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The Role of Highly Conserved Single-Stranded Nucleotides of *Xenopus* 5S RNA in the Binding of Transcription Factor IIIA†

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ABSTRACT: The role of highly conserved single-stranded sequence elements of *Xenopus* 5S RNA in the binding of transcription factor IIIA (TFIIIA) was studied. A series of mutant 5S RNA genes were constructed with defined block sequence changes in regions corresponding to each of the single-stranded loops of the transcribed 5S RNA. The interaction of the resulting mutant 5S RNA molecules with TFIIIA was determined both by a direct binding assay and by a competition assay. With one exception, the substitution of highly conserved single-stranded loop sequences had only a modest effect on the binding of TFIIIA. The single exception was loop A, which ironically is not part of the protected site of TFIIIA on 5S RNA. The possible involvement of loop A in the coaxial stacking of the helical domains of 5S RNA, and how this might affect TFIIIA binding, are discussed.

TFIIIA performs two essential functions in the *Xenopus* oocyte. It acts as a positive transcription factor, binding to an internal control region in the *Xenopus* 5S RNA gene and modulating the expression of these genes during oogenesis (Sakonju et al., 1980, 1981; Bogenhagen et al., 1980; Engelke et al., 1980). TFIIIA also binds to 5S RNA in the cytoplasm of immature oocytes, forming a 7S RNP storage particle that stabilizes the 5S RNA for later use in ribosome assembly (Picard & Wegnez, 1979; Pelham & Brown, 1980). Several recent discoveries have intensified the desire to understand how TFIIIA can interact specifically with two different nucleic acid targets. Studies on the structure of TFIIIA have shown that this protein contains approximately 9 mol equiv of Zn^{2+} ions essential for its function as a transcription factor (Hanas et al., 1983; Miller et al., 1985). Analysis of the cDNA sequence (Ginsberg et al., 1984) for the protein revealed the presence of nine imperfectly repeating sequence elements (Brown et al., 1985; Miller et al., 1985), containing highly conserved cysteine and histidine residues. It has been proposed that these residues coordinate to a Zn^{2+} ion, folding each repeat element into a novel structural domain best described as a protein "finger", and it is thought that each finger acts independently in binding to DNA (Miller et al., 1985). The presence of these fingers may provide properties required for the function of TFIIIA as a transcription factor, and finger structures appear to be a common motif in the putative nucleic acid binding domains of a wide range of eukaryotic regulatory proteins (Berg, 1986; Vincent, 1986; Evans & Hollenberg, 1988). Additionally, the protein fingers may have characteristics that facilitate the

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interaction of TFIIIA with 5S RNA (Berg, 1986).

The function of TFIIIA as a transcription factor has been studied intensively, and recently the TFIIIA–5S RNA interaction has been investigated by several groups. Thermodynamic and kinetic parameters have been determined for the TFIIIA–5S RNA interaction by using a nitrocellulose filter binding assay that has been demonstrated to measure the equilibrium resulting in 7S RNP formation (Romaniuk, 1985). TFIIIA binds to *Xenopus* oocyte 5S RNA with a dissociation constant (K_d) of 1 nM, the complex having a half-life of ca. 45 min (Romaniuk, 1985). Formation of the TFIIIA–5S RNA complex is favored by both enthalpy and entropy. The majority of the free energy of the interaction is derived from nonelectrostatic interactions, although approximately five ionic contacts are formed between TFIIIA and 5S RNA (Romaniuk, 1985). Several studies have shown that a number of eukaryotic 5S RNAs bind to TFIIIA with similar affinities (Pieler et al., 1984; Hanas et al., 1984; Romaniuk, 1985; Andersen & Delihas, 1986).

Footprinting experiments have been conducted on both native 7S RNP and reconstituted particles, and the results indicate that TFIIIA primarily interacts with the helix II/loop B and helix IV/loop E/helix V structural domains (Pieler & Erdmann, 1983; Andersen et al., 1984; Romaniuk, 1985; Huber & Wool, 1986; Christiansen et al., 1987). Studies with deletion mutants of 5S RNA indicated that full binding activity with TFIIIA was lost once deletions were made from either terminus past helix I (Andersen & Delihas, 1986; Romaniuk et al., 1987b). However, the decrease in binding can in some cases be attributed to major structural rearrangements resulting from the deletions, and there is some evidence from these experiments to suggest that the helix II/loop B domain represents the primary interaction site with TFIIIA (Romaniuk et al., 1987b).

Although the boundaries of the RNA equivalent of the internal control region have been defined, relatively little is known about functionally important regions within this binding site in comparison to knowledge about such regions of the DNA binding site. Nucleotides 53–56 have a demonstrated role in TFIIIA binding, since somatic-specific substitutions at these positions enhance the affinity of the RNA for TFIIIA by 3-fold (Romaniuk et al., 1987b). Three-dimensional mapping techniques have been applied to the analysis of the chemical footprinting data, and the results suggest that the contacts formed with TFIIIA are clustered in three specific areas of the 5S RNA, similar in distribution to the three elements of the internal control region on the gene (Christiansen et al., 1987). These elements occur at "hinge" regions between loops and helices in the 5S RNA; however, there is no direct evidence to support an essential role for the nucleotide sequences in these proposed RNA elements.

In the present study, a series of mutant 5S RNA genes have been constructed with defined block sequence changes in regions corresponding to each of the single-stranded loops of the transcribed 5S RNA. The interaction of the mutant 5S RNA molecules with TFIIIA has been determined both by a direct binding assay and by a competition assay. With one exception, substitution of highly conserved single-stranded loop sequences has only a modest effect on the binding of TFIIIA. The single exception is loop A, which ironically is not part of the protected site of TFIIIA on 5S RNA.

