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Defining the binding site of *Xenopus* transcription factor IIIA on 5S RNA using truncated and chimeric 5S RNA molecules

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ABSTRACT

The interaction of TFIIIA with deletion fragments of *Xenopus* 5S RNA has been quantified using a nitrocellulose filter binding assay. TFIIIA binding was found to be more sensitive to the deletion of nucleotides from the 5' terminus of the 5S RNA as opposed to the 3' terminus. These effects have been correlated to the changes in RNA secondary structure resulting from the deletions. Nucleotides 11-108 of the intact 5S RNA provide the necessary sequence and conformational information required for the binding of TFIIIA. Synthetic 5S RNA genes have been constructed so that *in vitro* transcription with T7 RNA polymerase yields mature 5S RNA. The transcription factor has a higher affinity for somatic vs. oocyte 5S RNA, similar to the differential affinity of TFIIIA for the two genes. Binding studies with chimeric 5S RNA molecules indicated that the increased binding strength of somatic 5S RNA is conferred by nucleotide substitutions in the 5' half of the molecule.

INTRODUCTION

TFIIIA has a dual biological role in the *Xenopus* oocyte. It acts as a positive transcription factor, binding to an internal control region and modulating the expression of the *Xenopus* 5S RNA genes during oogenesis [1-4]. The protein also forms a specific 7S RNP complex with newly synthesized 5S RNA, stabilizing the RNA for storage in the cytoplasm of immature oocytes [5,6]. As a result of these functions, TFIIIA binds specifically to DNA and RNA, two nucleic acids which have distinctly different conformations. However, there is now evidence which suggests that the DNA binding site for TFIIIA may adopt an A'-RNA like double helix [7,8]. Recent studies on the sequence and structure of TFIIIA indicate that this protein contains a repeating domain [9,10] which forms an unusual structure described as a 'metal-binding finger' [10]. This structure may account in particular for properties required for the function of TFIIIA as a transcription factor [10], and appears to be a conserved feature of other putative DNA binding proteins [11-14]. Such a structure may also facilitate the interaction of the protein with 5S RNA [14].

The DNA binding properties of TFIIIA have been studied extensively and recently several groups have begun to investigate the RNA binding properties of this protein. Chemical and nuclease probes have been used to map the protein binding region on 5S RNA in the isolated 7S RNP particle [15-18], and also in reconstituted particles [18]. Several assays have been used

to demonstrate that TFIIIA is capable of binding to a variety of eukaryotic 5S RNA molecules [18-21]. Thermodynamic and kinetic parameters for the RNA binding activity of the transcription factor have been determined using a nitrocellulose filter binding assay [18]. The protein binds *Xenopus* oocyte 5S RNA with a dissociation constant (K_d) of 1 nM, and complex formation is favoured by both enthalpy and entropy. Approximately 5 ionic bonds are formed between the protein and RNA, although ca. 70% of the free energy of complex formation is derived from non-ionic interactions [18].

In the present study, we have used the filter binding assay to compare several aspects of the RNA binding properties of TFIIIA with those already determined for the DNA binding activity. We have measured the effect that deleting nucleotides from the 5' and 3' ends of *Xenopus* oocyte 5S RNA has both on the structure of the RNA and on its interaction with TFIIIA. A series of cloned *Xenopus* 5S RNA genes have been constructed which can be transcribed with T7 RNA polymerase to produce µg amounts of fully mature 5S RNA. Several synthetic 5S RNAs have been used to investigate the structural basis for the difference in the TFIIIA binding affinities of somatic and oocyte 5S RNA.

MATERIALS AND METHODS

Materials

Reagents required for the chemical synthesis of oligonucleotides by the β-cyanoethylphosphoramidite method were purchased from Biosearch. Restriction enzymes were purchased from either Pharmacia or Boehringer-Mannheim, T4 polynucleotide kinase was purchased from USB Biochemicals, T4 DNA ligase was purchased from New England Biolabs, and AMV reverse transcriptase was purchased from Life Sciences. [α-^{32}P] GTP and [γ-^{32}P] ATP were purchased from New England Nuclear. T7 RNA polymerase was purified by a published procedure [22] from *E. coli* strain BL21/pAR1219 kindly provided by Dr. F. W. Studier.

Preparation of TFIIIA

The 7S RNP particle was isolated from the ovaries of immature *X. laevis* (Xenopus 1, Ann Arbor, MI) using a published procedure [23]. TFIIIA was prepared from the 7S RNP using a modification [18] of the procedure of Smith *et al* [22].

Binding Studies with TFIIIA

Nitrocellulose filter binding assays were conducted as described elsewhere [18]. For competition assays, the TFIIIA concentration was constant at 1 nM, the concentration of ^{32}P-labelled wheat germ 5S RNA was 0.5 nM, and competitor RNA concentration was varied between 0.1-20 nM. All other details of the competition assays are described in Ref. 18.

Preparation of fragments of *Xenopus* 5S RNA

Xenopus oocyte 5S RNA was prepared from 7S RNP by phenol extraction and purified by Sephacryl S-200 column chromatography [25]. An aliquot of 5S RNA (45 µg) was dissolved in 20 µl of a buffer containing 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 0.2 M NaCl and digested

for 60 min. at 4 °C with either 1.25 ng of RNase A, 1.25 U of RNase T_2, or 50 U of RNase T_1. The reactions were immediately frozen on dry ice and lyophilized. Each digest was reconstituted in 10 µl of a sample buffer containing 8M urea and 0.02% xylene cyanol and bromophenol blue and then was purified on a 7M urea - 15% polyacrylamide gel. Fragments were detected by UV shadowing, and gel slices containing RNA were eluted for 12 hours at room temperature in 0.5 ml of 0.5 M ammonium acetate, 0.1% SDS and 1 mM EDTA. Fragments were recovered by ethanol precipitation.

These RNA fragments were 5'-end labelled using [γ-^{32}P] ATP and polynucleotide kinase [26], and were then repurified on denaturing polyacrylamide gels. The identity of each fragment was obtained by partial enzymatic sequencing [27] and terminal nucleotide analysis [28].

