Binding of TFIIIA to derivatives of 5S RNA containing sequence substitutions or deletions defines a minimal TFIIIA binding site

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ABSTRACT

The repetitive zinc finger domain of transcription factor IIIA binds 5S DNA and 5S RNA with similar affinity. Site directed mutagenesis of the *Xenopus borealis* somatic 5S RNA gene has been used to produce a series of derivatives of 5S RNA containing local sequence substitutions or sequence deletions. Gel mobility shift analyses of the binding of TFIIIA to these altered 5S RNAs revealed that all three of the helical stems of the 5S RNA secondary structure are required for binding. TFIIIA was observed to bind with normal affinity to RNAs lacking 12 nucleotides at either the loop c or loop e/helix V regions of 5S RNA, as well as to a double mutant containing both deletions. The secondary structure of the resulting 96-nucleotide RNA, studied using structure-specific ribonucleases, was found to resemble the central portion of 5S RNA.

INTRODUCTION

Transcription factor TFIIIA contains a nucleic acid binding domain consisting of nine consecutive repeats of a zinc finger motif (1,2). Although zinc finger proteins are generally regarded as DNA binding proteins, TFIIIA is known to bind with high affinity and specificity to 5S RNA as well as to 5S DNA. It has been a matter of some interest to compare the detailed interactions that TFIIIA makes with 5S RNA to those made with 5S DNA.

The nine zinc fingers of TFIIIA protect residues 45 to 95 in the center of the 5S RNA gene (the intragenic control region, or ICR) from attack by nuclease or chemical probes (3–7). Although a pattern of repeated amino acid residues in the finger and linker segments of the zinc fingers of TFIIIA has been observed (2) the nine zinc finger motifs do not contribute equally to the affinity or specificity of DNA binding. Smith et al. (8) showed that a tryptic fragment of TFIIIA estimated to contain only the amino-terminal seven fingers could bind tightly to the distal portion of the ICR to protect gene residues 67 to 95. Vrana et al. (9) studied the DNA binding abilities of a more extensive series of deleted versions of TFIIIA obtained by in vitro transcription and translation of deletion mutants of the TFIIIA

gene. These experiments confirmed the result that progressive carboxy-terminal deletions of TFIIIA resulted in progressively smaller footprints restricted to the distal portion of the ICR. More recently, two groups have shown that a recombinant fragment of TFIIIA containing only the amino-terminal three zinc fingers could bind to residues 77 to 95 of the 5S RNA gene with a several-fold reduction in apparent binding affinity (10, 11). This result and the methylation interference experiments of Sakonju and Brown (5) indicate that the distal end of the ICR may be considered a tight binding site for TFIIIA. Studies using linker scanning (12) and point mutants of the 5S RNA gene (13, 14) have shown that extensive base pair changes are tolerated in the center of the ICR. Interaction of fingers 8 and 9 of TFIIIA with the proximal portion of the ICR and the presence of the carboxyl-terminal non-finger domain are essential for transcription of the 5S RNA gene (8, 9). Thus, clusters of a few zinc fingers may grasp the ends of the ICR and the intermediate element more tightly than other residues in the center of the gene. This model is reinforced by the recent analysis of the cocrystal of the three-finger domain of Zif 268 with a consensus DNA binding site (15).

It is remarkable that TFIIIA binds 5S RNA and 5S DNA with approximately the same affinity. A number of studies have attempted to determine whether TFIIIA binds in a similar fashion to 5S RNA as it does to 5S DNA. This has proven to be a difficult question since it is necessary to consider the folded structure of 5S RNA, which is only defined as a computer generated model at present (16). Pieler et al. (17) and Christiansen et al. (18) have noted that the folded 5S RNA contains helical stems that could stack upon one another to resemble the ICR of the 5S RNA gene. They have suggested that TFIIIA may bind this portion of 5S RNA in much the same manner as it binds 5S DNA. This would require that the amino terminal zinc fingers of TFIIIA should interact with an RNA analogue to the tight binding site in 5S DNA noted above. The simplicity of this 'RNA mimics DNA' model is appealing, despite the obvious differences in structure between duplex DNA and a folded RNA. TFIIIA does bind with essentially the same affinity to 5S RNA as it does to 5S DNA (19). Using the same proteolytic fragments initially employed by Smith et al. (8), Sands and Bogenhagen (20) showed that the