Materials and Methods

Plasmids. The 5S RNA genes used in these experiments were constructed from a series of synthetic oligonucleotides by a modification of the microscale shotgun ligation method (Grundstrom et al., 1985). The same methods were used in the construction of the wild-type *Xenopus* oocyte 5S RNA gene in the plasmid pUC18 (Romaniuk et al., 1987b), and the mutant genes were constructed by using the appropriate oligonucleotide replacements. Each gene was constructed to be under the control of a promoter for T7 RNA polymerase, in such a fashion that the first nucleotide of the resulting in vitro transcripts is the mature 5′ terminus of the 5S RNA (Romaniuk et al., 1987b).

Transcription of Mutant 5S RNA Genes. In vitro transcription was carried out by using T7 RNA polymerase purified by a published procedure (Davanloo et al., 1984) from *Escherichia coli* strain BL21/pAR1219, kindly provided by Dr. F. W. Studier. Prior to transcription, each plasmid containing a 5S RNA gene was digested with the restriction enzyme *Dra*I, which defines the 3′ terminus of the transcripts as nucleotide +121 of the gene. For the direct binding assay, internally labeled RNA was synthesized in an assay mixture containing 40 mM Tris-HCl, pH 8, 15 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 100 μg/mL BSA, 1000 units/mL RNasin, 0.5 mM each ATP, CTP, and UTP, 0.0125 mM GTP, 50 μCi of [α-^{32}P]GTP (600 Ci/mmol), 1 μg of linearized template DNA, and 0.6 μg of T7 RNA polymerase in a final volume of 10 μL. After incubation for 2.5 h at 37 °C, 10 μL of urea–dye sample buffer was added, and the 5S RNA was purified on a 8 M urea 12% polyacrylamide gel.

For use in the competition assay, unlabeled RNA was synthesized in an assay mixture containing 40 mM Tris-HCl, pH 8, 30 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 100 μg/mL BSA, 8% PEG8000, 0.01% Triton X-100, 5 mM NTP, 20 μg of linearized template DNA, and 10 μg of T7 RNA polymerase in a final volume of 200 μL. After incubation for 4 h at 37 °C, the reaction was extracted with 200 μL of phenol:chloroform, followed by extraction with 200 μL of chloroform. The crude RNA was recovered by ethanol precipitation and purified by gel permeation HPLC chromatography (Romaniuk et al., 1987b). Yields of pure 5S RNA were typically 100 μg.

Binding Assays. Preparation of 7S RNP and the purification of TFIIIA were performed as described previously (Romaniuk, 1985). Fractional activity of each protein preparation was determined by comparing the apparent association constant (K_a) measured for the preparation with the value of 1.3×10^9 M^{-1} determined by Scatchard analysis to be the apparent K_a obtained with 100% active TFIIIA (Romaniuk, 1985). Only those preparations that were >90% active were used to study the binding of mutant 5S RNAs. The equilibrium constants for the binding of mutant 5S RNAs to TFIIIA were determined by a standard nitrocellulose filter binding assay (Romaniuk, 1985) and compared in each case with the apparent K_a for the wild-type 5S RNA determined in parallel. For the determination of competition strengths, the TFIIIA concentration was held constant at 1 nM, the concentration of ^{32}P-labeled wheat germ 5S RNA was 0.5 nM, and competitor 5S RNA concentrations were varied between 0.1 and 20 nM. All other details of the competition assays have been described previously (Romaniuk, 1985; Romaniuk et al., 1987b).

Gel Mobility Shift Assays. Complexes between 5S RNAs (1 nCi, 0.06 μM) and TFIIIA (0.9 μM) were formed in 5 μL of a buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 M KCl, 40 units of RNasin, and 1 mM DTT. After incubation for 10 min at 20 °C, 1 μL of glycerol–dye buffer was added, the samples were loaded onto a 10% nondenaturing polyacrylamide mini gel (50 mM Tris–borate, pH 8.3, buffer),

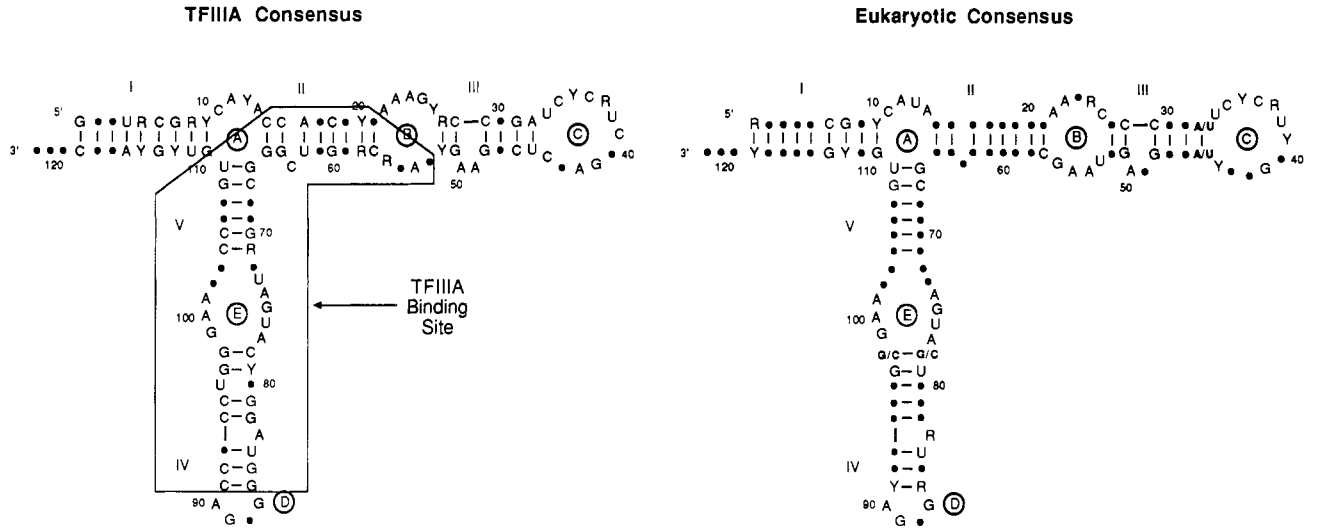

FIGURE 1: Consensus sequences of eukaryotic 5S RNA. (Left) Sequence elements common to all eukaryotic 5S RNAs known to bind TFIIIA have been drawn in the universal secondary structure. The box indicates the putative binding site for TFIIIA on *Xenopus* 5S RNA. (Right) The general eukaryotic consensus sequence [adapted from Delihas and Andersen (1982)].

and subjected to electrophoresis at 165 V for 1.5 h at 4 °C. The gels were then fixed for 10 min in methanol:acetic acid (10%:10% v/v), dried, and subjected to autoradiography.

