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A difference in the importance of bulged nucleotides and their parent base pairs in the binding of transcription factor IIIA to *Xenopus* 5S RNA and 5S RNA genes

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ABSTRACT

Individual bulge loops present in *Xenopus* 5S RNA (positions 49A-A50 in helix III, C63 in helix II and A83 in helix IV), were deleted by site directed mutagenesis. The interaction of these mutant 5S RNA molecules with TFIIIA was measured by a direct binding assay and a competition assay. The results of these experiments show that none of the bulged nucleotides in *Xenopus* 5S RNA are required for the binding of TFIIIA. The affinity of the mutant 5S RNA genes for TFIIIA was also studied by a filter binding assay. In contrast to the effect that deleting bulged nucleotides had on the TFIIIA-RNA binding affinity, deletion of the corresponding A-T base pair at position +83 in 5S DNA was found to reduce the apparent association constant of TFIIIA by a factor of four-fold.

INTRODUCTION

In immature oocytes of *Xenopus laevis*, TFIIIA carries out two essential functions. It promotes the accurate transcription of 5S RNA genes by binding to an internal control region of these genes (1-4) and subsequently binds to the resulting 5S RNA transcripts to form a 7S ribonucleoprotein storage particle (7S RNP). This 7S RNP stabilizes 5S RNA in the cytoplasm of immature oocytes until it is required for ribosome assembly. Therefore TFIIIA has the unusual ability to interact specifically with two different nucleic acids, a property which has come under extensive scrutiny.

Analysis of the primary sequence of TFIIIA has revealed the presence of 9 tandem repeats of ca. 30 amino acids (5,6), folded in a loop or finger of ca. 12 amino acids. Each domain contains two invariant pairs of histidines and cysteine residues, coordinated in a tetrahedral arrangement to a zinc ion. This "zinc finger" structure appears to be a common motif in the putative nucleic acid binding sites of a variety of eukaryotic regulatory proteins (7-9). It has been suggested that the finger structure may facilitate the interaction of some of these proteins with RNA (7).

Chemical and nuclease probes were used to determine that the binding region of TFIIIA on 5S RNA encompasses the helix II/loop B and helix V/loop E/helix IV domains (10-14). A number of studies showed that a variety of eukaryotic 5S RNAs bind to TFIIIA with similar affinities (12, 15-17). Thermodynamic and kinetic parameters have been measured for the TFIIIA-5S RNA interaction using a nitrocellulose filter binding assay (12). TFIIIA was found to bind *Xenopus*

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laevis oocyte 5S RNA with a dissociation constant (K_d) of 1 nM. 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 (17,18). 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 (18). A recent study has shown that there are no strong contacts formed between TFIIIA and any of the highly conserved nucleotides in single stranded loops lying within the TFIIIA binding region, although nucleotides in loop A are essential for binding of the protein (19).

More detailed information is available about the binding of TFIIIA to the internal control region of the 5S RNA gene. DNase footprinting experiments placed the TFIIIA binding region as extending from about nucleotide 45 to nucleotide 96 (4). Subsequently, transcription studies with deletion mutants of the gene showed that the essential binding site includes nucleotides 50-83 (1-3). Studies with linker scanning mutants and point mutants have lead to a proposal of an extended two box model for the internal control region (20-23), with nucleotides 80-86 being particularly important for the binding of TFIIIA.