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'20 kDa' tryptic fragment of TFI_{IIA} binds 5S RNA nearly as tightly as intact TFI_{IIA}. However, two lines of experimentation have emphasized the dissimilarity between the binding of TFI_{IIA} to 5S RNA and 5S DNA. First, mutagenesis experiments presented by Sands and Bogenhagen (21) and Romaniuk's laboratory (22–24) have provided several examples of sequence substitutions in the 5S RNA gene and 5S RNA that have differential effects on the binding of TFI_{IIA}. Second, comparison of the 'footprints' observed on 5S RNA with intact and '20 kDa' TFI_{IIA} showed that the most significant differences resulting from removal of the carboxy-terminal portion of TFI_{IIA} resulted from altered cleavages in the tip of helix IV/V of 5S RNA (20).

In this paper we present the results of experiments to study the binding of TFI_{IIA} to mutagenized derivatives of 5S RNA. These experiments do not support the model that '5S RNA mimics 5S DNA' as a ligand for binding TFI_{IIA}. Changes in all three helical stems of 5S RNA affect the binding of TFI_{IIA}. In the course of these experiments, we have generated a derivative of 5S RNA with significant deletions in helix V and loop c that binds TFI_{IIA} tightly. The availability of this deleted RNA may simplify future efforts to use physical techniques to study the structure of the 7S particle.

MATERIALS AND METHODS

Materials

Most of the materials used in this study were from the same sources reported previously (20). The Klenow fragment of DNA polymerase I was from Boehringer-Mannheim (Indianapolis, IN).

Mutagenesis

Oligonucleotide-directed mutagenesis was performed as described by Kunkel (25). One mutant (Del 3) was produced by R. Cipriani (SUNY Stony Brook) using the method of Taylor et al. (26). All mutations were confirmed by dideoxynucleotide sequencing using the Sequenase 2.0 kit (US Biochemicals, Cleveland OH).

Figure 1. Comparison of 5S RNA with the ICR. This representation of the *X. borealis* somatic 5S DNA and 5S RNA sequences is adapted from that of Christiansen et al. (18). However, the nomenclature regarding helix IV and V is based on the model of Anderson et al. (41) which considers that this region is one continuous helix containing non-Watson-Crick base pairs. The dark bars at the top of the figure indicate the extent of protection afforded to the DNA by amino-terminal fragments of TFI_{IIA} that retain the indicated numbers of zinc fingers (ref. 8–11).

RNA Synthesis and Mobility Shift Analysis

RNAs were synthesized by transcription with T7 RNA polymerase of Dra I-digested plasmid DNA containing derivatives of the *X. borealis* somatic 5S RNA gene. The dominant sequence of the *X. borealis* and *X. laevis* somatic 5S RNAs are identical, although 5S RNA multigene families include sequence variants (27). The detailed conditions were as adapted from Romaniuk et al. (28) by Sands and Bogenhagen (20) except that the RNAs were internally labelled with low specific activity α -³²P-GMP during transcription. RNAs were adjusted to a final concentration of 0.5 nM or 1 nM in 15 μ l binding reactions. TFI_{IIA} was prepared as described by Sands and Bogenhagen (20) and was diluted to 100 nM and 10 nM stocks in binding buffer (60 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.4, 2 mM DTT, and 10 μ M ZnCl₂) containing 0.05% Triton X-100 and 300 μ g/ml bovine serum albumin immediately before use. TFI_{IIA} concentrations used in binding reactions varied between 0.5 and 20 nM. Each binding titration employed 5 to 8 different concentrations of TFI_{IIA}. After incubation at 20°C for 10 min, complexes were separated from free 5S RNA by electrophoresis in 1.2% agarose gels containing 44.5 mM Tris-borate, 7 mM 2-mercaptoethanol, 0.01% Triton X-100 and 10 μ M Zn acetate essentially as described (29). Gels were dried onto DE81 paper and exposed to X-ray film (Fuji or Kodak XAR-5) without intensifying screens. An internal control consisting of a binding titration with wild type Xbs 5S RNA was performed in parallel with each binding titration using mutant RNAs. Bound and free 5S RNA were quantified by densitometry using either a Molecular Dynamics or LKB Ultrosan densitometer, and the fraction of RNA bound RNA_b/RNA_t was plotted as a function of the total TFI_{IIA} concentration, [A]. The K_D for binding of TFI_{IIA} to RNA was determined by fitting the experimental data to the equation RNA_b/RNA_t = [A]/(K_D + [A]) using Tablecurve and