RESULTS

Choice of Target Sites for Mutagenesis of Xenopus Oocyte 5S RNA. Figure 1 shows a preliminary "consensus" TFIIIA binding site on 5S RNA derived both from sequence comparison of those eukaryotic 5S RNAs known to form a strong interaction with TFIIIA (Pieler et al., 1984; Hanas et al., 1984; Romaniuk, 1985; Andersen & Delihas, 1986) and by taking into consideration binding data determined with truncated and chimeric 5S RNAs (Romaniuk et al., 1987b). For comparative purposes, the general eukaryotic 5S RNA consensus model (Delihas & Andersen, 1982; de Wachter et al., 1982) is also shown. In the general consensus model, the most highly conserved sequences occur within the single-stranded loops of the 5S RNA, and similar strong conservation of sequence is found in these areas in the TFIIIA consensus binding model. The only difference results from the nonstandard sequence elements found in the 53–56 region of loop B in *Xenopus* oocyte 5S RNA. As demonstrated in an earlier study, substitution of these nucleotides with the somatic-specific nucleotides, which are identical with the eukaryotic consensus in this region, enhances TFIIIA binding by approximately 3-fold (Romaniuk et al., 1987b). The apparent conservation of sequences in the helical regions of the TFIIIA consensus model simply reflects the smaller subset of 5S RNA molecules for which TFIIIA binding data are available. The fact that TFIIIA is capable of forming strong binding interactions with eukaryotic 5S RNAs from such diverse sources as plants, yeast, and amphibia suggested that the highly conserved single-stranded nucleotides common to all eukaryotic 5S RNAs would be excellent targets for mutagenesis.

Binding Affinities and Competition Strengths of Mutant 5S RNAs. Each mutant was constructed by synthesizing the appropriate oligonucleotides (for both strands) and substituting them for the corresponding wild-type oligonucleotides in a shotgun ligation reaction (Romaniuk et al., 1987b). The 5S RNAs were obtained by transcribing *Dra*I-digested plasmid DNA with T7 RNA polymerase in vitro. The mutants that were constructed are indicated on the secondary structure model of *Xenopus* oocyte 5S RNA (Figure 2). Excluding the 53–56 region of the oocyte 5S RNA, which has been

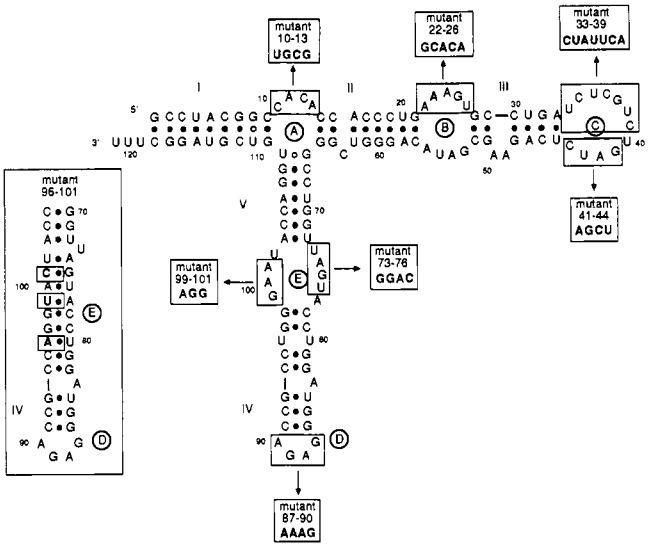

FIGURE 2: Eight mutant 5S RNA molecules used in the present study.

examined in a previous study (Romaniuk et al., 1987b), there are 28 conserved nucleotides in single-stranded loops common to the TFIIIA and eukaryotic 5S RNA consensus models. Rather than test each position individually by making point mutations, the relative importance of each loop for TFIIIA binding was determined by making block sequence changes. This type of sequence replacement is similar to linker scanning mutagenesis, with the added advantage that precise sequence replacements can be introduced. Seven of the mutants shown in Figure 2 have sequence substitutions in one loop, which should maintain the single-stranded secondary structure, although effects on tertiary interactions cannot be predicted. The final mutant, 96–101, has introduced three nucleotide substitutions with the intention of increasing the amount of Watson–Crick base pairing in the loop E–helix IV region of the 5S RNA in order to test whether the unusual conformation in this region of the molecule (Romaniuk et al., 1988) is required for the binding of TFIIIA.

