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Characterization of the RNA binding properties of transcription factor IIIA of *Xenopus laevis* oocytes

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ABSTRACT

A nitrocellulose filter binding assay has been developed to study the interaction of Xenopus transcription factor IIIA with 5S RNA. The protein binds Xenopus oocyte 5S RNA with an association constant of 1.4×10^9 M^{-1} at 0.1 M salt, pH 7.5 at 20°C. TF IIIA binds wheat germ 5S RNA with a two-fold higher affinity, E. coli 5S RNA with a four-fold weaker affinity, and has a barely detectable interaction with yeast $tRNA^{phe}$. The preference for binding eukaryotic 5S RNA is enhanced in competition assays. The homologous reconstituted complex contains one molecule each of protein and 5S RNA and is indistinguishable from native 7S RNP in mobility on non-denaturing polyacrylamide gels. The conformation of the RNA in reconstituted particles is identical to the conformation of RNA in native 7S RNP. Further analysis of the homologous interaction reveals that complex formation is favoured both by enthalpy and entropy. The 5S RNA binding activity has a broad pH optimum spanning pH 6.0 to pH 8.0. Determination of the salt dependence of K_a reveals that as many as 5 lysine-phosphate type ionic bonds may be formed in the homologous complex. Approximately 68% of the free energy of complex formation is contributed by non-electrostatic interactions between TF IIIA and Xenopus 5S RNA.

INTRODUCTION

Immature oocytes of Xenopus laevis accumulate a 7S ribonucleoprotein particle that is a 1:1 complex of 5S RNA and a 40 Kd protein [1]. The protein component of this particle has been shown to be one of the RNA polymerase III-associated transcription factors, TF IIIA, required for expression of the 5S RNA gene [2]. By binding to an intragenic control region in the DNA, TF IIIA promotes the initiation of transcription [3-6]. The 7S storage particle is then formed by the transcription factor and the 5S RNA transcript, and may have a potential role in feedback regulation of 5S gene expression [2].

TF IIIA therefore binds specifically to both DNA and RNA, and the interaction of the protein with DNA has been studied extensively [7-13]. The protein has a high, nonspecific affinity for single stranded DNA [7]. One TF IIIA molecule binds in a specific mode to the intragenic control region of the 5S RNA gene [8,9], with a dissociation constant of approximately 1 nM [7].

Chemical modification studies indicate that the protein interacts primarily with the non-coding strand of the gene [10], without causing a conformational change in the DNA [11,12]. The protein contains tightly bound Zn^{2+} ions required for the specific interaction with the 5S RNA gene [13] and apparently is organized into several structural domains [9]. The amino acid sequence of TF IIIA has been deduced from a cDNA clone [14].

The RNA binding properties of this molecule are less well understood. Chemical and nuclease probes have been used to map the protein binding region on 5S RNA in the isolated 7S particle [15-17]. The use of RNA molecules as competitors in DNA footprinting assays has demonstrated that eukaryotic 5S RNA molecules compete with DNA for binding to TF IIIA, while other RNA molecules do not [7]. Similar studies have recently been reported on the inhibitory effect of a variety of 5S RNA molecules on *in vitro* transcription [18]. In the work reported here, a nitrocellulose filter binding assay has been used to study the specificity of the RNA binding activity of TF IIIA. Thermodynamic and kinetic parameters have been measured for the interaction of TF IIIA with *Xenopus* 5S RNA. The homologous reconstituted complex has a mobility on non-denaturing polyacrylamide gels that is indistinguishable from native 7S RNP.

MATERIALS AND METHODS

Isolation of the 7S RNP particle

The 7S RNP particle was isolated from the ovaries of immature *X. laevis*, purchased from Xenopus I, Ann Arbor, MI. The procedure used was basically that of Picard and Wegnez [1] as modified by Hanas *et al.* [19].