Protein-DNA interactions classically involve the recognition of nucleotide sequences in the major groove of a B-DNA double helix (24). In contrast, the nucleotide functional groups in the major groove of the A-type double helical stems found in RNA are essentially inaccessible to proteins. The evidence from studies of several RNA-protein complexes suggests that proteins bind to RNA *via* interaction with single stranded nucleotides, irregularities in double helical stems and features of tertiary structure (19, 25-29). A common irregularity found in an RNA double helix is a bulged nucleotide, which has been proposed to be a special nucleation site for the formation of specific RNA-protein complexes (26). In the present study, three deletion mutants have been constructed: deletions of bulges ${}_{49}A$-A_{50} (ΔA49,50), C_{63} (ΔC63) and A_{83} (ΔA83) in *Xenopus* 5S RNA have been constructed by deletion of the corresponding base pairs in the 5S DNA. The consequence of these mutations on the binding of both DNA and RNA to TFIIIA has been tested by a direct binding assay (both RNA and DNA) and a competition assay (RNA only). All of the 5S RNA mutants bind TFIIIA with the same affinity as the wild type, indicating that bulged nucleotides are not nucleation points for the binding of TFIIIA to 5S RNA. However, the ΔA83 DNA mutant decreases the binding of TFIIIA by four fold compared to the wild type. This result suggests that TFIIIA has different modes of binding to 5S DNA and RNA, rather than recognizing a common structure in both nucleic acids.

MATERIALS AND METHODS

Construction of mutants

The wild type *Xenopus* oocyte 5S RNA gene, under the control of a promoter for T7 RNA polymerase, was originally constructed in the plasmid pUC18 (18). To facilitate the site-directed

mutagenesis experiments, the 5S RNA gene was inserted into M13mp18 by the same method. Mutations in the 5S RNA gene were introduced using a protocol from Amersham based upon a modified single primer method developed to produce high yields of mutants, as described by Eckstein et al. (30-32). Oligonucleotides used to produce the mutants were: TGTATCGCCTGAGATCA (ΔA49,50); CCAGGCCCACCCTGTAT (ΔC63); GGTCTCCCACCAGGTAC (ΔA83). For each mutant, phage were grown from twenty plaques randomly chosen from the resulting transformation, and then screened by dot blot hybridization with the mutant oligonucleotide under stringent conditions. Each mutant was verified by dideoxynucleotide sequencing of the double stranded phage DNA, as described previously (18).

Preparation of TFIIIA

The 7S RNP particle was isolated from ovaries of immature *Xenopus laevis* (Xenopus I, Ann Arbor, MI) by a standard procedure (33,34). Pure TFIIIA was obtained from the 7S particle by ammonium sulfate precipitation as described in (35). The protein pellet was resuspended in a buffer containing 50 mM Hepes KOH pH 7.5, 5 mM $MgCl_2$, 500 mM KCl, 1 mM DTT, 2 mM benzamidine and 20 % glycerol (v/v) and stored at 4°C. Protein concentrations were determined by the Bradford method (36). Fractional 5S RNA binding 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 X 10^9 M^{-1} determined by Scatchard analysis to be the apparent K_a obtained with 100% active TFIIIA and *Xenopus* oocyte 5S RNA (12). Only those preparations which were >90% active were used to study the binding of mutant 5S RNAs.

Transcription of mutant 5S RNA genes

In vitro transcription was carried out using T7 RNA polymerase purified by a published procedure (37) from *E. coli* strain BL21/pAR1219, kindly provided by Dr. F.W. Studier. Prior to transcription, each double stranded M13 phage DNA 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. Digested DNA samples were used as templates to produce internally labeled 5S RNAs for the direct binding assays, and unlabeled 5S RNAs for the competition assays, by protocols described in detail elsewhere (18,19).

Isolation and end labeling of 5S DNA

A 177 bp 5S RNA gene fragment can be excised from the M13mp18 constructs by restriction digest with Eco RI and Hind III. DNA (100 µg) was digested with 100 units each of Hind III and Eco RI for 2h at 37°C in a buffer containing 20mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 50mM KCl. After extraction with 100 µl of phenol-chloroform (1:1, v/v), the DNA was recovered from the aqueous phase by ethanol precipitation. The DNA was then subjected to 5' dephosphorylation with 2 units of calf intestinal phosphatase (30 min at 37°C in 50mM Tris-HCl pH 8, 0.1 mM EDTA), and recovered by ethanol precipitation. The 177 bp gene fragments were purified on a 10% non-denaturing polyacrylamide gel. The fragment corresponding to the 5S RNA genes were