Construction of cloned 5S RNA genes

5S RNA genes were constructed using a modification of the method of 'micro-scale ligation' developed by Chambon *et al* [29]. The cloned genes were synthesized as a series of oligonucleotides which were subsequently annealed and ligated into pUC18 (Figure 1A). Oligonucleotides were synthesized by the phosphite method [30] using a Biosearch 8600 DNA synthesizer and were purified by gel electrophoresis and reverse phase chromatography [31]. An aliquot of each oligomer (5 µg) was phosphorylated by incubating at 37 °C for 60 min. in 60 µl of a buffer containing 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 50 µg/ml BSA, 0.1 mM ATP and 3 units of T4 polynucleotide kinase. The reaction was diluted into 1 ml TE (10 mM Tris-HCl pH 8, 1 mM EDTA) buffer, and the oligonucleotides were repurified by reverse phase chromatography [31], and stored as 10 µM solutions at -20 °C. Oligonucleotides (0.5 pmol) were annealed by incubating for 1 hour at 37 °C in 8 µl of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20 mM DTT, 0.1 mM spermidine and 1 mM ATP. pUC18 (0.1 pmol) that had previously been digested with Eco RI and Bam HI was added along with 200 units of T4 DNA ligase, and incubation was continued overnight at 20 °C. The reaction was then heated to 70 °C for 10 min. to inactivate ligase, and then 20 µl of Kpn I buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.01% Triton-X100) was added along with 10 units of the restriction nuclease Kpn I. After incubation at 37 °C for 60 minutes, 5-10 µl of the reaction was used to transform *E. coli* strain JM105 made competent by $CaCl_2$ treatment [32]. Bacteria carrying plasmid DNA were selected on the basis of ampicillin resistance. For screening purposes, minipreparations of plasmid DNA were made from 10 isolates [32], and the DNA was analyzed both for the presence of a new Dra I restriction site, and the ability to produce 5S RNA transcripts. The correct sequence of each clone was verified by dideoxy nucleotide sequencing of super coiled plasmid DNA using the M13 reverse sequencing primer and reverse transcriptase [33]. In general, we found that 40-70% of plasmids from minipreparations contained the correct sequence.

Synthesis of RNA from cloned genes

a) Linearization of Plasmid DNA. In the case of the pXIs, pXIo, pXIs-5'chimer and pXIo-

5'chimer plasmids, 25 µg of plasmid DNA was dissolved in 100 µl of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 100 µg/ml BSA and digested with 50 units of the restriction endonuclease Dra I for 60 minutes at 37 °C. The extent of digestion was analyzed by electrophoresis of an aliquot of the reaction mixture on a 1% agarose gel. The reaction was then extracted with 100 µl of phenol:$CHCl_3$ followed by two extractions with 100 µl of $CHCl_3$. The DNA was recovered by ethanol precipitation and redissolved in TE to give a solution of 1 µg/µl.

In order to produce suitable templates for the production of 3' truncated 5S RNA fragments, pXlo-108 was digested to completion with either Stu I, Fok I or Bst NI, following the protocol outlined above for Dra I digestion, but using buffers optimal for each restriction enzyme. These were: Stu I, 6 mM Tris-HCl pH 7.9, 50 mM NaCl, 6 mM $MgCl_2$, 5 mM DTT, 0.01% Triton X-100; Fok I, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 100 µg/ml BSA; Bst NI, 10 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 100 µg/ml BSA.

b) Synthesis of internally labelled RNA. A final volume of 10 µl contained: 40 mM Tris-HCl pH 8, 15 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 100 µg/ml BSA, 1000 U/ml RNasin (Promega Biotec), 0.5 mM each ATP, CTP, UTP, 0.0125 mM GTP, 50 µCi [α-^{32}P] GTP (600 Ci/mmol), 1 µg of linearized template DNA, and 0.6 µg T7 RNA polymerase. The reaction was incubated for 2.5 hours at 37 °C, then 10 µl of urea-dye sample buffer was added and the RNA was purified on a 8M urea, 12% polyacrylamide gel.

TABLE 1. Relative Binding Strengths of Truncated Fragments of *Xenopus* oocyte 5S RNA

RNA	Binding Strength[a]
1-121	1.00
5' Truncates:	
10-121[b]	0.75
40-118	0.14
50-118	0.08
74-118	0.06
3' Truncates:	
1-108	0.90
1- 98	0.80
1- 69	0.40
Other RNAs:	
E. coli 5S RNA	0.25
Yeast $tRNA^{phe}$	<0.01

[a]Relative binding strength is defined as the ratio of K_a(fragment)/K_a(oocyte 5S RNA)

[b]Derived from somatic 5S RNA. Relative binding strength compared to intact somatic 5S RNA

c) Synthesis of unlabeled RNA. A final volume of 200 µl contained: 40 mM Tris-HCl pH 8, 15 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 100 µg/ml BSA, 1000 U/ml RNasin, 1 mM each ATP, CTP, UTP, GTP, 8 µg linearized template DNA, and 3 µg T7 RNA polymerase. After incubation for 90 min. at 37 °C, the reaction was extracted with 200 µl of phenol:$CHCl_3$ followed by extraction with 200 µl of $CHCl_3$. The crude RNA was ethanol precipitated, and then redissolved in 50 µl TE. 5S RNA was separated from template DNA and unincorporated nucleotides by gel permeation HPLC using a BioRad TSK-125 column (7.5 mm X 30 cm) and an elution buffer of 0.2 M sodium acetate, 1% methanol pH 6. Yields of pure 5S RNA were typically 30-50 µg.

Structural Analysis of RNA Fragments

An aliquot of 5'-^{32}P labelled RNA was incubated with various nucleases for 20 minutes at

