. 7S RNP complexes formed by binding TFIIIA to 5S RNA in vitro have a mobility in PAGE gels identical to that of naturally occurring 7S particles. We synthesized a series of RNAs with specific base changes throughout the 5S RNA sequence by in vitro transcription of a series of mutant 5S RNA genes containing synthetic linkers substituted for sections of the gene. The RNAs derived from the linker substitution mutations were then examined for their ability to bind TFIIIA to form RNPs in the gel mobility assay. These studies support two general conclusions. First, there is a marked discrepancy between sequences required for TFIIIA binding to RNA and to DNA. Second, TFIIIA appears to involve mainly the helical regions of the 5S RNA in and surrounding helices IV and V.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., and were used as specified by the manufacturer. Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences, Inc., St. Petersburg, Fla. Sequencing chemicals and reagents were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and P-L Biochemicals, Piscataway, N.J. Radioisotopes were obtained from Amersham-Searle, Arlington Heights, Ill., or ICN Pharmaceuticals Inc., Irvine, Calif. Immature and mature *Xenopus laevis* were purchased from Xenopus I (Ann Arbor, Mich.) or Nasco (Fort Atkinson, Wis.).

Linker substitution mutagenesis. Methods for constructing plasmids containing linker substitution mutants of the *X. borealis* somatic 5S RNA gene have been described previously. The 3' deletion mutants were described by Bogenhagen et al. (6). More extensive 3' deletion mutations were

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also generated by an additional round of deletion mutagenesis. The 5' deletion mutants were constructed as described by Bogenhagen et al. (6) except that flush-ended deleted DNAs were ligated directly into the *Hinc*II site of pUC9 to avoid using oligonucleotide linkers. Deletion endpoints were determined by dideoxy sequencing (34) of the *Bam*HI-*Hind*III inserts recloned in M13mp9 (22). Ligation of the appropriate 3' and 5' deletions gave rise to genes with 10 nucleotides derived from the linker sequences. The nomenclature of the mutants is described by Bogenhagen (5). Linker mutant LS 8/15 was constructed by ligating the DNA derived from pXbs $\Delta 5'+15$, with the *Bam*HI site filled in with reverse transcriptase, to the *Hae*III site at residue +8 of the 5S RNA gene. Mutant LS 92/105 was constructed by recombining the appropriate 5' and 3' deletions with decameric *Bam*HI linkers on both ends. The *Bam*HI site in the linkers for pXbs $\Delta 3'+92$ was filled in with dGTP and dATP and trimmed with nuclease S1; the *Bam*HI site in the linker for pXbs $\Delta 5'+105$ was filled in with all four deoxynucleotide triphosphates. The two flush-ended fragments were ligated to produce a gene with 12 nucleotides derived from the linker sequences. Linker mutants LS 57/67 and LS 67/78 have been described previously (5). All of the genes had the same 5' deletion endpoint with an *Hind*III linker joined to residue -48 in the 5'-flanking region. LS 86/97, LS 92/105, and LS 107/118 have 138 base pairs (bp) of 3'-flanking 5S DNA ending at a naturally occurring *Hind*III site. All other mutants have an *Hind*III linker joined to a *Pvu*II site 69 bp after the gene. Mutant 5S RNA genes were ligated at the *Hind*III site of pUC8 and pXbsMT-H (5). The orientation and fidelity of the constructions were confirmed by restriction analysis. The constructions cloned in the "maxigene" test vector have the designation MT added to the name; thus, LS 46/57 refers to the gene cloned in pUC8, while LS 45/57MT refers to the gene cloned in tandem with the maxigene.

Quantitative and preparative transcription. Quantitative transcription assays were conducted with the mutants cloned in the same vector with a 5S RNA maxigene as described by Bogenhagen (5). Reaction conditions were as described by McConkey and Bogenhagen (21) except that an S-150 extract derived from mature *X. laevis* ovaries (12) was used exclusively. The resulting transcripts were analyzed by PAGE on 6% polyacrylamide-8 M urea gels. Autoradiograms were obtained on Kodak XAR-5 film, and the RNA was quantified by Cerenkov counting of the samples excised from the gel, using the autoradiogram as a guide.

Preparative transcription of the mutants was carried out under the same conditions except that the individual reaction volumes were doubled and the genes were cloned in pUC8 with no competing maxigene present. The resulting transcripts were isolated by PAGE on 6% polyacrylamide-0.2% sodium dodecyl sulfate (SDS) gels. Autoradiograms of the gels were obtained, and the corresponding bands were excised. Radiolabeled RNA was electroeluted from the gel in $0.1 \times$ TBE buffer ($1 \times$ TBE buffer is 50 mM Tris, 50 mM boric acid [pH 8.3], 1 mM disodium EDTA) containing 0.2% SDS at 100 V for approximately 60 min. RNA was then concentrated by ethanol precipitation and washed with 70% ethanol to remove any traces of SDS. To obtain sufficient labeled RNA from mutant genes with poor template activity, the reaction volume and specific activity of the radiolabeled nucleotide were increased. The RNA was suspended in TE buffer (10 mM Tris, 1 mM disodium EDTA), and the volume was adjusted to yield 100 cpm/ μ l.