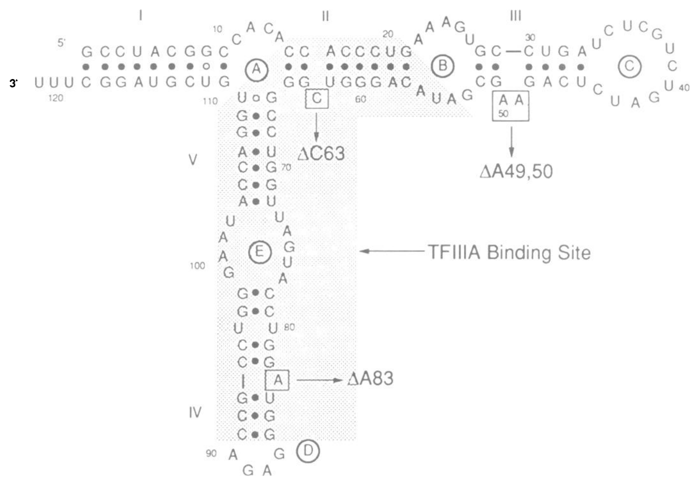

Figure 1. The secondary structure of *Xenopus* oocyte 5S RNA, showing the deletion mutants constructed for this study and indicating the TFIIIA binding site.

excised and eluted from the gel slice overnight at room temperature in 0.6 M ammonium acetate, 1mM EDTA pH 7.5. The DNA was recovered from the eluant by ethanol precipitation, dissolved in TE buffer and stored frozen at -20 °C. For filter binding studies, the 5S DNA fragments were labeled at the 5' ends using [γ–^{32}P] ATP and T4 polynucleotide kinase (38) and purified on a 8% non denaturing polyacrylamide gel. Two Bst NI restriction fragments from pUC18 were prepared for binding studies by the same method.

Filter binding assay

The ability of each mutant 5S RNA to bind TFIIIA was determined by a nitrocellulose filter binding assay (12), in which a constant concentration of labeled 5S RNA was titrated with increasing concentrations (0.12nM to 30nM) of highly purified protein. The same assay was used to measure the apparent K_a values for the binding of 5S DNA fragments to TFIIIA, by including 5 µg/ml of unlabeled poly d[I-C] in the binding buffer. Under these conditions, TFIIIA exhibits a specificity ratio of >100 for binding to DNA fragments containing the oocyte 5S RNA gene as opposed to binding to unrelated restriction fragments containing vector sequences (see Figure 5). The use of this assay for characterizing the DNA binding properties of TFIIIA will be discussed elsewhere (P.J. Romaniuk, in preparation).

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 (ca. 10 000 cpm) and the competitor 5S RNA concentrations were varied between 0.1-60 nM (18).

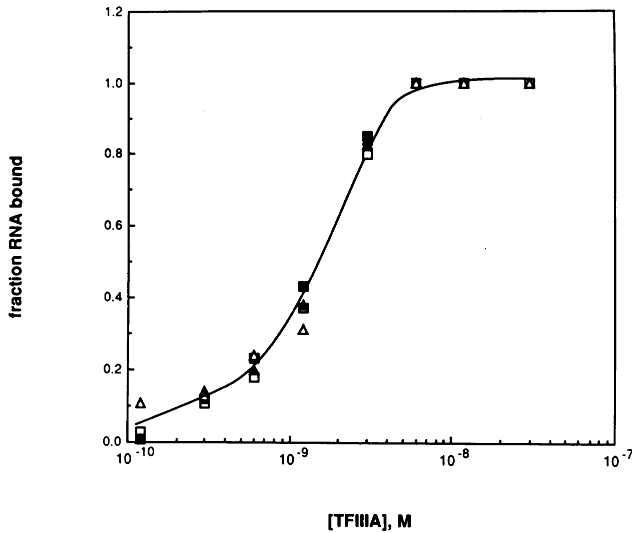

Figure 2. Determination of the apparent association constants for the binding of TFIIIA to wild type and mutant 5S RNAs: wild type (open squares), ΔA49,50 (closed squares), ΔC63 (open triangles), ΔA83 (closed triangles).