The ability of each mutant 5S RNA to bind TFIIIA was measured using a nitrocellulose filter binding assay, in which a constant concentration of labeled 5S RNA was titrated with increasing concentrations of highly purified TFIIIA (Figure 3). Of the mutants tested, several had binding constants identical with that measured for wild-type oocyte 5S RNA,

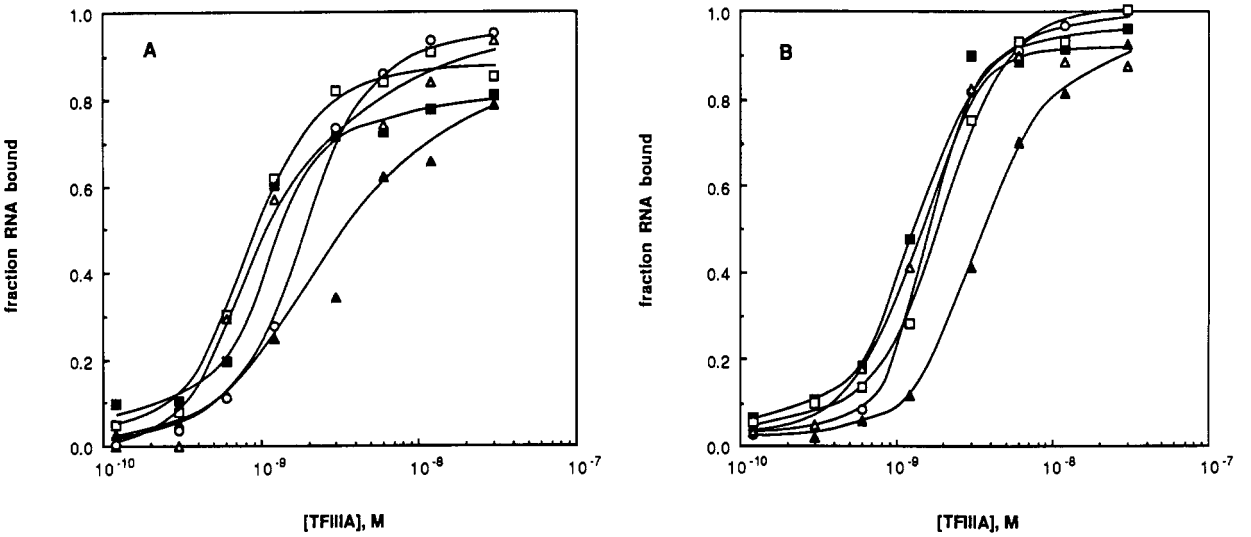

FIGURE 3: Determination of the association constants for each mutant 5S RNA with TFIIIA by the nitrocellulose filter binding assay. (A) (■) Wild-type 5S RNA; (△) 22–26 mutant; (□) 33–39 mutant; (▲) 41–44 mutant; (○) 73–76 mutant. (B) (■) Wild-type 5S RNA; (▲) 10–13 mutant; (△) 87–90 mutant; (○) 99–101 mutant; (□) 96–101 mutant.

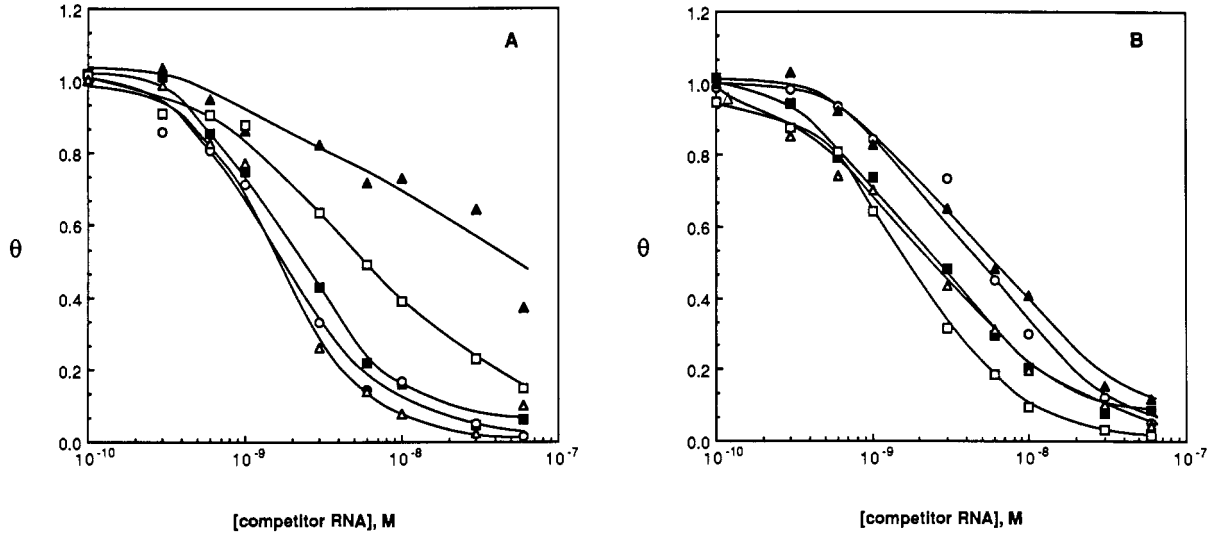

FIGURE 4: Determination of the competition strength of each mutant 5S RNA. θ is the ratio of RNA bound in the presence and absence of competitor RNA. (A) (■) Wild-type 5S RNA; (▲) 10–13 mutant; (○) 22–26 mutant; (□) 73–76 mutant; (△) 87–90 mutant. (B) (■) Wild-type 5S RNA; (□) 33–39 mutant; (○) 41–44 mutant; (△) 99–101 mutant; (▲) 96–101 mutant.

while others had slightly decreased K_a values (Table I). Only replacement of the sequences in loop A (nucleotides 10–13) and loop C (nucleotides 41–44) resulted in significantly larger decreases in the K_a, the effect being largest for the loop A mutant.

A competition assay was used to investigate further the effect that the substitution of single-stranded nucleotides in the mutant 5S RNAs had on TFIIIA binding (Figure 4). Although competition strengths will not necessarily be equal to the ratio of association constants, seven of the mutants have competition strengths that are similar to their relative binding affinities and generally within a factor of 2–3 compared to the wild-type 5S RNA (Table I). Only the loop A mutant has a dramatically decreased competition strength, being ca. 20-fold less effective in competing for TFIIIA binding compared to the wild-type 5S RNA.