TFIIIA preparation. 7S RNPs were isolated from imma-

ture *X. laevis* ovaries by a modification of the procedures described elsewhere (13, 24, 25). Immature ovaries were excised, placed in ice-cold buffer (60 mM NH_4Cl , 7 mM MgCl_2 , 10 μM ZnCl_2 , 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 2.5 mM dithiothreitol), and homogenized with a loose-fitting pestle. Nuclear and cellular debris were removed by centrifugation at 3,000 rpm for 5 min in a Sorval RT6000 table-top centrifuge. Smaller debris and mitochondria were removed by centrifugation for 15 min at $20,000 \times g$ in a Beckman J2-21 centrifuge. The supernatant at this stage contains large quantities of ribosomes and 42S particles (7 in addition to 7S particles). An S-100 extract was then prepared from the supernatant by centrifugation by $100,000 \times g$ in a Beckman ultracentrifuge. The resulting extract was then subjected to chromatography on DEAE-Sephacel (Pharmacia, Inc.), and 7S particles were eluted with KCl as described previously (13). The S-100 extract eliminated the need for glycerol gradient purification prior to the DEAE column purification. TFIIIA was purified from 7S particles by the method of Smith et al. (37).

DNase I protection assays. Plasmids containing a linker substitution mutant inserted into pXbsMTH were linearized with *Eco*RI, 3'-end labeled, and recut with *Ava*I to permit isolation of a singly end-labeled 2,100-bp fragment by agarose gel electrophoresis. DNase I protection assays were performed as described by McConkey and Bogenhagen (21).

RNA binding assay. Reconstitution of 7S particles was accomplished by the addition of TFIIIA to 5S RNA in buffer (80 mM NH_4Cl , 8 mM MgCl_2 , 10 μM ZnCl_2 , 12 mM HEPES, pH 7.4, 3 mM dithiothreitol) to a final reaction volume of 20 μ l. The binding reaction mixtures were incubated for 20 min at 20°C. At the end of the incubation, 2 ng of K-heparin (ICN) was added to each reaction mixture with 4 μ l of loading solution (80% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol). The reaction mixtures were then loaded on 6% polyacrylamide gels and electrophoresed in TBE buffer at room temperature at 300 V for approximately 2 h. An extensive series of control reactions were performed to determine the optimal concentration of heparin required to prevent RNA from sticking to the gel wells. In some reactions with widely varied total RNA concentrations, the heparin concentration was varied, as noted in the figure legends. Our control experiments indicated that excessive quantities of heparin could affect the binding equilibrium. Thus, while we believe that the conditions we used can give estimates of relative binding affinities, they cannot be used to derive rigorous binding constants. Transcripts resulting from the linker substitution mutations were analyzed in a similar manner except that a trace amount of radiolabeled transcript was added to the appropriate reaction mixture. The gels were dried, and radioactivity was visualized by autoradiography. The fraction of RNA bound to TFIIIA in 7S particles was determined by densitometry with an LKB laser densitometer.

RESULTS

Linker substitution mutations of the *X. borealis* somatic 5S RNA gene. A series of 11 linker substitution mutations encompassing the entire 5S RNA gene were constructed (Fig. 1). Synthetic DNA linkers were inserted into a wild-type *X. borealis* somatic gene as described in Materials and Methods. Individual mutants are designated with reference to the wild-type gene residues immediately 5' and 3' of the linker sequences. For example, linker substitution mutant

FIG. 1. Collection of linker substitution mutations. The series of linker substitution mutations covering the length of the *X. borealis* somatic 5S RNA gene are diagrammed. Linker substitution mutants are designated by the wild-type nucleotides immediately 5' and 3' of the linker sequences. Hatched regions represent inserted nucleotides. The gene sequences replaced are indicated to the right. The top row is the sequence of the noncoding strand of the linker DNA, and the bottom row represents the wild-type sequence. Uppercase letters indicate the bases that were changed, and lowercase letters represent bases that were conserved between the gene and the linker sequences.

LS 8/15 contains foreign sequences that exactly replace nucleotides +9 to +14 of the gene. Exact base pair changes resulting from each linker substitution are shown in Fig. 1. Mutations LS 57/67 and LS 67/78, located in the center of the intragenic control region, have been characterized previously (5).

Quantitative transcription of the series of linker substitution mutants. In vitro transcription of the mutant genes was carried out in an S-150 extract derived from mature *Xenopus laevis* oocytes as described in Materials and Methods. Each mutant was cloned in tandem with a 5S RNA maxigene, which served as an internal control for transcription (5). Figure 2A shows the RNAs transcribed from the series of mutants as analyzed by polyacrylamide-urea gel electrophoresis. Some slight variability in the mobility of the RNAs (designated 5S RNA) was observed due to incomplete denaturation of the transcripts in the urea gel. A transcription index was calculated for each mutant by dividing the 32 P radioactivity in the 120-nucleotide RNA by the total 32 P radioactivity in the maxigene transcript plus the 120-nucleotide RNA. Each transcription index was then normalized to the transcription index of the wild-type gene in the same maxigene vector. Transcription efficiencies of the mutant 5S RNA genes relative to the wild-type 5S RNA gene are illustrated in Fig. 2B.