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 RNasin and 1 mM DTT. After incubation for 10 min at 4 °C, 1 μl of glycerol-dye buffer was added, the samples were loaded onto a 10% non-denaturing polyacrylamide mini gel (50 mM Tris-borate pH 8.3 buffer), 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.

The 5S RNA deletion mutants that were constructed are displayed on the secondary structure model shown in Figure 1. Two of the mutations (ΔC63, ΔA83) lie within the TFIIIA "footprint", while the third (ΔA49,50) is found outside this region. Bulged nucleotides were chosen for mutagenesis, because it has long been thought that these sites "nucleate" the interaction between proteins and RNA (26), and there was considerable evidence from modification/protection studies (10-14) and mutagenesis experiments(18,45) to suggest that both C_{63} and A_{83} were situated in regions of the 5S RNA particularly important for the binding of TFIIIA (see discussion section). Bulged nucleotides may affect the binding of proteins both by the formation of direct bonds

TABLE 1. Relative Binding and Competition Strengths of Mutant 5S RNAs and DNAs.

	RNA		DNA
mutant	Binding[a]	Competition[b]	Binding[c]
wild type	1.00	1.00	1.00
ΔA49,50	1.00±0.02	1.00±0.03	1.00±0.02
ΔC63	1.00±0.02	1.00±0.02	1.00±0.02
ΔA83	1.00±0.05	1.00±0.02	0.25±0.05

[a]ratio of K_a(mutant 5S RNA)/K_a(wild type 5S RNA). Average of two or more determinations.
[b]ratio of concentrations of mutant 5S RNA and wild type 5S RNA giving 50% competitive inhibition. Average of two or more determinations.
[c]ratio of K_a(mutant 5S DNA)/K_a(wild type 5S DNA). Average of two or more determinations.

between an accessible nucleotide and protein side groups, and also by virtue of providing a structural irregularity in a double helical stem (26). Therefore, in order to initially screen for both of these functions in the TFIIIA-5S RNA interaction, we chose to create deletion rather than substitution mutants.

Binding Affinities and Competition Strengths of Mutant 5S RNAs

The ability of each 5S RNA mutant to bind TFIIIA was determined using a nitrocellulose filter binding assay, in which labeled RNA at a constant concentration is titrated with increasing concentrations of pure TFIIIA (Figure 2). All of the mutant 5S RNAs had binding constants that were identical to that measured for the wild type 5S RNA (Table 1). A competition assay was used to investigate further the effects that deleting bulged nucleotides had on TFIIIA binding (Figure 3). This assay has been shown previously to more accurately reflect the effect that conformational changes resulting from mutations have on TFIIIA binding (19). As the values in Table 1 indicate, deletion of each bulged loop has no effect on the competition strength of the 5S RNA compared to *Xenopus* oocyte wild type 5S RNA. These results indicate that any conformational changes which have occurred upon deletion of the bulged nucleotides do not impair TFIIIA binding.

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 4 shows, all of the mutant 5S RNAs are capable of forming complexes with TFIIIA that have

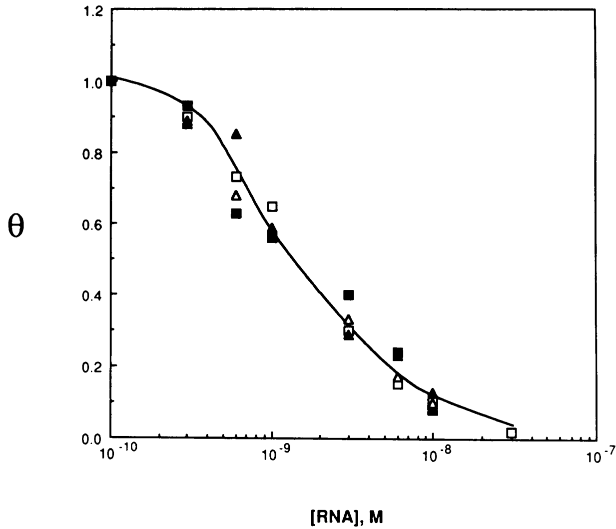

Figure 3. Determination of the competition strengths of wild type and mutant 5S RNAs: wild type (open squares), ΔA49,50 (closed squares), ΔC63 (open triangles), ΔA83 (closed triangles). θ is the ratio of labelled RNA bound in the presence and absence of competitor RNA.

mobility shifts identical to the wild type complex. This result is consistent with the fact that there are no differences in K_a values and competition strengths measured for the wild type and mutant 5S RNAs.