Preparation of TF IIIA

TF IIIA was prepared by two methods. The first method used was a modification of the procedure of Hanas *et al.* [7]. A solution of 7S RNP (1.5 mg/ml) was incubated for 90 min at room temperature with 10 µg/ml of RNase A. The incubation was then loaded on a 30 ml Sephadex G-50 column (0.7 × 40 cm) equilibrated in 20 mM Tris, 250 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DDT, pH 7.5 (G-buffer) and the proteins were eluted with the same buffer. TF IIIA eluted in the exclusion volume of the column, free of 90% of the input A_{260} absorbing material. The concentration of protein solutions was determined by the Bradford assay [20]. These TF IIIA preparations were judged to be 85-90% pure by SDS-PAGE and could be stored in the elution buffer at either 4°C or -70°C for 10-14 days before a loss in RNA binding activity was observed.

The second method used for preparing TF IIIA was modified from the procedure of Smith *et al.* [9] in which RNase A digested 7S RNP is purified by

chromatography on BioRex 70. In their procedure, 7S RNP is digested for 5 min with RNase A, and then 10 M urea is added to completely dissociate the RNA fragments from the protein before loading onto the column. We have found that by extending the digestion time from 5 min to 1 h, virtually all of the TF IIIA is retained on the column without the need for urea treatment. TF IIIA prepared by this method was typically 98-99% pure [9] and was stored at 4°C in the elution buffer.

Isolation and end labelling of RNA

Yeast $tRNA^{phe}$ was purchased from Boehringer. Xenopus 5S RNA was isolated either by phenol extraction of 7S RNP, or by phenol extraction of crude ovary homogenates followed by purification on a Sephacryl S-200 column [21]. Other 5S RNA samples were isolated from the appropriate ribosomes and purified in a similar manner. RNA was radiolabelled with ^{32}P at the 3' end using standard methods [22], repurified on 8.3 M urea - 15% polyacrylamide gels and stored as an aqueous solution at -20°C. Greater than 99% of the 5S RNA molecules were intact, as determined by subsequent denaturing gel analysis. For nuclease digestion experiments, native 7S RNP was radiolabelled with ^{32}P at the 3' end of the RNA by the procedure of Andersen et al. [17].

Filter binding assays

The standard TMK buffer for the filter binding assays was 20 mM Tris, 5 mM $MgCl_2$, 100 mM KCl, 100 µg/ml BSA, 1 mM DTT, adjusted to pH 7.5 at the incubation temperature. TF IIIA was serially diluted in 180 µl of TMK buffer to give final concentrations ranging from 0.4 nM to 0.6 µM and equilibrated for 10 min at the indicated temperature. The assay was started by the addition of 20 µl of end labelled RNA (3-5 nCi, ca. 0.1-0.5 nM final RNA concentration) and allowed to equilibrate for 15 min. A 180 µl aliquot was then removed and filtered through a pre-soaked nitrocellulose filter which was then dried and counted in a toluene based scintillant. Retention of free RNA on the filters was typically 5-10% of input and this value was used to correct measurements of complex formation.

Some TF IIIA preparations were found to be contaminated by small traces of RNase A, which could be completely inhibited by adding 200 U/ml RNasin (Promega Biotec) to the assay buffer. The addition of RNasin prevented the loss of RNA bound in the plateau region of the binding curve where the TF IIIA concentration was greater than 1×10^{-8} M. Control experiments indicated that RNasin did not interfere with the binding equilibrium between TF IIIA and RNA. RNasin was therefore added to those assays which required elevated protein concentrations or temperature, to ensure that the RNA was intact.

Gel electrophoresis of reconstituted complexes

Complexes between 5S RNA (5 nCi, ca. 0.02-0.03 μM final RNA concentration) and TF IIIA (0-0.6 μM) were formed by incubating the components in 10 μl G-buffer at 4°C for 10 min. The samples were diluted by the addition of 5 μl of loading buffer (50 mM Tris borate pH 7.5, 1 mM EDTA, 10% glycerol, 0.05% bromphenol blue and xylene cyanol) and then applied to a non-denaturing 10% polyacrylamide gel [17]. After electrophoresis at 300V for 4.5 h at 4°C the gel was autoradiographed. Markers for 7S RNP were provided either by following the exchange of labelled Xenopus 5S RNA into unlabelled 7S RNP under similar incubation conditions [17], or by applying 3' end labelled 7S RNP to the gel.