Most of the mutations that significantly affected transcription efficiency were located inside the ICR. Linker substitution mutations LS 46/57 and LS 86/97, with substitutions at the 5' and 3' borders of the ICR, respectively, decreased transcription to only 2 to 5% of wild-type transcription. As described previously (5), mutations in the middle of the ICR, LS 57/67 and LS 67/78, decreased transcription to 50 to 60% of wild type. Interestingly, two mutations near the 5' border of the ICR, which might be predicted to transcribe nearly as efficiently as the wild-type gene, showed substantially decreased transcription. Transcription efficiencies of 35 and 9% were observed for mutations 34/45 and 38/49, respectively. Linker substitution mutation LS 8/15, which also lay outside the ICR, had a transcription efficiency of approxi-

mately 75% relative to that of the maxigene. Pulse-chase experiments revealed no difference in the stability of any of the transcripts from the series of linker substitution mutants.

Binding of TFIID to the mutant genes. DNase I protection assays were performed with the linker substitution mutants to determine the effects of the base changes on the binding of TFIID. In some cases, as for LS 46/57, the decrease in

FIG. 2. Transcription of linker substitution mutants. (A) Autoradiogram of a 6% polyacrylamide-8 M urea gel analysis of transcripts synthesized from linker substitution mutants cloned in tandem with the 5S RNA maxigene. Mobilities of the maxigene (MAXI) and 5S transcripts are indicated. Lane wt contains the transcription products of a wild-type *X. borealis* somatic 5S RNA gene cloned in tandem with the maxigene. Lanes 1 to 11 contain the transcription products of the linker substitution mutants LS 8/15MT, LS 18/29MT, LS 26/37MT, LS 34/45MT, LS 38/49MT, LS 46/57MT, LS 57/67MT, LS 67/78MT, LS 86/97MT, LS 92/105MT, and LS 107/118MT, respectively. The diagram below the autoradiogram schematically represents the 5S RNA gene and the approximate position of the ICR with respect to the linker substitution mutations. (B) Graph showing the transcription efficiencies of the linker substitution mutants relative to the wild-type gene. Bars correspond to the lanes in panel A and represent the average and standard error of the mean (SEM) of five experiments. A transcription index was calculated from the 32 P label incorporated into the 5S-sized and maxigene RNAs as described in Materials and Methods. The transcription indices for the mutants were compared with that of the wild-type gene to yield the percentage of the wild-type index. The average transcription index for the wild-type gene was 0.428 ± 0.014; this value was considered 100% for normalization.

FIG. 3. DNase I protection analysis of the binding of TFIIIA to wild-type and mutant 5S RNA genes. Autoradiograms are shown of sequencing gel analyses of DNase I footprinting assays of various 5S DNAs cloned in tandem with the 5S RNA maxigene. The coding strand of each of the genes was end labeled as described in Materials and Methods. End-labeled DNA at a concentration of 1 nM (containing 2 nM 5S RNA genes, including the test gene plus the 5S RNA maxigene) was subjected to DNase I treatment either with or without prior binding of TFIIIA. (A) Footprints of the wild-type *X. borealis* somatic 5S RNA gene, LS 34/45MT, LS 38/49MT, and LS 46/57MT. Lanes 1 to 4 of each group contained 0, 2, 4, and 8 nM TFIIIA, respectively. Lanes 5 show the end-labeled products of *Bam*HI cleavage for the respective DNAs. (B) Footprints of the wild-type 5S RNA gene, LS 86/97MT, and LS 92/105. Lane designations are as described for panel A.

transcription efficiency was correlated with the inability of the gene to bind TFIIIA normally (Fig. 3A). In this example, the region of the ICR replaced with linker DNA was accessible to DNase I, and the DNA appeared to have a reduced affinity for TFIIIA. However, several other mutants appeared to bind TFIIIA in a nearly normal fashion. TFIIIA protected the entire ICR in LS 86/97MT but with a decreased binding affinity (Fig. 3B). This result is similar to results obtained previously for mutants LS 57/67MT and LS 67/78MT (5). The DNase I footprinting method did not detect altered binding of TFIIIA to mutations LS 34/45MT and LS 38/49MT. As demonstrated in Fig. 3, both genes behaved similarly to the wild-type gene with respect to protection pattern and affinity for TFIIIA binding. DNase I protection analysis of the remaining mutants, LS 8/15MT, LS 18/29MT, LS 26/37MT, LS 92/105MT, and LS 107/118MT, showed no evidence of altered binding of TFIIIA (data not shown). The overall pattern of results obtained with these linker substitution mutants was similar to that obtained by footprint analysis of point mutants (21). Drastic changes in transcription efficiency can result from base changes that do not cause marked alteration in DNase I protection by TFIIIA.

Analysis of reconstituted 7S RNPs by PAGE. 7S RNPs composed of TFIIIA and 5S RNA, purified from immature *X. laevis* oocytes, survived PAGE under nondenaturing conditions, migrating more slowly than free 5S RNA. We performed an extensive series of control experiments to vary the conditions for gel electrophoresis, including the running

buffer, temperature, and varied 7S particle concentrations. Under all conditions, there was a tendency for 7S particles to dissociate to yield free 5S RNA when low quantities of 7S particles were subjected to electrophoresis, with an apparent dissociation constant of approximately 100 to 200 nM. The conditions described in Materials and Methods reflect the optimal conditions for electrophoresis according to our experience.