Binding of TFIIIA to 5S DNA Deletion Mutants.

In order to measure the binding specificity of TFIIIA with the 5S DNA mutants, the binding assay used to measure the binding of 5S RNAs was modified by adding 5 μg/ml poly d[I-C] to the

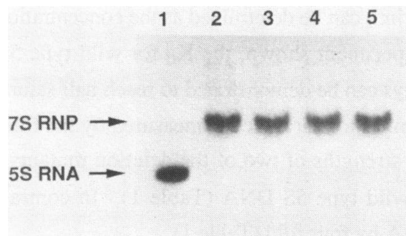

Figure 4. Gel mobility shift assay of the complexes formed between TFIIIA and the mutant 5S RNAs. Lane 1: 5S RNA marker; lanes 2-5: complexes formed between various 5S RNAs and TFIIIA; lane 2: wild type 5S RNA; lane 3: ΔA49,50 mutant; lane 4: ΔC63 mutant; lane 5: ΔA83 mutant.

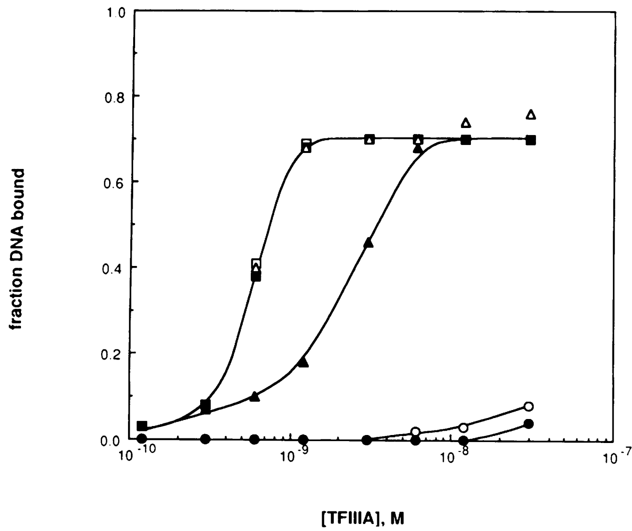

Figure 5. Determination of the apparent association constants for the binding of TFIIIA to wild type and mutant 5S DNAs: wild type (open squares), ΔA49,50 (closed squares), ΔC63 (open triangles), ΔA83 (closed triangles), 191 bp Bst NI fragment of pUC18 (open circles), 288 bp Bst NI fragment of pUC18 (closed circles).

binding buffer. Under the conditions used for the filter binding assay, TFIIIA binds to a 177 bp restriction fragment containing the oocyte wild type 5S RNA gene with high affinity, but has virtually no affinity for 191 bp and 288 bp restriction fragments from pUC18 (Figure 5). Unlike the RNA binding curves, the DNA binding curves reach saturation at 70% of input DNA bound (compare Figures 2 and 5). However, it has been demonstrated that for filter binding assays, it can be assumed that the percent of DNA bound at the plateau is representative of the complete binding of active DNA molecules to the protein (46,47), and that the dissociation constant (K_d) for a simple bimolecular equilibrium can be determined as the concentration of TFIIIA required to give 50% saturation. For the experiment shown, the K_d for wild type 5S DNA is 0.56 nM. Semi-quantitative footprinting assays can be demonstrated to reach half saturation at approximately 1 nM TFIIIA (23), a value very similar to that which is measured by the filter binding assay used in this study. The relative binding strengths of two of the deletion mutants (ΔA49,50 and ΔC63) were indistinguishable from the wild type 5S DNA (Table 1). In contrast, deletion of base pair 83 reduced the affinity for TFIIIA by four fold (Table 1).