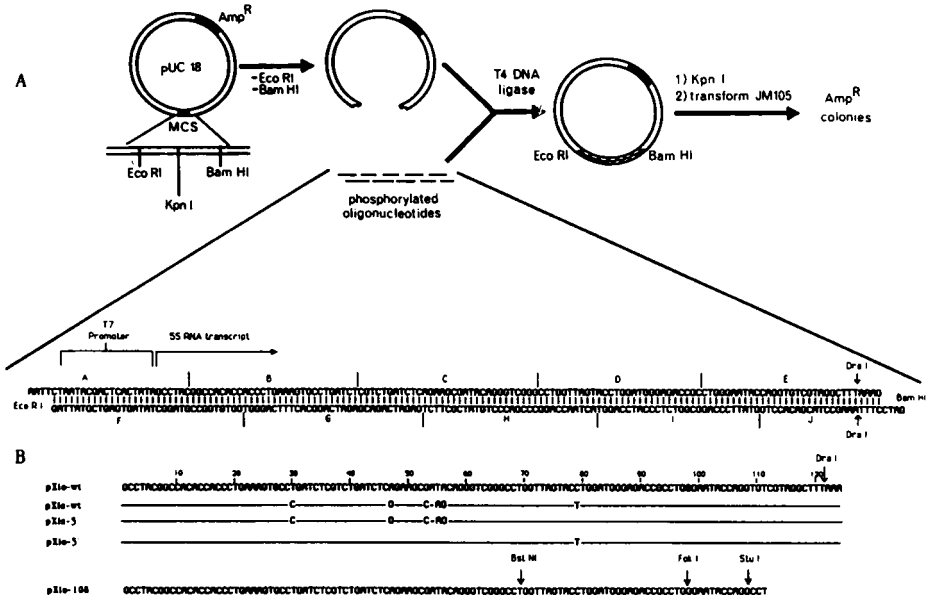


Figure 1. A. *Xenopus* 5S RNA genes were constructed from oligonucleotides A-J using the microscale shot gun ligation method [29]. Ligation points are indicated by the vertical lines. After ligation, the reaction mixture is digested with Kpn I to linearize any re-sealed pUC18 vector before transformation. B. Sequence of the non-coding strand from constructs used in this study. The top four lines represent the four 5S RNA genes. The horizontal line in pXls-wt, pXls-5' and pXlo-5' indicate the identical sequence to pXlo-wt. Cleavage of these plasmids with Dra I and subsequent *in vitro* transcription with T7 RNA polymerase yields a 121 nucleotide long 5S RNA transcript. The bottom line indicates the sequence of the non-coding strand for pXlo-108, a construct used to produce 5S RNA fragments truncated at the 3' terminus. The sequence is identical to pXlo-wt from positions +1 to +108. Vertical arrows indicate the end point of transcripts produced when this plasmid is digested with Bst NI (1-69), Fok I (1-98) and Stu I (1-108).

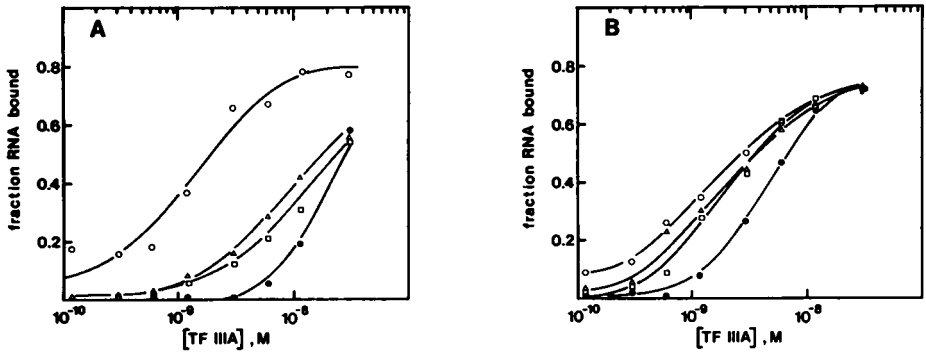


Figure 2. Titration of RNA fragments with TFIIIA under equilibrium binding conditions. RNA molecules were: A. 5S RNA (○), 40-118 (△), 50-118 (□), and 74-118 (●). B. 5S RNA (○), 1-108 (△), 1-98 (□), 1-69 (●).

20 °C in a 1.5 ml eppendorf tube containing 100 µl of TMK buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM KCl) and 10 µg carrier tRNA. The nucleases employed were: T_1 (0.1, 1 unit), T_2 (0.01, 0.1 unit), A (0.1, 1 ng) and V_1 (0.35, 0.7 unit). The reaction mixtures were then extracted with 100 µl of phenol:$CHCl_3$, and the RNA was recovered from the aqueous phase by ethanol precipitation. The RNA was redissolved in 5 µl of urea-dye buffer, heat denatured and analyzed on a 8M urea, 12% polyacrylamide sequencing gel.

RESULTS

1. Binding of Truncated 5S RNA Molecules to TFIIIA

The results of footprinting experiments reported by several groups indicate that TFIIIA binds oocyte 5S RNA in the region between nucleotides 52-110 [15-18]. In order to determine whether this region of the RNA molecule contains all of the structural and sequence information required for the specific binding of TFIIIA, we have measured the K_a values for the binding of truncated *Xenopus* oocyte 5S RNA molecules to the transcription factor. Figure 2A shows the results of a nitrocellulose filter binding assay used to measure the affinity of 5' truncated RNA molecules for TFIIIA. It is apparent that even 5S RNA fragments containing all of the putative binding sequence have a significantly reduced affinity for TFIIIA. The reduction in the binding of RNA fragments is not the result of degradation of the RNA upon incubation with TFIIIA: the integrity of RNA fragments after incubation with 10 nM TFIIIA was checked by denaturing gel electrophoresis, and in each case less than 2% of the RNA was degraded. The results obtained from several determinations with all of the 5' truncated fragments are presented in Table 1. While the loss of 10 nucleotides results in a molecule that has retained 75% of the binding activity of the intact 5S RNA, the further loss of 30 nucleotides has reduced the binding affinity to less than 15%. The loss of additional nucleotides simply reduces the binding activity further,

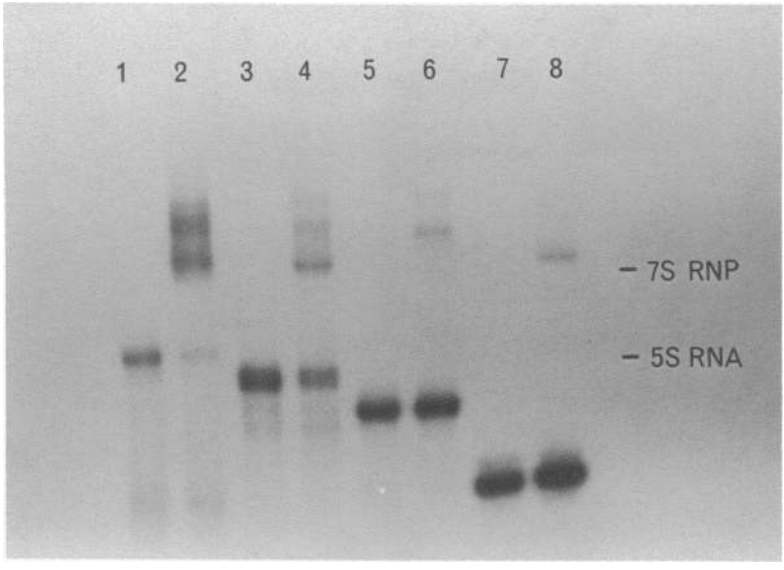


Figure 3. Autoradiogram of a non-denaturing polyacrylamide gel showing the exchange properties of 3' deletion fragments of 5S RNA. Incubations were: 1. 5S RNA, 2. 5S RNA + 7S RNP; 3. 1-108, 4. 1-108 + 7S RNP; 5. 1-98, 6. 1-98 + 7S RNP; 7. 1-69, 8. 1-69 + 7S RNP.

until the K_a approaches that measured for the nonspecific binding of yeast $tRNA^{phe}$ to TFIIIA [18].