Reconstitution of 7S particles in vitro was performed by mixing purified TFIIIA and 5S RNA in a buffer suitable for in vitro transcription. We used two general sets of conditions to permit reconstitution of 7S particles. The simplest approach was to add increasing concentrations of TFIIIA to a fixed quantity of approximately 1 μ M 5S RNA. At these high concentrations, the 7S particles could be followed by staining the native gel with ethidium bromide (Fig. 4A). These conditions were not appropriate for studying the binding of TFIIIA to transcripts of linker substitution mutants, since micromolar concentrations of altered 5S RNA were not available. Therefore, we used a second approach to study the binding of TFIIIA to altered 5S RNAs. Trace quantities of radioactively labeled RNA were mixed with nonradioactive carrier 5S RNA, and a binding titration was performed by adding increasing quantities of TFIIIA to the RNA. An example of such a titration is shown in Fig. 4B.

Ability of transcripts of the linker substitution mutants to bind TFIIIA. Radiolabeled transcripts from the linker substitution series were prepared in an S-150 extract and puri-

fied on native gels as described in Materials and Methods. TFIIA was added to final concentrations from 0 to 2 μ M to reaction mixtures containing a trace quantity of radiolabeled transcript and 0.5 μ M unlabeled wild-type 5S RNA. The reaction was allowed to proceed for 20 min at 20°C, at which time 2 ng of K-heparin and loading dyes were added. The resulting complexes were analyzed by PAGE (Fig. 5). Base changes in the 5' portion on the 5S RNA due to mutations in the 5' half of the 5S RNA gene (LS 8/15 to LS 46/57; Fig. 5B to G) did not drastically affect the binding of TFIIA. In contrast, transcripts resulting from most of the 3' mutations (e.g., LS 57/67, LS 67/78, LS 92/105, and LS 107/118; Fig. 5H, I, K, and L, respectively) bound TFIIA poorly. Even at higher TFIIA/5S RNA ratios (4:1; lanes 4, Fig. 5A to L) little or no specific binding was observed. An interesting exception was provided by the transcript of LS 86/97, which

bound TFIIA nearly as well as the wild-type 5S RNA (Fig. 5J).

Figure 6 presents the binding efficiencies of the transcripts relative to that of wild-type *X. borealis* 5S RNA. Mutant genes LS 46/57 and LS 86/97 bound TFIIA poorly, whereas the transcripts were capable of binding the protein. In contrast, mutations in the gene which had no effect on TFIIA binding, LS 92/105 and LS 107/118, supported the synthesis of transcripts which did not bind TFIIA. In addition, mutant genes LS 57/67 and LS 67/78, with linkers inserted in the center of the ICR, bound TFIIA relatively well and were efficiently transcribed to yield RNAs that failed to bind TFIIA. We conclude that clusters of base pair changes have different effects on the binding of TFIIA of 5S RNA compared with that to the 5S RNA gene.

DISCUSSION

Altered transcriptional phenotypes of certain linker mutations. In this study we constructed a series of linker substitution mutations of the 5S RNA gene to compare the effects of the base substitutions on the binding of TFIIA to both the mutant 5S RNA genes and the resulting transcripts. Linker substitution mutagenesis allowed us to study nearly the entire gene by embedding synthetic linkers within wild-type sequences while conserving the length of the gene. Minimal effects on transcription were generally observed with base substitutions outside the ICR. There was one exception to this generalization. The mutation LS 8/15, clearly outside the TFIIA binding site, was transcribed only 75% as efficiently as a wild-type *X. borealis* 5S RNA gene. The basis for this impaired transcription is not understood, although it is interesting that this region shows homology to the consensus sequence for RNA polymerase II enhancer sequences (39). To date, we have only transcribed these mutants in S-150 extracts. It is conceivable that the relative transcription efficiencies would be altered if the genes were transcribed in other extracts containing different relative concentrations of factors.

Mutants with linkers substituted within the ICR had transcription efficiencies consistent with the two-domain model proposed for the binding of TFIIA (5, 33). Mutations LS 46/57 and LS 86/97, located in the 5' and 3' borders of the ICR, respectively, supported transcription at a minimal level. These mutations presented a sharp contrast to two mutant genes (LS 57/67 and LS 67/78) which were transcribed with an efficiency of 50 to 60% relative to the wild-type gene (Fig. 2) (5). Surprisingly, two mutations which lay primarily outside the ICR, LS 34/45 and LS 38/49, substantially decreased transcription efficiency. The impairment of transcription resulting from these two linker substitutions was comparable to that resulting from deletion of the gene from the 5' side to residue +50 (32).