Mobility of Mutant 5S RNA–TFIIIA Complexes on Native Gels. The gel mobility shift properties of the complexes formed between TFIIIA and each mutant 5S RNA were measured to determine whether any of the mutant complexes had conformational properties different from those of the wild-type 7S RNP particle. As the autoradiogram in Figure 5 shows, all of the mutant 5S RNAs are capable of forming complexes with TFIIIA that have mobility shifts identical with the wild-type complex. It is evident that the differences in K_a

Table I: Relative Binding and Competition Strengths of the Mutant 5S RNAs

RNA	binding strength[a]	competition strength[b]
wild type	1.00	1.00
10–13	0.30 ± 0.01	0.06 ± 0.01
22–26	1.00 ± 0.02	1.24 ± 0.04
33–39	1.00 ± 0.02	1.75 ± 0.13
41–44	0.40 ± 0.10	0.52 ± 0.05
73–76	0.57 ± 0.02	0.38 ± 0.05
87–90	0.71 ± 0.10	1.45 ± 0.05
99–101	0.61 ± 0.08	0.85 ± 0.20
96–101	0.59 ± 0.01	0.52 ± 0.05
$tRNA^{phe}$	<0.02 ± 0.01[c]	<0.01 ± 0.01[c]

[a] Expressed as the ratio of the apparent association constant (K_a) measured for the mutant divided by the K_a measured for wild-type 5S RNA. The numbers represent the mean value of a minimum of two independent determinations. [b] Expressed as the ratio of mutant and wild-type RNA concentrations required to provide a 50% competition value. The numbers represent the mean value of a minimum of two independent determinations. [c] Data taken from Romaniuk (1985).

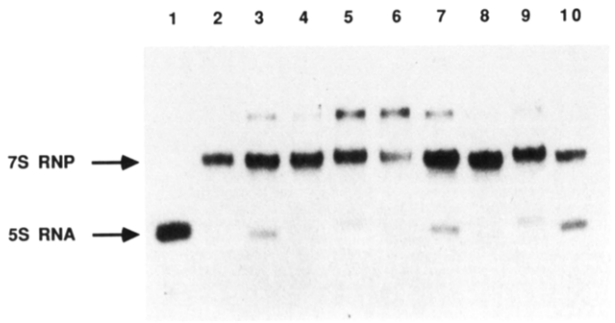

FIGURE 5: Gel mobility shift assay of the complexes formed between TFIIIA and the mutant 5S RNAs. (Lane 1) 5S RNA marker; (lanes 2–10) complexes formed between various 5S RNAs and TFIIIA, (lane 2) wild-type 5S RNA, (lane 3) mutant 10–13, (lane 4) mutant 22–26, (lane 5) mutant 33–39, (lane 6) mutant 41–44, (lane 7) mutant 73–76, (lane 8) mutant 87–90, (lane 9) mutant 99–101, (lane 10) mutant 96–101.

values and competition strengths measured for the mutant 5S RNAs do not reflect gross conformational changes in the complexes formed with TFIIIA.

Discussion

Most Highly Conserved Single-Stranded Nucleotides of 5S RNA Are Not Essential for TFIIIA Binding. TFIIIA is capable of binding with roughly equal affinity to a variety of eukaryotic 5S RNAs (Pieler et al., 1984; Hanas et al., 1984; Romaniuk, 1985; Andersen & Delihas, 1986). In addition to a common three-dimensional structure, the most highly conserved sequence elements among eukaryotic 5S RNAs occur in the single-stranded loops. I constructed seven "block" mutants of *Xenopus* 5S RNA to test the relative importance of 28 highly conserved single-stranded nucleotides for TFIIIA binding. On the basis of the association constants and competition strengths measured for these mutants, it is apparent that 25 of the 28 highly conserved nucleotides do not make a major contribution to the free energy of TFIIIA binding. With the exception of the loop A mutant, substitution of conserved nucleotides in the other loops resulted in a decrease in the association constant for TFIIIA binding by a factor of 2 or less, representing the net loss of less than one hydrogen-bonding interaction in each case. The implication of these results for the interpretation of chemical protection data for 7S RNP is discussed below.

The eighth mutant tested for TFIIIA binding was designed to alter the local structure of loop E. The conformation of this region in the 5S RNA has been the subject of considerable speculation: although the potential for base pairing within loop E is limited in most 5S RNAs, this region is completely base paired in the 5S RNA from *Sulfolobus acidocaldarius* (Stahl et al., 1981) and is resistant to cleavage by single strand specific nucleases in most 5S RNAs tested. A detailed study of the solution structures of *Xenopus* oocyte and somatic 5S RNAs has lead to the proposal, based upon chemical reactivity data, of an extended, base-paired conformation for loop E formed by noncanonical hydrogen bonding (Romaniuk et al., 1988). Three point mutations were introduced into *Xenopus* 5S RNA to convert loop E to a fully Watson–Crick base paired, A-type RNA double helix. The structural conversion in this mutant also reduced TFIIIA binding affinity by less than 2-fold, suggesting that the protein does not require the wild-type conformation in this region of the molecule in order to bind efficiently to *Xenopus* 5S RNA.

Another assay that can be used to investigate the complexes formed between TFIIIA and 5S RNA is a mobility shift assay on a nondenaturing polyacrylamide gel (Romaniuk, 1985; Andersen & Delihas, 1986; Sands & Bogenhagen, 1987). The results obtained are qualitative rather than quantitative: in order to observe a mobility shift, it is necessary to increase the TFIIIA concentration in the assay 3 orders of magnitude above the apparent dissociation constant. However, this assay is capable of detecting differences in the stoichiometry or conformation of heterologous TFIIIA–RNA complexes compared to 7S RNP (Romaniuk, 1985). The complexes formed between TFIIIA and the eight mutants had mobility shifts that were identical with that of the wild-type complex, indicating that each block substitution did not significantly alter either the conformation or stoichiometry of the TFIIIA–5S RNA complex.