Name	Sequence	Relative TFI _{IIA} Binding
WT XBS RNA	CCUGGUUAGUACUUGGAUGGGA GGACC - AUAAGGGUCC - GCCAG	1.0
XBS 86/97	CCUGGUUAGUACUUGGAUGGGA GGACC - AUAAGGGUCC - GCCAG	1.0
XBS 82/91	CCUGGUUAGUACUUGGACGACGAC GGACC - AUAAGGGUCC - GCCAG	0.72
XBS 79/87	CCUGGUUAGUACUUGGACGACGAC GGACC - AUAAGGGUCC - GCCAG	0.215
XBS 68/78	CCUGGUUAGUACUUGGACGACGAC GGACC - AUAAGGGUCC - GCCAG	0.178
XBS 68/78-98/108	CCUGGUUAGUACUUGGACGACGAC GGACC - AUAAGGGUCC - GCCAG	0.329

Figure 2. The effect of sequence substitutions in helix IV/V on binding of TFI_{IIA}. The sequences of the helix IV/V regions of a series of derivatives of 5S RNA are shown. Sequence alterations are printed in reverse lettering. The right hand column shows the relative affinity for binding of TFI_{IIA} expressed as K_D(wild type)/K_D(mutant).

Sigmaplot curve fitting software (Jandel Scientific). The average K_D observed for wild-type 5S RNA in these experiments was 1.34 ± 0.3 nM.

Probing of the secondary structure of 5' and 3' labeled RNAs was performed as described (20). Cleavage patterns were interpreted using nuclease ladders generated by cleavage with T1 and T2 nuclease in the presence of 7 M urea as described (30).

RESULTS

Effect of sequence substitutions in helix IV–V on TFIIIA binding

The sequence of *X. borealis* 5S RNA can be drawn in a conformation that maximizes its similarity to the ICR of the 5S RNA gene as shown in Figure 1 (adapted from Christiansen et al., (18)). The experiments reported here are intended to directly address the model that TFIIIA binds with the same orientation

to the stacked RNA helices as it does to 5S DNA. In 5S DNA, the tightest interactions with TFIIIA are between the distal portion of the ICR beginning with residue 67 or 68 and the amino-terminal fingers of TFIIIA. As shown in Figure 1, this region may be considered to resemble helix IV/V in 5S RNA. Since sequence changes in this region of 5S DNA dramatically reduce the affinity for TFIIIA, we have constructed a set of sequence substitution mutants in this region of 5S RNA. The sequences of the helix IV/V region of these mutants are shown in Figure 2. The collection of sequence substitution mutants includes two mutants, Xbs 86/97 and 68/78, originally described by Sands and Bogenhagen (21). Experiments with these mutants are included here since the earlier paper presented only a rather qualitative assessment of the TFIIIA binding abilities of these RNAs. Both mutants were transferred to the T7 expression vector to allow synthesis of RNAs for the present studies. RNAs were synthesized by transcription with T7 RNA polymerase. The

Figure 3. Effects of deletions in helix V on binding of TFIIIA. A. Binding data derived from mobility shift analyses using RNAs transcribed from wild type (●), mutant V1 (▽) and mutant V2 (▲) templates. The curve represents the computer fit to the binding data for wild-type 5S RNA. B. Binding data derived from mobility shift analyses using RNAs transcribed from wild type (●), mutant V3 (□) and mutant V4 (■) templates. The three curves represent computer fits to the data for wild-type (upper curve), mutant V3 (center curve) and mutant V4 (lower curve) RNA. C. Sequences of the mutagenized regions of the RNAs are shown along with the relative TFIIIA binding affinities as described in the legend to Figure 2.

ability of each RNA to bind TFIIIA was assessed using a gel mobility shift assay. Examples of the sort of raw data obtained with this assay are presented below. The autoradiograms were analyzed by densitometry to generate a graph of the fraction of RNA bound as a function of the TFIIIA concentration. The dissociation constants were determined by a curve fitting procedure described in Materials and Methods.