We examined the binding of TFIIA to linker substitution mutants of the 5S RNA gene by DNase I protection assays. Mutants with linker substitutions outside the ICR bound TFIIA with a normal affinity and protection pattern. Mutants with linkers substituted in the center of the ICR (LS 57/67 and LS 67/78) showed a normal protection pattern but had a slightly lower affinity for TFIIA (5). The two linker substitution mutations at the 5' and 3' borders of the ICR, LS 46/57 and LS 86/97, bound TFIIA less tightly than the wild-type gene. In the case of the mutant LS 86/97, TFIIA binding was only slightly impaired and the size of the footprint was not affected. This is consistent with the early observation that 3' deletion to residue 83 of the gene still

FIG. 4. Reconstituted 7S particles analyzed by PAGE. (A) Photograph of an ethidium bromide-stained polyacrylamide gel demonstrating the mobility of 5S RNA (lane 1), reconstituted 7S RNPs (lanes 2 to 6), and purified 7S RNPs (lane 7). The samples loaded in lanes 1 and 7 contained 1.2 μ M 5S RNA and 2.5 μ M 7S RNPs, respectively, in a 20- μ l volume. The 20- μ l samples loaded in lanes 2 to 6 each contained 1.2 μ M 5S RNA and 0.3, 0.6, 1.2, 2.5, and 5.0 μ M TFIIA, respectively. (B) Autoradiogram demonstrating the in vitro binding of increasing concentrations of TFIIA to trace amounts of radiolabeled 5S RNA to form 7S particles. The migration positions of 5S RNA and 7S particles (7SP) are indicated to the left. Each lane contained a trace amount of radiolabeled RNA mixed with 25 nM unlabeled 5S RNA. Lanes 1 to 14 contained 0, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, and 600 nM TFIIA, respectively. Reaction conditions were the same as described above, except that K-heparin was added as follows: lanes 1 to 3, no heparin; lanes 4 to 7, 80 pg; lanes 8 to 10, 800 pg; and lanes 11 to 14, 2.5 ng.

FIG. 5. Binding of transcripts from linker substitution mutants to TFIIIA. Radiolabeled transcripts from the linker substitution mutants were incubated with TFIIIA, and the resulting 7S RNPs were analyzed by PAGE. The positions of 5S RNA and 7S RNP are indicated. Panels A to L represent the wild-type 5S RNA and transcripts resulting from mutations LS 8/15, LS 18/29, LS 26/37, LS 34/45, LS 38/49, LS 46/57, LS 57/67, LS 67/78, LS 86/97, LS 92/105, and LS 107/118, respectively. Lanes 1 to 4 of each panel contained 0.5 μ M unlabeled wild-type 5S RNA and a trace amount of the corresponding radiolabeled transcript with 0, 0.5, 1.0, and 2.0 μ M TFIIIA, respectively.

permits detectable transcription (6). TFIIIA failed to protect the linker region of LS 46/57. Altered binding of TFIIIA due to mutations in the borders of the ICR is probably responsible for the altered transcriptional phenotypes of those genes. In contrast, DNase I footprinting did not detect any significant alteration in the binding of TFIIIA to mutants LS 34/45 and LS 38/49, although both were transcribed poorly. These findings lead us to speculate that the region near the 5' border of the ICR may be involved in the binding of additional factors necessary for the 5S RNA gene transcription. Similar effects on transcription and in DNase I protection assays have been observed by McConkey and Bogenhagen (21) with a series of point mutations in and around the 5' border of the TFIIIA binding site.

Assembly of 7S particles monitored by native PAGE. We have developed an in vitro reconstitution assay to study the

interactions of TFIIIA with 5S RNA. Native gel electrophoresis has been used previously to study 7S particles (7). Anderson and Delihias (1) have used electrophoresis to monitor the ability of heterologous 5S RNAs to exchange with *X. laevis* 5S RNA in 7S particles. In our hands, such exchange assays have a tendency to permit the formation of higher-order complexes that do not comigrate with authentic 7S particles.

Reconstitution of 7S particles was accomplished by the addition of purified TFIIIA to 5S RNA under buffer conditions capable of supporting 5S RNA transcription. Reconstituted complexes were analyzed by nondenaturing PAGE. Under the conditions used, reconstituted 7S RNPs migrated with the same mobility as 7S particles purified from immature *X. laevis* oocytes. Results obtained with this mobility shift assay varied with the concentration of 5S RNA used in

the binding reactions. When very low concentrations of 5S RNA were used (<10 nM), any 7S particles that were formed did not appear to survive electrophoresis. Also, at very high ratios of TFIIIA to 5S RNA, some of the RNA can be trapped within the gel well. These artifacts can be minimized by adding small quantities of heparin. The addition of heparin to the reaction mixtures may reduce the amount of nonspecific binding (18), which can trap the radiolabeled 5S RNA in the gel wells. Once particles have entered the gel, they appear to be stable, as indicated by the lack of streaking between the positions of free 5S RNA and 7S particles. At high concentrations of 5S RNA (1 μ M or greater), the binding of TFIIIA can be studied by staining the RNA with ethidium bromide. The sensitivity of the assay can be increased by using radiolabeled 5S RNA.

The 7S particle reassociation data shown in Fig. 4 can be used to derive an apparent dissociation constant. In this titration series, approximately 50% of the 5S RNA is bound by 200 to 300 nM TFIIIA. This is in good agreement with the apparent dissociation constant determined by electrophoresis of 7S particles isolated from immature oocytes (7; Bogenhagen, unpublished observations). This estimation of the dissociation constant is higher than estimates made by using other techniques. Hanas et al. (14) used footprint competitions to study the relative affinity of TFIIIA for 5S RNA compared with 5S DNA. These experiments suggested a dissociation constant for TFIIIA and 5S RNA in the range of 10^{-8} M. Recently, Romaniuk (30) used a filter-binding assay to measure a dissociation constant for TFIIIA and 5S RNA of 1 nM. These data were obtained by using a filter-binding technique which can be quite harsh to protein-nucleic acid complexes and will not distinguish between authentic 7S particles and other complexes or aggregates. Even though the complexes analyzed by the gel assay migrate to the same position as authentic 7S particles, the dissociation data should be interpreted cautiously because electrophoresis may also disrupt the 7S particles. In any case, it is clear that dilute samples (<10 nM) of naturally

occurring or reassociated 7S particles do not survive native gel electrophoresis under a wide range of conditions. This fact limits the utility of gel electrophoresis as a technique to investigate the occurrence of 7S particles in somatic cells or the formation of 7S particles in *in vitro* transcription extracts.