DISCUSSION

The mutants used in this study are shown on the secondary structure model for *Xenopus* oocyte 5S RNA (Figure 1). The conserved structural features of 5S RNA are believed to be im-

portant for the formation of specific interactions with various 5S RNA-binding proteins (26, 28,29,41). In the general consensus model of eukaryotic 5S RNA structure, the three bulged loops are conserved as a structural element (39) and have a phylogenetically conserved sequence among those eukaryotic 5S RNAs known to bind TFIIIA (12, 15-17). By stacking out of the double helix, bulged nucleotides are expected to offer unique opportunities for the formation of bonding contacts with the amino acid side chains of proteins, and are thus considered to be strategically important for RNA-protein interactions. The first evidence that bulged nucleotides were essential to an RNA-protein interaction came from studies on the binding of ribosomal protein L18 to *E. coli* 5S RNA (26). This damage-selection experiment showed that methylation of the bulged adenosine-66 in helix II significantly reduced the binding of L18. Comparison with putative protein binding sites on other RNAs suggested that bulged nucleotides were a feature common to many RNA-protein interactions (26). Bulged nucleotides may contribute to the stability of an RNA-protein complex either by the formation of specific bonds with the protein, or by providing a conformation in the RNA which facilitates protein binding. In the case of the *E. coli* 5S RNA-L18 interaction, the formation of direct contacts has been proposed between a glutamine residue on the protein, and the N6 position of the bulged A_{66}, and N7 position of the neighboring G67 (28). Studies on the interaction of R17 coat protein with the bulged A in the RNA hairpin target have shown that a combination of direct contacts, and an intercalated conformation, are involved in the binding of the protein at this site (25,40).

Footprinting of TFIIIA on the 5S RNA (Figure 1) indicated that the bulged nucleotides at positions 63 and 83 lay within the putative protein binding region, while the double bulged nucleotides at positions 49 and 50 lay outside of this region (10-14). Studies on the solution conformation of oocyte 5S RNA using a large variety of chemical and enzymatic structure-specific probes (14,41,42) have shown that all three loops are susceptible to nuclease attack, and can be chemically modified at functional groups normally involved in Watson-Crick base pairing. Because of their reactivity towards probes, these nucleotides are not implicated in helix stacking or in long range tertiary interactions and therefore are presumably available for specific interaction with TFIIIA.

The possibility that the bulged A at position 83 is directly involved in TFIIIA binding was strongly suggested by the observations that nuclease cleavage points in the naked 5S RNA at G_{82} and A_{83} were protected when TFIIIA was bound (12,14), and that chemical modification of exocyclic amino group at position 2 of G_{82} and U_{84} N3 was also prevented when the protein was bound (14). Indeed, the only protection from modification in the 5' side of helix IV provided by TFIIIA is clustered specifically at the bulged A_{83} and the two flanking nucleotides (14). In addition, strong protection from modification was observed on the opposite strand for residues C_{94} to G_{98} (14), which form the base pairs immediately adjacent to the bulged nucleotide. Deletion of helix IV significantly reduces the affinity of TFIIIA binding to the RNA (18). From these data it was clear that TFIIIA contacts the 5S RNA in the region immediately adjacent to both

sides of the bulged A_{83}, implying that the bulged conformation may be essential for protein binding. Was there evidence that TFIIIA makes a direct bonding contact with the bulged A? Chemical modification studies had shown that TFIIIA does not protect the bulged A_{83} itself from modification at either N7 or N1 (10,14). However, chemical modification methods do not test every potential interaction site on a nucleotide: for example the exocyclic amino group at the C6 position of the adenine would be an excellent candidate for the formation of a direct TFIIIA-RNA bond, but such an interaction cannot be detected using the available chemical probes. Therefore a deletion mutant of the bulged A was constructed in order to completely test the importance of this bulged nucleotide for the binding of TFIIIA to the 5S RNA.