Figure 2 provides a summary of the TFIIIA binding data obtained for RNAs containing sequence substitutions in helix IV/V. Sequence changes in mutants Xbs 86/97 and Xbs 82/91 that are expected to disrupt the structure of the tip of helix V were found to have little effect on the affinity of the RNA for TFIIIA. Sequence changes located closer to the junction between the three helical stems in 5S RNA, in mutants Xbs 79/87 and Xbs 68/78, resulted in an increase in the apparent dissociation constant to 6.2 and 7.5 nM, respectively. Figure 2 also shows the sequence of an additional mutant derived from Xbs 68/78, designated Xbs 67/78–98/108, with compensatory base changes expected to restore helical structure in helix IV/V. TFIIIA does indeed bind more tightly to this RNA than to the transcript of Xbs 68/78, although wild type binding affinity is not fully restored. These results suggest that binding of TFIIIA requires an intact helical stem in helix IV/V. Similar results have been reported by You and Romaniuk (23). These authors reported that smaller sequence substitutions in helix IV/V that were designed to preserve the RNA helix had little effect on TFIIIA binding. These authors suggested that binding was relatively independent of the nucleotide sequence in this region. However, the impaired binding observed due to the more extensive sequence changes in mutant Xbs 68/78–98/108 is consistent with the possibility that some base-specific contacts have been disrupted in this mutant. The most significant conclusion provided by the data in Figure 2 is that extensive sequence changes in helix IV/V in 5S RNA can have relatively little effect on the binding of TFIIIA.

TFIIIA binding to RNAs with internal deletions that shorten helix IV/V

Results with the sort of sequence substitution mutants studied in Figure 2 must be interpreted cautiously since there is no guarantee

that the sequence changes would only affect the local RNA structure. Our analyses of the secondary structures of mutants 86/97 and 67/78 suggests that the alterations in the secondary structure are indeed localized to the vicinity of the sequence substitutions (M. Sands, Ph.D. Thesis, SUNY at Stony Brook). Results from other laboratories have also suggested that small sequence changes in 5S RNA frequently have only localized structural effects (31, 32). Since directly studying the secondary structure of a large series of mutants would be an imposing task, we have taken an alternate approach to further define RNA sequences required for binding TFIIIA. First, we have designed mutants with reference to the secondary structure of 5S RNA in order to shorten the helical stems of the RNA. As shown below, these experiments have allowed the construction of deletion mutants that still bind TFIIIA with high affinity. Then, we have used structure-specific enzymes to probe the secondary structure of the smallest deletion derivative that retains the wild-type affinity for TFIIIA.

Oligonucleotide-directed mutagenesis was used to create a series of mutants with progressive deletions of the tip of helix V. The RNAs were synthesized *in vitro* and tested for the ability to bind TFIIIA in mobility shift experiments. Data derived from densitometry of the resulting autoradiograms is presented in Figure 3. The deletion of 8 or 13 residues from the tip of helix V, in mutants V1 and V2, did not result in a detectable reduction in the affinity for the binding of TFIIIA. Deletion of 17 nucleotides reduced binding affinity only about 2-fold. These results show that TFIIIA binds with normal or near-normal affinity to derivatives of 5S RNA lacking essentially all of the RNA analogue to the tight binding site in 5S DNA. These results are in striking contrast to the prediction of the 'RNA mimics DNA' model for the binding of TFIIIA.

TFIIIA binding to RNAs deleted in helix III and loop c

A similar series of deletion mutants was constructed in which portions of loop c and, ultimately, helix III were deleted. The sequences of these mutants and their TFIIIA binding affinities are shown in Figure 4. Mutant C1 has a significant reduction in the size of loop c. Mutant C2 lacks four additional residues,

B	Name	Sequence	Relative TFIIIA Binding
	WT	<pre> CUGAUCUCGGAAGC - CAAGCAGGUCGG GCUAUAAGCC CGUGAAAGUCCCA - CC </pre>	1.0
	DEL C1	<pre> UCUCGGAAGC - CAAGCAGGUCGG AUAGCC CGUGAAAGUCCCA - CC </pre>	1.06
	DEL C2	<pre> UCCGGGC - CAAGCAGGUCGG AAGCC CGUGAAAGUCCCA - CC </pre>	1.31
	DEL III	<pre> C - CAAGCAGGUCGG GUGAAAGUCCCA - CC </pre>	0.34

Figure 4. Effects of deletions in helix II/III on binding of TFIIIA. A. Binding data derived from mobility shift analyses using RNAs transcribed from wild type (●), mutant C1 (▽), mutant C2 (△) and mutant Del III (□) templates. The curve represents the computer fit to the binding data for wild-type 5S RNA. B. Sequences of the mutagenized regions of the RNAs are shown along with the relative TFIIIA binding affinities as described in the legend to Figure 2.