Binding of TFIIIA to 5S RNAs containing linker substitutions. We used gel electrophoresis to analyze the ability of trace amounts of labeled, altered 5S RNA to bind TFIIIA to form 7S particles. RNAs transcribed from 5S RNA genes containing linker substitution mutations were purified and tested for their ability to bind TFIIIA. Base changes in the 5' portion of the transcripts, as contained in mutants LS 8/15 through LS 46/57, had a much less pronounced effect on the binding of TFIIIA than did changes in the 3' portion. Changes in the 3' portion of the transcripts eliminated or substantially decreased the binding of TFIIIA, with the exception of LS 86/97. The regions of 5S RNA affected by these linker substitution mutations are shown with reference to the secondary structure of *X. laevis* 5S RNA (2) in Fig. 7. Base changes that decreased binding were located primarily in or around helices I, IV, and V. Our results with reconstitution of 7S particles are consistent with results obtained by digesting end-labeled 7S particles with specific nuclease probes (3, 17). Indirect assays measuring the protection of adenine residues in the 7S particle from chemical modification (27) or the ability of fragments generated from nuclease-treated 5S RNA to inhibit 5S RNA gene transcription have also implicated the helix IV/V region in the binding of TFIIIA (28).

Conserved structural features of 5S RNA are believed to be important for proper interactions with various 5S RNA-binding proteins (3, 8, 26). At present we cannot rule out the possibility that some of the linker substitution mutations used here could result in extensive refolding of the 5S RNA secondary structure. However, a number of mutations would be expected to affect only regions of the RNA that are in single-stranded loops in the established secondary structure. Estimation of free energy values by the rules defined by Tinoco et al. (38) also suggests that all of the transcripts derived from the linker mutations would be unlikely to exhibit extensive changes in the secondary structure. We believe that secondary-structure changes caused by base substitutions are likely to be localized to the region of 5S RNA containing the altered sequence.

The results of our experiments and of the nuclease cleavage studies mentioned above are consistent with the possibility that TFIIIA may interact mainly with double-stranded regions of the 5S RNA. This result is somewhat surprising, because unpaired bases in RNA would be expected to permit additional opportunities for hydrogen bond formation between the RNA and amino acid residues of the protein. A detailed understanding of the interactions of TFIIIA with 5S RNA will require elucidation of the tertiary structure of 5S RNA. The secondary structure shown in Fig. 7 may not represent many aspects of the tertiary structure. A three-dimensional model of *Escherichia coli* 5S RNA has been proposed by Pieler and Erdman (26). This model shows helix I aligned with the region encompassed by helices IV and V. The sequence within the helix I region of the 5S RNA bears some homology to the 5' boundary of the ICR. Base substitutions caused by LS 57/67, LS 67/78, LS 92/105, and LS 107/118 would be expected to disrupt the helical structure of the 5S RNA.

How does TFIIIA bind specifically to both 5S RNA and 5S DNA? The collection of linker substitution mutations presented here offers a unique opportunity to compare the

FIG. 6. TFIIIA binding to altered 5S RNAs. This diagram graphically represents the binding of TFIIIA to radiolabeled wild-type 5S RNA and transcripts derived from the series of linker substitution mutants. Bars correspond to the lanes described in the legend to Fig. 2A. The autoradiograms in Fig. 5 were scanned with a laser densitometer to determine the fraction of total 5S RNA bound in the 7S particle. The histogram was generated from data obtained from the lanes containing 2 μ M TFIIIA. The fraction of RNA bound within 7S particles was normalized to the binding of wild-type *X. borealis* somatic 5S RNA.

FIG. 7. Location of linker substitutions, with reference to the secondary structure of 5S RNA. The regions of 5S RNA affected by linker substitutions are diagrammed with reference to the secondary structure of 5S RNA (2). For the purpose of this diagram, we assume that each mutation only affects the local secondary structure, as described in the text. Areas containing base pair changes in particular mutants are indicated by a bold line traced around the sequence. (A) Regions of the 5S RNA affected by linker substitutions that did not drastically reduce binding of the RNA in 7S particles (binding, >50% of the wild-type level). The specific gene mutations responsible for the changes were LS 8/15, LS 18/29, LS 26/37, LS 34/45, LS 38/49, LS 46/57, and LS 86/97. (B) Regions of the 5S RNA affected by linker substitutions that significantly impaired binding of the RNA in 7S particles (binding <25% of wild type). The base changes in this diagram were derived from mutations LS 57/67, LS 67/78, LS 92/105, and LS 107/118. Helices I to V are indicated.