The 5' truncated fragments were produced by limited nuclease cleavage of *Xenopus* oocyte 5S RNA. This method did not yield any useful 3' truncates of 5S RNA, and a different approach was used to study the effects of 3' deletion on TFIIIA binding. A synthetic gene was constructed with a unique Stu I site introduced at nucleotide +108 of the *Xenopus* oocyte 5S RNA sequence (Figure 1B). Cleavage of plasmid pXlo-108 by Stu I followed by *in vitro* transcription with T7 RNA polymerase yielded a derivative of the oocyte 5S RNA molecule containing only nucleotides 1-108. Cleavage of the same clone at the Fok I site at nucleotide +98, and a Bst NI site at nucleotide +69 produced the templates used to prepare the other 3' truncated molecules. The results of a typical binding assay with the 3' deleted fragments are shown in Figure 2B, and a summary of the data are presented in Table 1. The loss of up to 23 nucleotides from the 3' end of the RNA molecule reduces the binding of TFIIIA only slightly, while the binding activity is significantly reduced by the loss of a further 28 nucleotides. The data in Table 1 suggest that the loss of structural and/or sequence information at the 3' end of the 5S RNA is less detrimental to TFIIIA binding than is the loss of nucleotides at the 5' end.

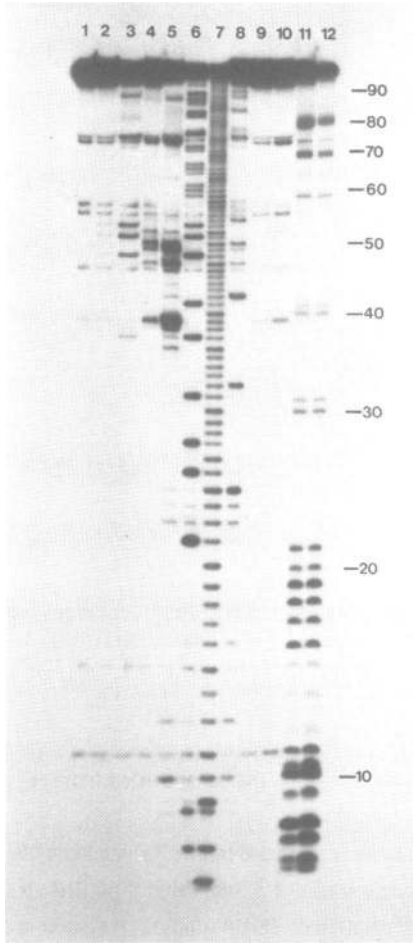
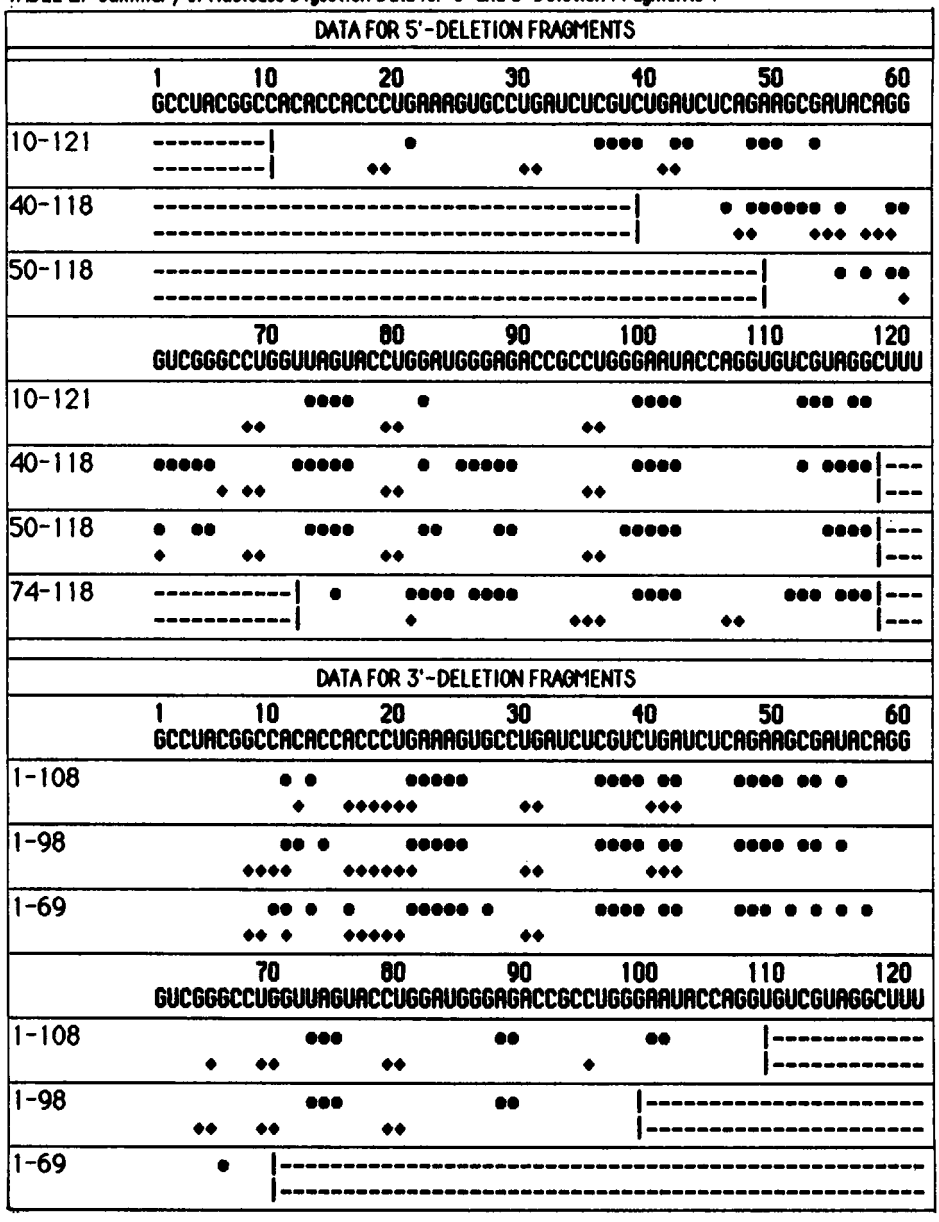


Figure 4. Nuclease probing of the RNA conformation in 5'-^{32}P end labelled 1-98 fragment. Incubations were: 1. control (no nuclease), 2. 0.1 U RNase T_1, 3. 1 U RNase T_1, 4. 0.01 U RNase T_2, 5. 0.1 U RNase T_2, 6. T_1 sequencing reaction, 7. base hydrolysis, 8. U_2 sequencing reaction, 9. 0.01 ng RNase A, 10. 0.1 ng RNase A, 11. 0.35 U cobra venom nuclease, 12. 0.7 U cobra venom nuclease. Numbers to the right indicate the sequence position.