the two bulged A residues in helix III and one base pair in helix III. Baudin and Romaniuk (33) have shown that the two bulged A residues are not required for binding of TFIIIA. Both of these deleted RNAs bind TFIIIA with normal affinity (Fig. 4). Additional deletion of the remaining portion of helix III and the enclosed loop c in mutant Del III significantly diminished the affinity for TFIIIA. This pattern of results is consistent with previous experiments showing that binding of TFIIIA subtly alters the pattern of cleavage by cobra venom nuclease in helix III (20). At present it is not clear whether this reflects a direct interaction of TFIIIA with helix III or a slight change in the structure of helix III resulting from tighter interactions of the protein with adjacent regions in the RNA.

Binding of TFIIIA requires specific residues in helix I

The foregoing experiments have shown that TFIIIA can bind to RNAs containing substantial deletions in helix IV/V and loop c/helix III. An attempt was made to make a slight deletion in helix I to achieve two goals. First, it was of interest to determine if the length of helix I could be reduced. Second, if the sequence of helix I could be altered, it might be possible to increase the strength of the T7 promoter. In all of the RNA expression constructs we have used in the experiments reported above, T7 RNA polymerase is required to initiate with the sequence pppGCCUAC... derived from 5S RNA. This sequence reduces the efficiency of the T7 promoter significantly. Two mutants were constructed as shown in Figure 5. It was anticipated that restoration of the helical structure in mutant I-3 would permit efficient binding of TFIIIA. However, the results shown in Figure 5 clearly indicate that the wild type sequence in helix I

Figure 5. Helix I is required for binding of TFIIIA. The sequence of helix I in wild type Xbs 5S RNA (1) is compared to the corresponding regions of two mutants: RNA I-2, an RNA containing base substitutions in the 5' arm or helix I, and RNA I-3, a mutant containing compensatory base changes in addition to the changes in mutant I-2 intended to restore base pairing in helix I. The upper portion shows an autoradiogram of a binding titration performed with these 3 RNAs using 0.5 nM RNA and increasing concentrations of TFIIIA. Each set of five lanes used 0, 1, 2, 4 and 8 nM TFIIIA.

promotes more efficient binding of TFIIIA than either of the mutants. Sands and Bogenhagen (20) reported that TFIIIA binding does protect residues in helix I from digestion by cobra venom nuclease.

Binding of TFIIIA to double mutants deleted in helix V and loop c

The foregoing results have shown that deletions of about 10% of the size of 5S RNA can be made at the tips of helix V and loop c without significantly impairing the binding of TFIIIA. One of the goals of this work is to produce an RNA of minimal size that binds TFIIIA with normal affinity. Therefore, we combined two pairs of deletions described above to produce two double mutants shortened in both loop c and helix V. These two double mutants were designated C1V1 and C2V2. Although both double mutant RNAs were observed to bind with normal affinity to TFIIIA, data is presented only for the more extreme deletion mutant, C2V2. Figure 6 shows a direct comparison of the binding of TFIIIA to wild type and C2V2 RNA using the mobility shift method.

The structure of the 96-nucleotide RNA transcribed from mutant C2V2 might be expected to differ from that of wild-type 5S RNA mainly in the regions surrounding the two deletions. We were interested in knowing whether the secondary structure of 5S RNA is largely preserved in the regions retained in this 96-nucleotide RNA. Therefore, we probed the end-labeled RNA with structure specific nucleases to allow a direct comparison of its structure with that of wild type somatic 5S RNA. Three nucleases were used in these experiments. Nuclease T1 cuts after G residues in single stranded loops in RNA. Nuclease T2 cuts in single stranded sequences with a less stringent base specificity that favors cleavage at A residues. Cobra venom nuclease (nuclease V1) cuts within duplex regions in RNA. In our hands, this nuclease tends to cut more efficiently at the ends of helical stems in RNA. 5' and 3' labeled C2V2 RNAs were probed with all three nucleases. In most experiments, end-labeled 5S RNA was included for the sake of comparison. The results of this analysis are summarized in Figure 7. In the native RNA, T1 cleavage occurs at G residues within or immediately adjacent to single stranded loops. Cleavage at G₆₉ tends to dominate the T1 digest pattern (see bold arrow in Fig. 7), as is observed at the corresponding site in 5S RNA. Nucleases V1 and T2 produced complementary cleavage patterns, as expected for an RNA with