Evidence that the bulged C at position 63 lies within a region of 5S RNA that is directly involved in TFIIIA binding was provided by the results of both protection studies and mutagenesis. For example, G residues on both sides of C_{63} (G_{61}, G_{64}, G_{66}) were protected from digestion by cobra venom nuclease, and modification by kethoxal, when TFIIIA was bound to the 5S RNA (14). In addition, strong protection effects were observed for the adjacent nucleotides on the opposite strand in positions 17 to 21 (14). Deletion of the opposite strand alters the conformation of the 5S RNA in this region, and decreases the binding constant for TFIIIA by almost 10-fold (18). Substitution mutants in positions 17 to 21 and 57 to 62 that disrupt the base pairs closing the bulged C result in a decreased affinity for TFIIIA binding of 2.5 to 3 fold (45). A double mutant which restores base pairing in this region restores wild type binding activity. These results indicate that the bulged C at position 63 lies within a region essential for TFIIIA binding, and that the bulged conformation may be a critical feature for protein binding. Very few data existed which would indicate whether a direct protein-RNA bond is formed at C_{63}: the only chemical modification point is the N3 position, and this position was not protected from modification in the presence of TFIIIA (14). However, chemical probes are not available to test other potential interaction sites such as the exocyclic amino and keto groups of C_{63} for involvement in TFIIIA binding. Therefore, deletion mutagenesis of the bulged C was used to determine how this nucleotide might be involved in the binding of TFIIIA to 5S RNA.

This evidence combined with the proposal that bulged nucleotides are an essential feature of RNA-protein interactions in general, suggested that at least two of the three bulged loops represented excellent candidates for the formation of specific interactions with TFIIIA. However, deletion of each bulged loop had no effect on the binding affinity of the resulting mutant 5S RNA for TFIIIA. These results clearly show that although the bulged nucleotides at positions 63 and 83 of *Xenopus* 5S RNA are found within regions important for protein binding, they do not contribute either direct bonding contacts or local conformations required for the specific binding of TFIIIA. In contrast, although substitution of the bulged A_{66} of *E. coli* 5S RNA does not affect the binding of protein L18 (29), deletion of this nucleotide does reduce the apparent binding constant for L18 by approximately seven fold (28), suggesting that the bulged nucleotide provides a conformation essential for protein binding. In the case of the R17 coat protein-RNA interaction,

nucleotide substitution of the bulged A reduces the binding affinity for the protein by as much as 1000-fold (25,40). Our results with TFIIIA indicate that a bulged nucleotide within a protein binding site on an RNA is not always essential for the specific binding interaction with a protein. In the case of *Xenopus* 5S RNA, it is more likely that the highly conserved bulged nucleotides play a role in the specific binding of the ribosomal protein rather than the transcription factor. Recently, it has been shown that substitution of highly conserved nucleotides in the single stranded loops of *Xenopus* oocyte 5S RNA also has little effect on the binding affinity of TFIIIA, with the exception of loop A nucleotides, which likely control the co-axial stacking of the helical stems (19). Taken together, these results consistently indicate that TFIIIA does not form strong individual contacts with extrahelical nucleotides of the 5S RNA. The protein either forms most of its strong contacts with base paired nucleotides in the helical stems of the 5S RNA, or it binds to the RNA using a combination of many weak bonding interactions within the three dimensional shape provided by the tertiary structure, giving rise to the observed specificity of the RNA binding activity. A detailed comprehension of the interactions of TFIIIA with 5S RNA will require further study with base pair mutants of the 5S RNA, and elucidation of the tertiary structure of the 5S RNA molecule.