2. Exchange of Truncated 5S RNA Molecules into 7S RNP

It has been reported that the loss of 4 nucleotides from the 3' end of *Xenopus* 5S RNA completely abolishes the ability of the RNA to exchange into native 7S RNP particles [21]. The results presented above obtained with the filter binding assay indicate that 5S RNA fragments lacking up to 23 nucleotides from the 3' end still bind quite strongly to purified TFIIIA. The

TABLE 2. Summary of Nuclease Digestion Data for 5' and 3' Deletion Fragments[a].



[a]Symbols indicate: ●, cleavage 3' of the indicated nucleotide by ribonuclease T_1, T_2 or A (single strand specific); ◆, cleavage 5' of the indicated nucleotide by ribonuclease V_1; |-, border of deleted region.

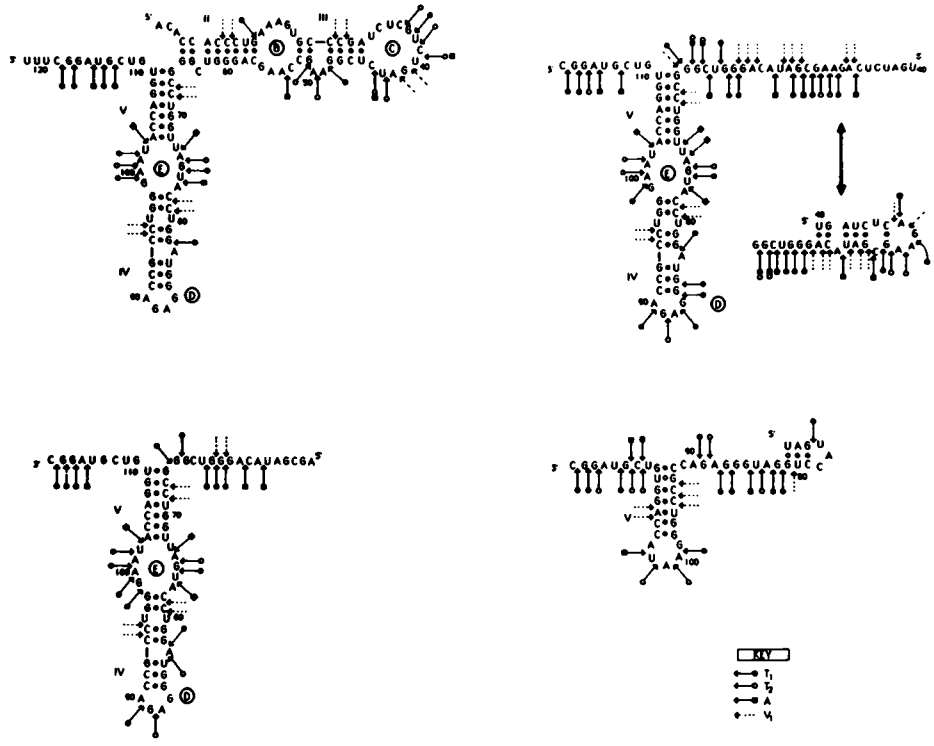


Figure 5. Summary of the results of the secondary structural analysis of 5' deletion fragments of *Xenopus* 5S RNA. TOP: 10-121 (left), 40-118, with an alternate conformation for the 40-65 region (right), BOTTOM: 50-118 (left), 74-118 (right).

ability of these fragments to exchange into 7S RNP was measured using the conditions described by Andersen and Delihas [21], and the results of this experiment are shown in Figure 3. It is obvious that all of the 3' truncated fragments did exchange into 7S RNP, although differences in the TFIIIA binding activity of the fragments were accentuated. Prolonged storage of the labelled 5S RNA and 1-108 fragment resulted in some degradation of the RNA from radionucleolytic damage (lanes 1-4). However, the degraded RNA did not exchange into the 7S RNP (compare lanes 1 and 2, for example) and therefore its presence did not affect the interpretation of the experimental results. The exchange of the 1-98 and 1-69 fragments gave rise to particles with a slower gel mobility than 7S RNP. This altered mobility probably reflects two consequences of deleting a substantial portion of the 5S RNA: a less compact conformation of the resulting RNP compared to 7S RNP and a decrease in the negative charge density.

3. Conformation of Truncated 5S RNA Molecules

The reduction in K_a observed when oocyte 5S RNA is truncated may result from a loss of