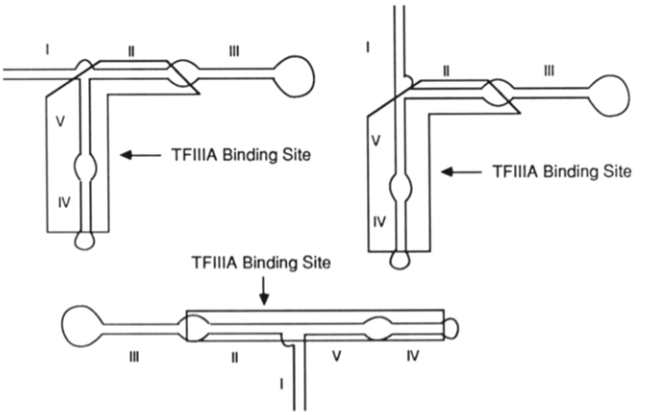

FIGURE 6: Possible coaxial stacking arrangements of *Xenopus* 5S RNA, indicating the position of the TFIIIA binding site on each.

Importance of Loop A for the Binding of TFIIIA to 5S RNA. Of the single-stranded mutants tested for TFIIIA binding activity, only the loop A mutant had a competition strength for TFIIIA that was reduced by over an order of magnitude. This mutant also had the lowest association constant for TFIIIA binding measured by the direct binding assay, although in this case the reduction observed was 3.3-fold compared to the wild-type 5S RNA. I have demonstrated previously that the competition assay provides a more sensitive indication of the specificity of the TFIIIA–RNA interaction (Romaniuk, 1985) and therefore conclude that the sequence of nucleotides 10–13 is essential for the binding of TFIIIA to *Xenopus* 5S RNA. The normal interpretation of such a result would be that the reduced binding affinity results from the loss of protein–RNA bonding interactions upon nucleotide substitution. However, this conclusion contradicts the data from numerous footprinting experiments conducted on the TFIIIA–5S RNA complex: by use of both chemical and enzymatic reagents, protection of loop A from modification has never been observed (Pieler & Erdmann, 1983; Andersen et al., 1984; Romaniuk, 1985; Huber & Wool, 1986; Christiansen et al., 1987). An alternative explanation that rationalizes both results would be that the nucleotides of loop A are critical to the 5S RNA conformation, and substitution in this region of the molecule has structural consequences that lead to the reduced affinity for TFIIIA. As pointed out by Christiansen et al., it has long been thought that loop A forms a hinge that controls the coaxial stacking of the three helical domains of the 5S RNA via the formation of noncanonical hydrogen bonding between one or more nucleotides in loop A with the highly conserved G at position 66. There are three possible coaxial stacking arrangements, one of which would provide a collinear arrangement of the TFIIIA binding site on 5S RNA by stacking the helix IV–V domain on the helix II–III domain (Figure 6). Perhaps the substitution of the nucleotides in loop A reduces the stability of this coaxial

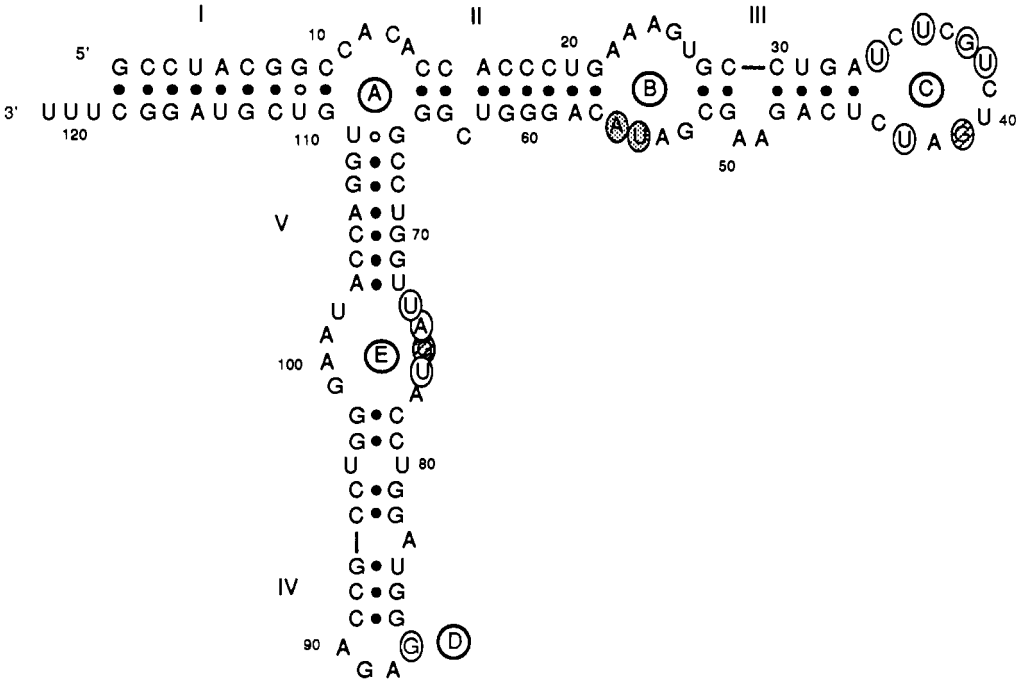

FIGURE 7: Comparison of chemical modification data (Christiansen et al., 1987) with the binding data for the mutant 5S RNAs. Open symbols indicate nucleotides where substitution in a mutant has altered the hydrogen-bonding nature of the chemically protected functional group, without significantly decreasing TFIIIA binding. Symbols with diagonal lines indicate nucleotides where substitution in the mutant has not altered the hydrogen-bonding nature of the chemically protected functional group. Gray symbols indicate nucleotides where substitution in the mutant has altered the hydrogen bonding of the chemically protected functional group and resulted in a significant (>2-fold) reduction in TFIIIA binding affinity.

stacking arrangement compared to the other two, to the detriment of the interaction with TFIIIA. The results from the gel mobility shift assay indicated that substitution of the loop A nucleotides does not lead to gross conformational changes either in the free 5S RNA or in the TFIIIA–5S RNA complex. Additional studies will be required to determine exactly how the loop A nucleotides affect both the structure of *Xenopus* 5S RNA and its interaction with TFIIIA.