TFIIIA has the unusual ability to bind specifically to both DNA and RNA, and the mechanism by which the protein interacts with the two nucleic acids has been subject to speculation. It has been suggested that TFIIIA is able to bind to both nucleic acids because the protein binding region on the 5S RNA can stack into a DNA-like conformation (14). There is a high degree of overlap in the TFIIIA binding sites on both DNA and RNA, particularly if helix II stacks on helix V (14). In this model, the bulged nucleotides and single stranded loops within the TFIIIA binding site on the 5S RNA are presumed to be highly stacked, resulting in a conformation that resembles a completely helical base paired stem (14). Studies on the solution structure of *Xenopus* oocyte 5S RNA indicate that many of the putative single stranded loop residues are highly stacked, particularly in loop E (42), and the sequence of loop A residues, essential for controlling the co-axial stacking of the 5S RNA helical domains, has been demonstrated also to be essential for TFIIIA binding (19).

The basic premise of the model, that the TFIIIA binding site on the 5S RNA adopts a DNA-like conformation, has not been tested directly. However, the model predicts that TFIIIA should interact in an identical fashion with both DNA and RNA. Although a study has not yet been undertaken to directly compare the DNA and RNA binding activities of TFIIIA under identical conditions, a comparison of some of the data available from numerous studies can be made and suggests that TFIIIA has different modes of interaction with the two nucleic acids. For example, TFIIIA forms only 5 ionic contacts with 5S RNA (12), while it forms 8 essential ionic contacts within the internal control region on the 5S DNA (43). The TFIIIA-5S DNA complex has a half life of approximately 6 min (15), while the TFIIIA-5S RNA complex has a complicated dissociation pathway, with an average half life of approximately 45 min (12). Deletion mutagenesis

has indicated another fundamental difference: within the approximate binding site from nucleotides 50 to 90, the 3' end of this site is more essential for DNA binding (2,44), while the 5' end is more essential for 5S RNA binding (18).

This comparison of some of the known properties of the DNA and RNA binding activities of TFIIIA suggests that the protein does not use a common mechanism to bind specifically to both nucleic acids. Further support for this view comes from the comparison of the binding of TFIIIA to the deletion mutants used in this study. The deletion of each bulged loop from the 5S RNA had absolutely no effect on the affinity of the RNA for TFIIIA. However, the deletion of base pair 83 from the 5S DNA did reduce TFIIIA binding affinity by four fold, while the other two deletions had no effect on the binding of TFIIIA to the 5S DNA. This result is consistent with what is already known about the DNA binding site from the study of point mutants by transcription and template exclusion assays. The 5S gene promoter has two functional domains, as defined by mutagenesis experiments: box A (+51 to +64), responsible primarily for interactions between the DNA and TFIIIC and box C (+80 to +89), with positions +80 to +86 essential for TFIIIA binding (21-23). Based upon this picture of the internal control region, the ΔA49,50 and ΔC63 mutants as expected did not have an effect on TFIIIA binding. In contrast, deletion of position 83 (ΔA83) which lies within the region of box C essential for TFIIIA binding, did in fact reduce the binding affinity, the magnitude of the effect being similar to that observed on transcriptional efficiency when A83 is substituted by point mutants (23).

If TFIIIA does not recognize a conformational feature common to both 5S RNA and 5S DNA, how does this protein encode specific binding activities for both nucleic acids? TFIIIA has nine "zinc fingers", which likely arose *via* gene duplication events (8), but which appear to have evolved independently. It seems possible that while each finger has retained a general nucleic acid binding capability, some fingers may have evolved to interact specifically with the 5S DNA and other fingers may have evolved to interact specifically with the 5S RNA.

CONCLUSION

Bulged nucleotides occurring within protein binding regions on RNA molecules have been regarded as essential elements for nucleating the formation of specific protein-RNA complexes. The results of this study give a clear example of a case where bulged nucleotides within the TFIIIA binding site on *Xenopus* 5S RNA are not essential for the binding of the transcription factor. Therefore the essential nature of bulged nucleotides in protein-RNA interactions cannot be considered a universal feature of such binding events. A comparison of the effects of these deletion mutations on the binding of TFIIIA to 5S DNA and 5S RNA, taken together with data from previous studies, strongly suggests that TFIIIA has different modes of interacting with the two nucleic acids.

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