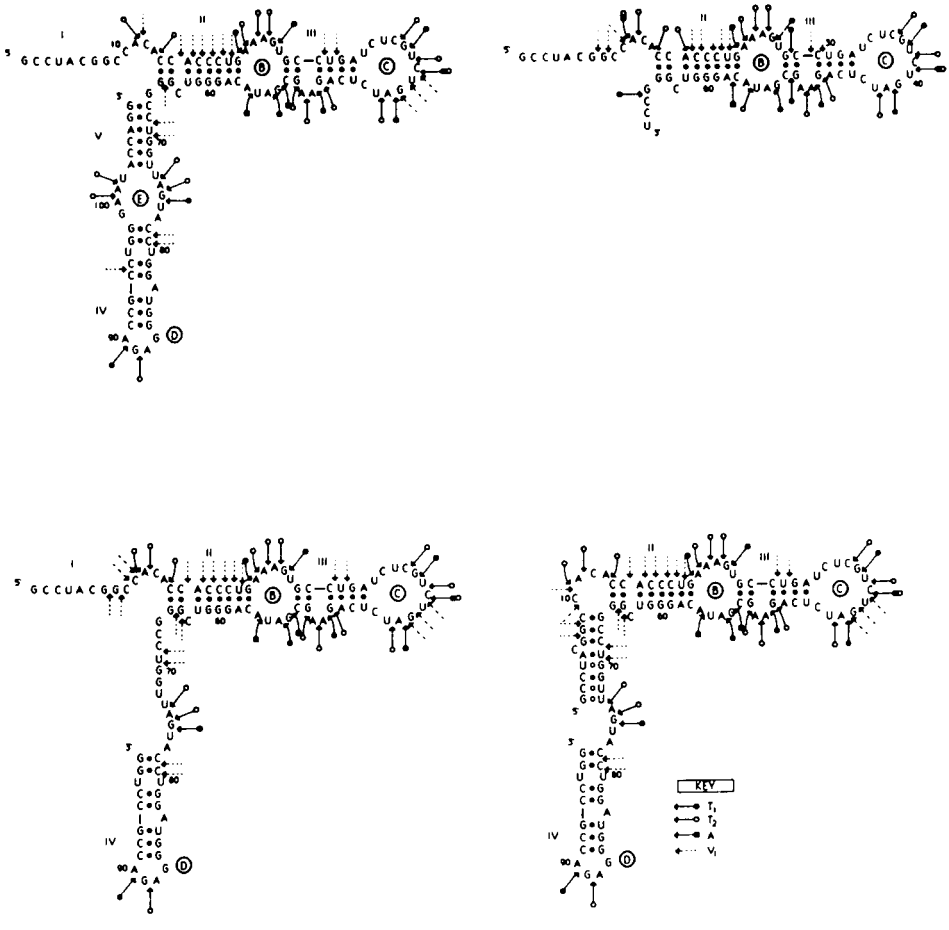


Figure 6. Summary of the results of the secondary structural analysis of 3' deletion fragments of *Xenopus* 5S RNA. TOP: 1-108 (left), 1-69 (right), BOTTOM: Two possible structures for the 1-98 fragment. The structure on the right is consistent with the lack of single strand nuclease cleavages in the 1-9, 66-72 regions (see text).

structural and/or sequence information required for the optimal binding of TFIIIA. However, it is also possible that some of the RNA fragments form unusual secondary structures which are unable to bind to the transcription factor. The structure of each fragment was analyzed using limited nuclease digestion to probe for single stranded (nucleases T_1, T_2, A) and double stranded (cobra venom nuclease V_1) structural features. An autoradiogram of a sequencing gel showing the analysis of the 1-98 fragment is presented in Figure 4. The results of similar studies for all of the RNA fragments are summarized in Table 2 and Figures 5 and 6.

The effect of 5' deletions on the relative stability of the remaining 5S RNA secondary structural features is indicated in Table 2 and Figure 5. The deletion of up to 49 nucleotides has no discernible effect on the structure of the helix IV-V domain (Table 2). The loss of 9 nucleotides from the 5' terminus of the intact 5S RNA apparently has no effect on the helix II-III structural domain either, and the structural analysis of the 10-121 fragment is consistent with the disruption of helix I by the loss of the 5' pairing region.

In comparison, the loss of an additional 30 nucleotides leads to a dramatic difference in the nuclease sensitivity of the 40-65 region of the 40-118 fragment (Table 2). These nucleotides are normally involved in the formation of helices II and III in intact 5S RNA, and were expected to be single-stranded in the 40-118 fragment. In fact, this region of the fragment was cleaved at a number of sites by the single strand specific nucleases T_1, T_2, and A. There were also eight cobra venom nuclease cleavages observed in this same region of the molecule, which is usually indicative of a double stranded structure. It has been demonstrated that cobra venom nuclease cleaves within areas of highly stacked single stranded RNA [34]. Therefore the top conformation of the 40-65 region (Figure 5) would be consistent with both the single stranded and cobra venom nuclease cleavage patterns. However, the data would also support an equilibrium between two conformations as shown in Figure 5 (top right). Since the helix IV-V domain is intact, and the 3' terminal portion of helix I is cleaved only by single strand specific nucleases, the 40-65 region can presumably only form a base pairing arrangement with itself. Of the various possible conformations, only the base paired structure shown is consistent with all of the cobra venom nuclease data and many of the single stranded nuclease data. Therefore the poor TFIIIA binding activity of the 40-118 fragment is explained by the altered conformation of the 40-65 region.

The 50-118 fragment similarly has an intact helix IV-V domain, but the 5' deletion in this fragment extends almost to the end of the helix II-III domain. The remaining 15 nucleotides of this domain apparently adopt a stacked single stranded conformation (Figure 5).

The nuclease digestion data indicate that the 74-118 fragment no longer retains any 5S RNA-like structural elements. The increased single stranded cleavages observed in the 78-88 region, combined with a loss of cobra venom sensitivity, suggests that helix IV is not formed in this fragment. The structural data are consistent with the structure shown in Figure 5. The pairing of nucleotides 92-98 with nucleotides 104-110 has also been observed with a 76-118 fragment of *Xenopus* 5S RNA [39].

The structural analysis of the 3' deletion fragments is summarized in Table 2 and Figure 6. These fragments maintain familiar 5S RNA structural elements. Only the conformational analysis of the 1-98 fragment revealed several unusual features. In intact 5S RNA nucleotides 66-72 base pair with 103-109 to form helix V, which lies within the region protected by TFIIIA from modification [15-18]. In the fragment nucleotides 1-9 and 66-72 were expected to be single stranded, yet only cobra venom nuclease cleaved within these regions (Table 2).

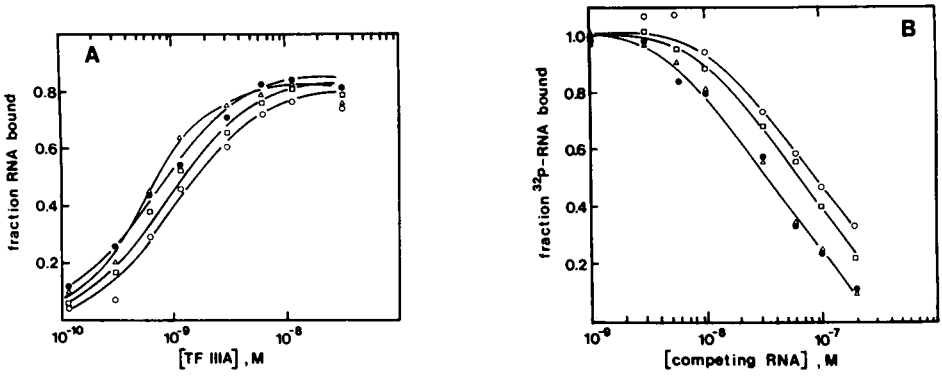


Figure 7. A. Titration of synthetic 5S RNAs with TFIIIA under equilibrium binding conditions. B. Competition between labelled wheat germ 5S RNA and unlabeled synthetic 5S RNAs for binding to TFIIIA. Symbols used are: somatic, wild type 5S RNA (●), 5'-somatic, 3'-oocyte chimeric 5S RNA (△), 5'-oocyte, 3'-somatic chimeric 5S RNA (□), oocyte wild type 5S RNA (○).