sequences required for binding of TFIIIA to 5S RNA and to 5S DNA. Much more progress has been made in studies of the binding of TFIIIA to 5S RNA than to 5S DNA. Smith et al. (37) suggested that a single molecule of TFIIIA binds the entire ICR, with the tightest contacts between a 20-kilodalton amino-terminal proteolytic domain of the protein

and the 3' half of the ICR, that is, the region downstream from residue 64. These tight-binding contacts were first observed by Sakonju and Brown (33) in methylation interference experiments. TFIIIA also associates with 5S RNA in a 1:1 complex. In these 7S RNPs, the amino-terminal half of the protein also makes tight contacts with the RNA (37). We suggest that this amino-terminal domain of TFIIIA contacts the helix IV/V region of 5S RNA within 7S particles. If this is the case, the tight binding site in 5S RNA will also be a helical region, beginning with residue 64. Our results suggest that TFIIIA interacts with a helical region within a single strand of RNA. This essential helix IV/V region involves portions of the RNA transcribed from regions of the gene that are outside the TFIIIA-binding site in 5S DNA.

Our data suggest that the binding of TFIIIA to both the 5S RNA gene and 5S RNA is not specific for any particular sequence. We have demonstrated two mutations, LS 46/57 and LS 86/97, which have deleterious effects on transcription that correlate clearly to abnormal binding of TFIIIA to the gene. Although both genes were transcribed very inefficiently, the transcripts of the genes bound TFIIIA as well as wild-type 5S RNA did. In contrast, several linker substitution mutations that bound TFIIIA well were transcribed to yield RNAs that did not bind the protein. These linker substitution mutations occurred in the 3' portion of the gene, except for the transcript derived from LS 86/97. It is not yet obvious how this result can be reconciled with the fact that TFIIIA appears to bind to helical regions in both 5S RNA and 5S DNA. We suggest that the RNA folds into a secondary structure that makes contacts available for TFIIIA binding to maximize the resemblance to 5S DNA.

It has been observed that TFIIIA involves Zn^{2+} in binding to both 5S RNA and 5S DNA (15). Following publication of the TFIIIA sequence (11), Miller et al. (23) suggested that TFIIIA is composed of nine repeating units which may coordinately bind Zn^{2+} ions to form "fingers" which interact with the DNA backbone. Rhodes and Klu (29) observed a repetitive character to the sequence within the ICR which has a periodicity of approximately 5.5 bp. It has been proposed that these stretches of DNA sequences may adopt the A-form conformation and interact with the individual fingers of the TFIIIA molecule (10, 29). A recent study has shown that the crystal structure of a synthetic oligodeoxynucleotide with the same sequence as nucleotides 81 to 89, at the 3' border of the ICR of the 5S RNA gene, occurs in an A-form conformation (20) that might resemble duplex RNA (31). The zinc finger model might provide an unusual conformational flexibility that would enhance the ability of TFIIIA to interact with both 5S RNA and 5S DNA.

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LITERATURE CITED

- Anderson, J., and N. Delihias. 1986. Characterization of RNA-protein interaction in 7S ribonucleoprotein particles from *Xenopus laevis* oocytes. *J. Biol. Chem.* **261**:2912-2917.
- Anderson, J., N. Delihias, J. S. Hanas, and C. W. Wu. 1984. 5S RNA structure and interaction with transcription factor A. 1. Ribonuclease probe of the structure of 5S RNA from *Xenopus laevis*. *Biochemistry* **23**:5752-5759.
- Anderson, J. A., N. Delihias, J. S. Hanas, and C. W. Wu. 1984. 5S RNA structure and interaction with transcription factor A. 2. Ribonuclease probe of the 7S particle from *Xenopus laevis*.