Figure 6. Comparison of binding of TFIIIA to wild type and deleted RNAs. The autoradiogram of a comparison of the mobility shift binding data for wild-type 5S RNA (panel A) and mutant RNA C2V2 (B) is shown. In each set, lanes 1 through 7 contained 0, 1, 2, 4, 6, 8 and 12 nM TFIIIA.

a homogeneous secondary structure. The results are consistent with the interpretation that the C2V2 RNA retains much of the secondary structure of the central region of *X. borealis* somatic 5S RNA.

DISCUSSION

The TFIIIA Binding Site in 5S RNA

The mutagenesis of 5S RNA presented in this paper has served to delimit the binding site for TFIIIA to the region surrounding the central crux of the RNA where the three helical stems of the RNA converge. Several previous studies have indicated that TFIIIA interacts with helices II and IV/V (18, 21, 34). The deletion mutagenesis experiments presented here clearly show that the tips of helix V and loop c are not required for binding TFIIIA. These results are in general agreement with the results of mutagenesis of the *X. laevis* oocyte 5S RNA by Romaniuk and coworkers (22, 23). One exception to this generalization is that Romaniuk (22) observed that sequence changes in residues 41–44 of oocyte 5S RNA reduced the binding of TFIIIA slightly. It is conceivable that differences between the sequence of oocyte and somatic 5S RNA could contribute to this apparent discrepancy. The sequence in loop c of the somatic 5S RNA might be considered to permit an extension of base pairing in helix III that would reduce the size of the single stranded loop (see Fig. 1). The potential for base pairing is reduced in the corresponding region of oocyte 5S RNA. Romaniuk et al. (34) and Westhof

et al. (16) have suggested that there is not a significant difference in the structures of these two RNAs in this region. Despite the potential differences in structure, our deletion mutagenesis suggests that sequences in loop c do not play a significant role in binding TFIIIA.

The results in this paper support the conclusion that all three of the helical stems of 5S RNA are required for binding TFIIIA. The observation in Figure 5, that binding of TFIIIA requires helix I, has not been widely recognized in previous studies. Sands and Bogenhagen (20) have shown protection by TFIIIA of cobra venom nuclease cleavage sites in helix I. However, Huber and Wool (34) and Christiansen et al. (18) did not include helix I in their summaries of the binding site for TFIIIA. There may be technical reasons why the earlier nuclease studies did not reveal protection in helix I. First, Huber and Wool (34) did not use cobra venom nuclease to probe 7S particles; second, the primer extension approach used by Christiansen et al. (18) did not permit a clear analysis of helix I, since the oligonucleotide primer was hybridized to the 3' end of 5S RNA and the 5' half of helix I was not resolved well in their gel system. Three earlier studies of the binding of TFIIIA to enzymatically truncated derivatives or mutants of 5S RNA have addressed the role of helix I. First, Anderson and Delihias (36) showed that deletions in helix I significantly impaired the ability of deleted RNAs to be exchanged into 7S particles. Second, Sands and Bogenhagen (21) showed that an RNA synthesized from a linker substitution mutant, Xbs LS 107/118 did not bind TFIIIA. The principle effect expected for this mutation is disruption of helix I. Third, Romaniuk et al. (28) obtained a mixed pattern of results in similar studies of truncated RNAs. In this case, nitrocellulose filter binding assays suggested that an RNA containing residues 1 to 108 of 5S RNA could bind TFIIIA with normal affinity, although this RNA was only poorly bound in an RNA exchange assay. These results, in combination with those shown in Figure 5, would suggest that weakly bound complexes lacking significant contacts may be retained in the nitrocellulose filter binding assay. In addition to these earlier studies, a recent chemical footprinting analysis of the interaction of TFIIIA with 5S RNA has concluded that TFIIIA does protect residues within helix I (37).