Although competition strengths and relative binding strengths are not directly comparable, the large discrepancy between these two values for the loop A mutant (Table I) may result from a fundamental difference between the two assays. TFIIIA binds to 5S RNA with an extremely rapid on-rate, and the resulting complex dissociates very slowly in comparison (Romaniuk, 1985). If a mutation like 10–13 disrupts the conformational equilibria of the 5S RNA, resulting in a lower concentration of the conformer to which TFIIIA preferentially binds, the on-rate for complex formation will similarly decrease. Since the resulting TFIIIA–5S RNA complex has a slow dissociation rate and TFIIIA is always present in great excess to free 5S RNA, the binding of TFIIIA will effectively pull the conformational equilibria of the mutant 5S RNA toward this less stable conformation because it is the preferred binding structure for the protein. Even when complexes dissociate, the resulting free 5S RNA may be rapidly bound by another TFIIIA molecule before it undergoes conformational rearrangement. Therefore, for conformational mutants that still interconvert between the forms shown in Figure 6, the direct binding assay will yield an apparent association constant that does not reflect simply the binding of the mutant conformation to TFIIIA but rather results from the effects that this mutation and TFIIIA binding have on the equilibrium concentrations of the different solution conformers for the 5S RNA. For mutants of this type, the competition assay provides a different measurement of the effects such mutations have on TFIIIA binding. In this assay, the decreased concentration of the preferred binding conformer in the mutant must compete with a high concentration of wheat germ 5S RNA with the correct conformation for binding to a fixed amount of TFIIIA (in slight excess over the concentration of wheat germ 5S RNA). Under these conditions, the amount of free TFIIIA remaining after the wheat germ 5S RNA has bound will be insufficient to affect the conformational equilibria of the mutant. Therefore, much higher input concentrations of the mutant 5S RNA are required to provide a concentration of the preferred binding conformer that can compete effectively with wheat germ 5S RNA for the binding of TFIIIA. Because TFIIIA cannot influence the 5S RNA conformational equilibria in the competition assay, the competition strength measured for the 10–13 mutant must reflect the relative equilibrium concentrations of the preferred binding conformer for the mutant vs the oocyte wild-type 5S RNA.

Comparison of the Binding Data with Chemical Protection Data. A number of nucleotide functional groups in *Xenopus* 5S RNA are protected from chemical modification when TFIIIA is bound (Christiansen et al., 1987). However, it is unclear whether protection from chemical modification results from the formation of a direct RNA–protein bond or from steric exclusion. In many of the mutants used in the present study, the hydrogen-bonding capabilities in the single-stranded loops are altered as a result of nucleotide substitution. Therefore, a comparison of the mutant binding data and the chemical modification data can identify potential sites of direct protein–RNA interactions.

Figure 7 shows the results obtained by comparing the binding data for the single-stranded mutants, the data from a previous study (Romaniuk et al., 1987b) on the somatic-specific nucleotide substitutions in loop B, and the chemical modification data (Christiansen et al., 1987). For example, in the case of nucleotides 33–39 of loop C, TFIIIA was found to protect the N3 of U_{33} and the N1 and N2 of G_{37} and to weakly protect the N3 of U_{38} from chemical modification when

the protein was bound to the 5S RNA (Christiansen et al., 1987). In mutant 33–39, substitution of U_{33} by a C, U_{35} by an A, G_{37} by a U, and U_{38} by a C substantially alters the hydrogen-bonding possibilities at each of these positions, but has no effect on the ability of the 5S RNA to bind to TFIIIA. Substitution of nucleotides 41–44 of loop C does decrease the binding affinity of the RNA by about 2-fold, but few protection effects were observed in this region of loop C. A potential weak protein–RNA interaction at the N7 position of G_{41} (Christiansen et al., 1987) would be maintained in the mutant, where an A has been substituted. However, a simiiar interaction at the N3 position of U_{43} (Christiansen et al., 1987) would presumably be disrupted by the C substitution in the mutant, which would convert the N3 position from a hydrogen-bond donor to a hydrogen-bond acceptor.

The oocyte 5S RNA differs from the consensus eukaryotic 5S RNA sequence at positions 53, 55, and 56 in the 3′ half of loop B. These nucleotides form part of the 5′ interaction site proposed by Christiansen et al. Substitution of these positions with the somatic-specific nucleotides, which restores the sequence to the eukaryotic consensus, enhances the affinity of the 5S RNA for TFIIIA by approximately 3-fold (Romaniuk et al., 1987b). The somatic-specific substitutions in loop B would disrupt the potential protein–RNA contacts at positions 55 and 56 suggested from the chemical modification studies. Therefore, substitution of U_{55} and/or A_{56} may provide new RNA–protein contacts that result in the enhanced binding of TFIIIA to the somatic 5S RNA (Romaniuk et al., 1987b). Point mutational analysis will be required to determine the exact effect of nucleotide substitution at these two positions.

When TFIIIA is bound to the 5S RNA, there are strong protection effects observed for nucleotides 72–76 of loop E, and this region was proposed to be the 3′ element involved in the binding of the protein to 5S RNA (Christiansen et al., 1987). As indicated above, loop E may adopt an extended, hydrogen-bonded structure composed entirely of noncanonical base pairs (Romaniuk et al., 1988). Such an extended structure may have evolved in the 5S RNA as an efficient binding site for proteins. Mutants 73–76 and 99–101 replace highly conserved sequences on either side of loop E, and mutant 96–101 incorporates three point mutants that keep the highly protected 72–76 nucleotide sequence intact but convert the conformation to an A′ RNA helix (see Figure 2). For each mutant, the nucleotide substitutions resulted in only a slight decrease in the TFIIIA binding affinity of between 1.5- and 2.5-fold. Therefore, neither the highly conserved sequences on either side of loop E nor the special conformational properties of this region are essential for the binding of TFIIIA to *Xenopus* 5S RNA. Of the many protein–RNA bonds that might be inferred from the chemical modification data on loop E, only the potential N7 interaction at position 75 would be maintained in these three mutants.