The first eight nucleotides of this fragment have a striking complementarity to the 66-72 sequence, and it is possible to draw a secondary structure for the 1-98 fragment which accounts for the nuclease digestion data (Figure 6). In addition, cobra venom cleavages are observed at residues 41-43 of loop C in fragment 1-98, but are not observed in intact 5S RNA, or any of the other fragments except for much weaker cleavages at the same site in the 1-108 fragment. The conformational adaptation of the 1-98 fragment clearly does not interfere with TFIIIA binding, and the formation of a pseudo helix V structure is probably responsible for the retention of protein binding activity.

4. Binding of Somatic and Chimeric 5S RNA Molecules to TFIIIA

TFIIIA has a differential affinity for the somatic and oocyte 5S RNA genes of *Xenopus* [35]. In order to determine whether a similar difference exists in the RNA-TFIIIA interaction, the binding of the protein to the somatic, oocyte and two chimeric 5S RNA molecules was measured. The RNAs were synthesized from the constructs shown in Figure 1B using T7 RNA polymerase. RNA labelled internally using [α-^{32}P] GTP was used for the direct binding assays with TFIIIA (Figure 7A). The relative binding affinities, averaged from several independent determinations, are listed in Table 3. The somatic 5S RNA binds to TFIIIA approximately 1.7 times tighter than the oocyte 5S RNA. Of the two chimeric RNA molecules, the 5'-Xls chimer has a binding affinity similar to somatic 5S RNA, while the 5'-Xlo chimer has a binding affinity similar to oocyte 5S RNA (Table 3).

In a competition assay, the somatic 5S RNA gene has a four fold higher affinity for TFIIIA compared to the oocyte gene [35]. Figure 7B shows the results of a similar competition assay

TABLE 3. Relative Binding and Competition Strengths of *Xenopus* 5S RNAs

RNA	Binding[a]	Competition[b]
somatic	1.00	1.00
5'-somatic chimer	0.98	0.85
5'-oocyte chimer	0.85	0.42
oocyte	0.60	0.33

[a]ratio of K_a(5S RNA)/K_a(somatic 5S RNA)

[b]ratio of concentrations of 5S RNA and somatic 5S RNA giving 50% competitive inhibition

which measures the ability of the somatic, oocyte and chimeric 5S RNAs to compete with labelled wheat germ 5S RNA for binding to TFIIIA. The relative competition strengths determined from this assay are listed in Table 3. In the competition assay, the somatic 5S RNA has a three fold higher affinity for TFIIIA than does the oocyte 5S RNA, and the 5'-Xls chimer has a competition strength similar to the purely somatic 5S RNA. In the DNA-TFIIIA interaction, the higher affinity of the somatic gene for the protein is also the result of somatic-specific nucleotides in the 5'-half of the gene [35].

DISCUSSION

The physical binding site for TFIIIA on the 5S RNA gene has been studied by deletion mapping [1,2,35], footprinting [3,4], chemical modification [36], and mutational analysis [37,38]. An exact definition of the borders of the binding site was obtained by a combination of footprinting experiments [3,4], and quantitative measurement of the effects that gene deletion had on the transcriptional activity [1,2] and competition strength of 5S genes [35]. Deletions from the 3' end of the gene up to nucleotide 98 had no effect either on transcriptional activity or on the binding of TFIIIA, but both activities decreased rapidly upon the deletion of further nucleotides [2,35]. Deletions from the opposite direction past nucleotide +28 of the coding region of the gene caused a significant reduction in competition strength and transcriptional activity [1,35]. These results indicated that the region essential for the binding of TFIIIA to the 5S gene is contained within nucleotides +47 to +97. Even when this internal control region (ICR) is removed from the context of the 5S RNA gene, TFIIIA still binds and directs transcription by RNA polymerase III [2].

The binding region for TFIIIA on 5S RNA has been investigated by chemical modification and nuclease digestion [15-18], and encompasses nucleotides 52-110, with the primary site of interaction centering upon the helix IV-V region of the molecule. In order to determine the functional consequences of removing the RNA binding site from the context of intact 5S RNA, we

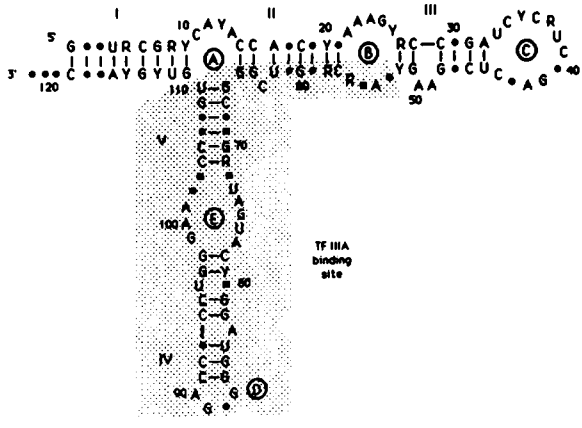


Figure 8. Consensus model derived by comparing the sequences of those eukaryotic 5S RNAs which bind to TFIIIA [18-21]. The shaded area represents the region of *Xenopus* oocyte 5S RNA protected from modification when TFIIIA is bound [15-18].

have measured the apparent association constants of a series of 5S RNA molecules which have been truncated from either the 3' or 5' terminus.

Deletions from the 3' end of oocyte 5S RNA up to position 98 had only a slight effect on the binding of TFIIIA. This result was surprising in view of the fact that the TFIIIA protection zone includes nucleotides 99-110 [15-18]. Further deletion of the RNA to nucleotide 69 resulted in a 2.5 fold reduction in the affinity of the RNA for TFIIIA. In contrast, a ten fold decrease in transcriptional activity was observed when 3' deletion of the 5S gene was extended past nucleotide 98 to nucleotide 83 [35].