- immature oocytes and RNA exchange properties of the 7S particle. *Biochemistry* **23**:5759–5766.
4. **Bieker, J. J., P. L. Martin, and R. G. Roeder.** 1985. Formation of a rate-limiting intermediate in 5S RNA gene transcription. *Cell* **40**:119–127.
 5. **Bogenhagen, D. F.** 1985. The intragenic control region of the *Xenopus* 5S RNA gene contains two factor A binding domains that must be aligned properly for efficient transcription initiation. *J. Biol. Chem.* **260**:6466–6471.
 6. **Bogenhagen, D. F., S. Sakonju, and D. D. Brown.** 1980. A control region in the center of the 5S RNA gene directs specific initiation of transcription. II. The 3' border of the region. *Cell* **19**:27–35.
 7. **Denis, H., and M. le Maire.** 1983. Thesaurisomes, a novel kind of nucleoprotein particle. *Subcell. Biochem.* **9**:263–297.
 8. **Douthwaite, S., A. Christensen, and R. A. Garrett.** 1982. Binding sites of ribosomal proteins on prokaryotic 5S ribonucleic acids: a study with ribonucleases. *Biochemistry* **21**:2313–2320.
 9. **Engelke, D. R., S. Ng, B. S. Shastri, and R. G. Roeder.** 1980. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. *Cell* **19**:717–728.
 10. **Fairall, L., D. Rhodes, and A. Klug.** 1986. Mapping of the site of protection on a 5S RNA gene by the *Xenopus* transcription factor IIIA. *J. Mol. Biol.* **192**:577–591.
 11. **Ginsberg, A. M., B. O. King, and R. G. Roeder.** 1984. *Xenopus* 5S gene transcription factor, TFIIIA: characterization of a cDNA clone and measurement of RNA levels throughout development. *Cell* **39**:479–489.
 12. **Glikin, G. C., I. Ruberti, and A. Worcel.** 1984. Chromatin assembly in *Xenopus* oocytes: in vitro studies. *Cell* **37**:33–41.
 13. **Hanas, J. S., D. F. Bogenhagen, and C. Wu.** 1983. Cooperative model for the binding of *Xenopus* transcription factor A to the 5S RNA gene. *Proc. Natl. Acad. Sci. USA* **80**:2142–2145.
 14. **Hanas, J. S., D. F. Bogenhagen, and C. W. Wu.** 1984. Binding of *Xenopus* transcription factor A to 5S RNA and to single stranded DNA. *Nucleic Acids Res.* **12**:2745–2758.
 15. **Hanas, J. S., D. J. Hazuda, D. F. Bogenhagen, F. H.-Y. Wu, and C.-W. Wu.** 1983. *Xenopus* transcription factor A requires zinc for binding to the 5S RNA gene. *J. Biol. Chem.* **258**:14120–14125.
 16. **Honda, B. M., and R. G. Roeder.** 1980. Association of a 5S gene transcription factor with 5S RNA and altered levels of the factor during cell differentiation. *Cell* **22**:119–126.
 17. **Hüder, P. W., and I. G. Wool.** 1986. Identification of the binding site on 5S rRNA for the transcription factor IIIA: proposed structure of a common binding site on 5S rRNA and on the gene. *Proc. Natl. Acad. Sci. USA* **83**:1593–1597.
 18. **Konarska, M. M., and P. A. Sharp.** 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. *Cell* **46**:845–855.
 19. **Lassar, A. B., P. L. Martin, and R. G. Roeder.** 1983. Transcription of class III genes: formation of preinitiation complexes. *Science* **222**:740–748.
 20. **McCall, M., T. Brown, W. N. Hunter, and O. Kennard.** 1986. The crystal structure of d(GGATGGGAG) forms an essential part of the binding site for transcription factor IIIA. *Nature (London)* **322**:661–664.
 21. **McConkey, G. A., and D. F. Bogenhagen.** 1987. Transition mutations within the *Xenopus borealis* somatic 5S RNA gene can have independent effects on transcription and TFIIIA binding. *Mol. Cell. Biol.* **7**:486–494.
 22. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**:269–276.
 23. **Miller, J., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *EMBO J.* **4**:1609–1614.
 24. **Pelham, H. R. B., and D. D. Brown.** 1980. A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. *Proc. Natl. Acad. Sci. USA* **77**:4170–4174.
 25. **Picard, B., and M. Wegnez.** 1979. Isolation of a 7S particle from *Xenopus laevis* oocytes: a 5S RNA-protein complex. *Proc. Natl. Acad. Sci. USA* **76**:241–245.
 26. **Pieler, T., and V. A. Erdman.** 1982. Three-dimensional structural model of eubacterial 5S RNA that has functional implications. *Proc. Natl. Acad. Sci. USA* **79**:4599–4603.
 27. **Pieler, T., and V. A. Erdman.** 1983. Isolation and characterization of a 7S RNP particle from mature *Xenopus laevis* oocytes. *FEBS Lett.* **157**:283–287.
 28. **Pieler, T., U. Guddat, S. L. Oei, and V. A. Erdman.** 1986. Analysis of the RNA structural elements involved in the binding of transcription factor IIIA from *Xenopus laevis*. *Nucleic Acids Res.* **14**:6313–6326.
 29. **Rhodes, D., and A. Klug.** 1986. An underlying repeat in some transcriptional control sequences corresponding to half a double helical turn of DNA. *Cell* **46**:123–132.
 30. **Romaniuk, P. J.** 1985. Characterization of the RNA binding properties of transcription factor IIIA of *Xenopus laevis* oocytes. *Nucleic Acids Res.* **13**:5369–5387.
 31. **Saenger, W.** 1984. RNA structure and DNA structure, p. 242–281. In C. R. Cantor (ed.), *Principles of nucleic acid structure*. Springer-Verlag, Berlin.
 32. **Sakonju, S., D. F. Bogenhagen, and D. D. Brown.** 1980. A control region in the center of the 5S RNA gene directs specific initiation of transcription. I. The 5' border of the region. *Cell* **19**:13–25.
 33. **Sakonju, S., and D. D. Brown.** 1982. Contact points between a positive transcription factor and the *Xenopus* 5S RNA gene. *Cell* **31**:395–405.
 34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 35. **Segall, J., T. Matsui, and R. G. Roeder.** 1980. Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III. *J. Biol. Chem.* **255**:11986–11991.
 36. **Setzer, D. R., and D. D. Brown.** 1985. Formation and stability of the 5S RNA transcription complex. *J. Biol. Chem.* **260**:2483–2492.
 37. **Smith, D. R., I. J. Jackson, and D. D. Brown.** 1984. Domains of the positive transcription factor specific for the *Xenopus* 5S RNA gene. *Cell* **37**:645–652.
 38. **Tinoco, I., O. C. Uhlenbeck, and M. D. Levine.** 1971. Estimation of secondary structure in ribonucleic acids. *Nature (London)* **230**:362–367.
 39. **Weiher, H., M. Konig, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. *Science* **219**:626–631.