It is interesting to consider the role of helix II in detail. Two studies have suggested that this is an important region for the interaction of TFIIIA with 5S RNA. First, Sands and Bogenhagen (20) have described a distinctive pattern of nuclease protection in helix II resulting from binding of TFIIIA. This has recently been confirmed in another laboratory (37). It would be interesting to determine whether this protection pattern would be observed for mutants in which the nucleotide sequence in helix II is altered without disrupting the helical structure (23). Second, Baudin et al. (39) suggested that helix II was a site of cross-linking of 5S RNA to TFIIIA with trans-platinum. It would be quite interesting to determine the precise site of TFIIIA cross-linked to this region of 5S RNA. Cross-linking has the potential of identifying specific near-neighbor interactions in the RNP that would permit the building of detailed models for the 7S particle.

Although the RNA binding domain of TFIIIA appears to be restricted essentially to the seven amino terminal zinc fingers (20), this domain of the protein may make an extensive set of weak contacts with the RNA. Localized mutations of either the RNA or protein may have little effect on the overall binding affinity as measured with filter binding or mobility shift techniques. An extensive collection of mutations prepared by Romaniuk and his collaborators has not revealed a tight binding site for TFIIIA within 5S RNA. These workers have shown that TFIIIA is more

Figure 7. The secondary structure of RNA C2V2 resembles a core structure for 5S RNA. Separate preparations of 5' and 3' end-labeled C2V2 RNA were partially treated with ribonucleases. Panel A is a summary of cleavages observed with the double strand specific cobra venom nuclease CV1. Panel B is a summary of cleavages observed with strand specific nucleases T1 and T2. The bold arrows indicate preferential sites of cleavage.

likely to recognize helical regions of 5S RNA than single stranded loops (22). More recently, You and Romaniuk (23) have shown that alterations of the sequence of 5S RNA that preserve the structure of helices II and IV/V do not block binding of TFIIIA. These results have been taken to indicate that TFIIIA binds in a relatively sequence non-specific manner to the helical stems of 5S RNA. This model for the binding of TFIIIA to 5S RNA contrasts sharply with the binding of TFIIIA to 5S DNA, which can be significantly reduced by methylation of single G residues (5) or by any of several single base pair changes (38). These critical residues in the distal portion of the ICR constitute a tight binding site for fingers 1 to 3 of TFIIIA (10, 11). It is remarkable that Liao et al. (11) observed only a 2.5-fold difference in the affinity of the ICR for the three finger peptide, Zf123, compared to intact TFIIIA. The recombinant Zf123 peptide binds specifically to a DNA fragment containing only residues 80 to 92 of 5S DNA (11). Two aspects of these results are particularly relevant to the comparison of TFIIIA binding to DNA and RNA. First, as noted by Liao et al. (11), it is surprising that deletion of six fingers of TFIIIA can make so little difference in the free energy of interaction with DNA. In our experiments, we have noted a similar phenomenon. When extensive deletions of 5S RNA do cause a reduction in binding affinity for TFIIIA the reduction is so slight that it is not possible to speculate on how many detailed interactions have been lost. A concise thermodynamic interpretation of the interaction of the multiple zinc fingers of TFIIIA with 5S RNA or 5S DNA is not possible at present. Second, the ability of TFIIIA to bind mutant RNA V2 as tightly as it binds 5S RNA clearly shows that the region in 5S RNA that is analogous to the tight binding site in DNA is dispensable for binding of TFIIIA.

Definition of the structure of the 7S particle

Ultimately the precise description of the RNA:protein contacts in the 7S particle will require high-resolution physical analysis. To our knowledge, efforts to crystallize the 7S particle have had only limited success to date. Crystals have been grown, but these evidently diffract poorly (40). There are several reasons why the crystallization of naturally-occurring 7S particles represents a difficult problem. First, the 7S particle is rather large, with a total molecular mass of about 80,000. Second, it might be anticipated that the naturally occurring 7S particles would be heterogeneous, since both oocyte and somatic 5S RNAs are contained in the 7S particles and the oocyte-type 5S RNA represents an average sequence of a fairly divergent multigene family. In addition, the non-finger domain of TFIIIA does not appear to bind tightly to 5S RNA. This domain is readily removed by treatment of the 7S particle with proteases, and may be somewhat disordered in the 7S particle. One way to circumvent some of these limitations and to reduce the size of the 7S particle might be to attempt to reconstitute a particle containing the deleted C2V2 RNA and a fragment of TFIIIA containing the 7 amino-terminal zinc fingers. Such a reconstituted RNP would be only 70% as large as the intact 7SP and should be substantially more compact. It may be that additional efforts to study the binding of smaller fragments of TFIIIA to 5S RNA will result in an even smaller derivative of the 7S particle.

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