Comparison with a Study of Linker Scanning Mutants of X. borealis 5S RNA. Recently, the binding of TFIIIA to linker scanning mutants of *X. borealis* 5S RNA was determined by using a qualitative gel shift assay (Sands & Bogenhagen, 1987). The position and large size of the nucleotide substitutions in these mutants will in most cases result in the disruption of the 5S RNA secondary structure, which must be maintained for the efficient binding of TFIIIA (Romaniuk et al., 1987b). However, a comparison of the results obtained with some of the linker scanning mutants and those reported here with the defined block mutants may help identify some secondary structure effects. For example, the 67/78 linker scanning mutant, which covers the 5′ sides of helix V and loop E, is defective for TFIIIA binding (Sands & Bogenhagen, 1987). The fact that the 73–76 block mutant has a small effect on TFIIIA binding (Table I) suggests that it is disruption of the base pairing in helix V, and not replacement of conserved nucleotides in loop E, that is responsible for the decrease in TFIIIA binding observed with the 67/78 linker scanning mutant. A similar conclusion is reached by comparing the 92/105 linker scanning mutant (defective for TFIIIA binding) and the 99–101 loop E block mutant (retains TFIIIA binding). One area where the results of the two studies appear to disagree concerns the effects of mutations in loop A. Substitution of nucleotides 10–13 in *X. laevis* 5S RNA has a significant effect on TFIIIA binding and a large effect on the competition strength of the 5S RNA (Table I). The substitution of nucleotides 8–15 of *X. borealis* 5S RNA by linker scanning mutagenesis has little effect on the binding of TFIIIA (Sands & Bogenhagen, 1987). However, the 8/15 linker scanning mutant not only substitutes nucleotides in loop A but also disrupts the two base pairs that close the loop, which perhaps restores conformational flexibility to the 5S RNA. In addition, different nucleotide substitutions are incorporated into loop A of each mutant: the wild-type *X. borealis* sequence CAUA was converted to AUCC in the linker scanning mutant, while the *X. laevis* wild-type sequence CACA was converted to UGCG in the 10–13 block mutant (Figure 2).

Comparison with Other RNA–Protein Interactions. The results of studying the TFIIIA binding properties of a number of mutants of *Xenopus* 5S RNA have demonstrated that, with the exception of the somatic-specific nucleotide substitutions in loop B (Romaniuk et al., 1987b) and the nucleotides of loop A (this study), the highly conserved nucleotides in the single-stranded loops of the 5S RNA are not major contact points for the binding of TFIIIA. How does this compare with the role of single-stranded nucleotides in other RNA–protein interactions? The most comprehensively studied system is the interaction of R17 coat protein with its translational operator at the initiation site of the replicase gene. The binding site for the coat protein consists of a 21 nucleotide long hairpin loop (Carey et al., 1983a), and the sequence specificity of coat protein binding has been investigated by using over 100 sequence variants of the 21-mer (Carey et al., 1983b; Uhlenbeck et al., 1983; Romaniuk et al., 1987a; Wu & Uhlenbeck, 1987). From these studies, it is apparent that only four nucleotides are essential for the formation of specific RNA–protein interactions and that these nucleotides are found in single-stranded regions of the 21-mer: three in the hairpin loop and a single bulged A nucleotide. There are no sequence requirements for the nucleotides that form the helical stem, other than the restriction that the base-paired structure be maintained in such a way that the single-stranded nucleotides are folded into the correct conformation. There is also evidence from nucleotide inhibition studies (Romaniuk & Uhlenbeck, 1985) and a U → C substitution in the hairpin loop (Lowary & Uhlenbeck, 1987) that a transient covalent bond may form between R17 coat protein and a single-stranded U residue on the RNA. In this RNA–protein interaction, single-stranded nucleotides are the critical elements involved in the formation of bonding contacts between the RNA and protein.

Studies of other RNA–protein interactions also suggest that the formation of specific contacts at single-stranded nucleotides may be a common feature of such interactions. Mutational analysis of the translational operator of *E. coli* ribosomal protein L1 indicates that specific contacts between the protein and RNA involve single-stranded nucleotides (Thomas & Nomura, 1987). Similar studies on the 16S rRNA binding

site for ribosomal protein S8 also indicate a requirement not only for a highly conserved loop but also for the sequence of nucleotides within this loop (Gregory & Zimmerman, 1986). Structural studies on a relevant fragment of the 16S rRNA indicate that the loop in the S8 binding site may adopt an unwound helical structure in which three essential adenines are looped out for interaction with the protein (Mougel et al., 1987).

The interaction of TFIIIA with 5S RNA appears to differ from the above RNA–protein interactions in terms of the strength of individual bonding interactions. Substitution of highly conserved nucleotides of 5S RNA has little effect on the binding affinity, the largest effect observed being a reduction in competition strength of 1 order of magnitude when nucleotides are substituted in loop A, which lies outside the putative binding site of TFIIIA. In contrast, the essential nucleotides in the R17 binding site were identified by the observation that substitution of any one of these nucleotides resulted in a reduction of the binding affinity for coat protein of greater than 2 orders of magnitude [e.g., Romaniuk et al. (1987a)]. The data presented here suggest that TFIIIA may achieve the necessary specificity in binding to RNA by recognizing the unique tertiary structure of 5S RNA and forming a number of relatively weak sequence-specific contacts with the nucleotides in single-stranded loops. This apparent lack of strong sequence-specific contacts between the protein and single-stranded nucleotides and the fact that TFIIIA also interacts specifically with DNA suggest that TFIIIA may form specific bonds with base pairs in the 5S RNA. Further study with mutant 5S RNAs should contribute to a better understanding of how TFIIIA specifically binds to 5S RNA.

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