Deletion of the first 10 nucleotides from the 5' end of the 5S RNA resulted in a small decrease in binding affinity, while deletion of nucleotides 1-39 had a dramatic effect on the binding of TFIIIA. This 40-118 fragment still contains the critical 52-110 region and is a reasonably strong competitive inhibitor of 5S gene transcription [19]. Deletion of the 5' end of the RNA up to nucleotides 50 and 74 resulted in a further decrease in TFIIIA binding activity. These effects are similar to those observed for comparable deletions of the 5S RNA gene [35].

The interpretation of the above binding results is not straightforward, since a decrease in binding affinity may result either from the loss of nucleotides which form direct bonding interactions with the protein, or from a change in the conformation of the RNA. This dilemma is perhaps best illustrated by the poor binding activity of several fragments which contain the intact 52-110 sequence, and the strong binding activity of the 1-98 fragment, which lacks 12 nucleotides of this sequence. The secondary structure of each fragment was therefore studied using single strand and double strand specific nucleases to provide information regarding the conformational consequences of these deletions.

The poor binding activity of 5' deletion fragments which still contain the 52-110 sequence is related to a conformational change of the 40(50)-65 region from the helix II-loopB-helix III structure found in 5S RNA. In the 40-118 fragment, this region may alternate between single stranded and partially base paired structures. In comparison, the strong binding activity of the 1-98 fragment apparently is the result of the formation of a pseudo helix V structure which pairs nucleotides 1-9 with 66-72. Therefore the loss of nucleotides 99-110 in this fragment is compensated for by a conformational change which maintains the binding interaction with TFIIIA.

Based upon the binding data obtained with the truncated 5S RNA molecules and an understanding of the conformational properties of these fragments, it is reasonable to conclude that unlike the DNA-TFIIIA interaction, the binding site on 5S RNA cannot be removed from its sequence context and retain a complete interaction with TFIIIA. This difference is a consequence of the fact that placement of the ICR in a plasmid does not remove it from the conformational context of supercoiled DNA, while the deletion of 5S RNA sequences often disrupts the tertiary structure of the molecule in a way that reduces TFIIIA binding. Although the results of footprinting TFIIIA on 5S RNA suggest that the primary site of interaction lies within nucleotides 52-110 of the RNA [15-18], the fragment binding studies suggest that conformational requirements define the minimum TFIIIA binding site to be nucleotides 11 to 108. The alteration of the helix II-III structure resulted in a more dramatic reduction in TFIIIA binding than the deletion of the helix IV-V region of the 5S RNA. This result suggests that helix II-III may play a more important role in the interaction with the protein than was indicated by the footprinting experiments [15-18].

The binding data that we have obtained with RNA fragments can be compared to a similar study using an exchange assay [21]. The exchange of labelled 5S RNA fragments into 7S RNP is abolished upon the deletion of even a few nucleotides from either terminus [21]. The fragments used in the present study also exchange into 7S RNP, although with a lower efficiency than intact 5S RNA. The discrepancy in the results obtained with the two assays is most likely related to the methods employed to detect RNA-TFIIIA complexes. In the exchange assay, 7S RNP complexes are separated from free RNA on non-denaturing polyacrylamide gels. However, the complexes can only be observed when the component concentration in the assay is greater than 1 μM, a concentration which is three orders of magnitude higher than the dissociation constant for 5S RNA-TFIIIA complexes as measured by filter binding [18] and DNase footprinting assays [20]. Apparently RNA-TFIIIA complexes are not stable under the conditions of electrophoresis employed, and the complexes formed by the exchange of RNA fragments are likely to be even less stable. In contrast, the stability of RNA-TFIIIA complexes to filtration has been clearly demonstrated [18].

While this manuscript was in preparation, a report was published on the analysis of the

competition strength of 5' deletion fragments of 5S RNA measured using a transcription assay [39]. In contrast to the results reported here, Pieler and co-workers have found that much of the 5' region of the 5S RNA can be deleted with only a slight decrease in the ability of the RNA to compete with the gene for the binding of TFIIIA [19,39]. These results were obtained using a single RNA concentration of ca. 0.1 - 1 μM, well above the K_d values measured for similar fragments using the filter binding assay (e.g. Figure 2A). Therefore, the RNA concentration used in the competition assay was saturating for most of the fragments studied, leading to an overestimate of the relative binding strengths. In comparison, the relative binding strengths measured in this paper are more precisely defined as the ratio of the apparent association constants for the RNA fragment and intact 5S RNA.

TFIIIA has a four fold higher affinity for the somatic 5S RNA gene compared to the oocyte 5S RNA gene [35]. We have synthesized somatic and oocyte 5S RNAs from cloned genes using T7 RNA polymerase. The synthetic oocyte 5S RNA is indistinguishable from natural oocyte 5S RNA in its binding interaction with TFIIIA and in its secondary structure as determined by nuclease digestion (I.S. and P.J.R. in preparation). The results of both direct binding experiments and competition experiments indicate that TFIIIA has a higher affinity for the somatic vs. oocyte 5S RNA. By reconstructing the synthetic genes, two chimeric 5S RNA molecules were created, and from the binding experiments, it is apparent that the increased affinity of TFIIIA for somatic 5S RNA is related to nucleotide differences in the 5' region of the two RNAs. A similar result was obtained in a study on the competition strength of chimeric 5S RNA genes [35]. In this respect the DNA and RNA binding properties of TFIIIA are identical.

The results of the experiments described here can be combined with data obtained for the binding of other eukaryotic 5S RNAs [18-21], in order to compile a preliminary consensus binding site for TFIIIA. It is apparent from the structure shown in Figure 8 that even given the limited amount of data, there is very little sequence homology in most stems. Highly conserved sequences occur primarily in the single stranded loops, and helix IV. The binding data obtained with truncated molecules of oocyte 5S RNA imply that helix I is not required for the binding of the transcription factor. It has been proposed that a repeating CCUGG base paired structure in helices IV and V is an important structural feature for the binding of TFIIIA to *Xenopus* 5S RNA [16]. The consensus structure shows however that the CCUGG sequence is conserved in helix IV, but not helix V. Of course, it is still possible that a CCUGG sequence is required in helix V of *Xenopus* oocyte 5S RNA, but its absence in other eukaryotic 5S RNAs is compensated for by other sequence changes.

Clearly, further study is required before a comprehensive consensus binding site for TFIIIA can be drawn. The development of an *in vitro* method for synthesizing altered *Xenopus* 5S RNA in the amounts necessary for detailed structural and functional studies will make it possible to determine which nucleotides of 5S RNA are essential for the interaction with TFIIIA.

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