Nuclease digestion of RNP complexes

The nuclease sensitivity of reconstituted complexes and native 7S RNP was measured in the following way. Reconstituted complexes formed between 3'-end labelled Xenopus 5S RNA (0.5 μCi, 1 nM final concentration) and TF IIIA (3 nM final concentration) or 3'-end labelled native 7S RNP (0.5 μCi, 1 μM final concentration) were incubated for 15 min at 20°C in standard TMK buffer containing 300 U/ml RNasin. Then RNase T_1 or T_2 was added to give final concentrations of 10 or 1 U/ml RNase T_2 or final concentrations of 1000 or 100 U/ml RNase T_1, and incubation was continued for a further 5 min. The reaction was then filtered through a pre-soaked nitrocellulose filter, and the filter was washed with a further 0.5 ml of TMK buffer lacking BSA. The filters were extracted for 10 min with 0.3 ml of 0.1 M Tris, 0.5 M ammonium acetate pH 8.0 and then 0.3 ml of neutralized phenol was added and the extraction continued for 15 min. Labelled RNA was recovered from the aqueous layer by ethanol precipitation in the presence of 20 μg E. coli bulk tRNA.

The nuclease sensitivity of free Xenopus 5S RNA was measured in parallel, using identical incubation conditions. In this case, the reactions were stopped by extracting with an equal volume of 50:50 phenol-chloroform. Labelled RNA was then recovered using the procedure outlined above.

The recovered RNA was dissolved in a sample buffer containing 8 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA and 0.2% each xylene cyanol and bromophenol blue, and then denatured by heating for 3 minutes at 90°C and immediately cooling on ice. The samples were analyzed by electrophoresis on 8.3 M urea, 12% polyacrylamide sequencing gels (40 cm × 35 cm × 0.03 cm) at 2500 V for 2.5 h. A geiger counter was used to ensure that an equal amount of radioactivity was loaded into each lane of the gel. The gels were autoradiographed for 24 h at -70°C with Kodak XK-1 x-ray film.

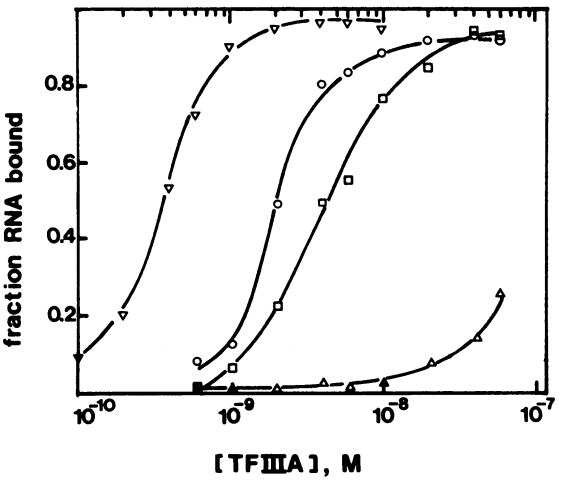


Figure 1. Titration of RNA with TF IIIA under equilibrium binding conditions. RNAs used were wheat germ 5S RNA (▽), Xenopus 5S RNA (○), E. coli 5S RNA (□), and yeast $tRNA^{phe}$ (△).

RESULTS

Equilibrium constants

The interaction between TF IIIA and various RNA molecules was measured using a nitrocellulose filter binding assay. In this assay, RNA at a constant, low concentration (ca. 0.1-0.5 nM) was incubated in TMK buffer at 20 °C with varying concentrations of TF IIIA (0.4 nM to 0.6 μM) for 15 min. The reactions were filtered without dilution or subsequent washing. The filters retain free protein and protein-nucleic acid complex, while allowing uncomplexed RNA to pass through. The results of one such assay are shown in Figure 1.

It is possible to analyze the data in Figure 1 using a simple bimolecular equilibrium model, provided that certain conditions in the assay are met [23]. It is important to determine whether the filtration process in any way alters the equilibrium. The primary concerns are whether complexes initially bound might dissociate during the filtration process, or if free protein bound to the filters might trap free RNA during filtration. The dissociation rate of complexes bound to filters was tested by filtering two separate 90 μl aliquots from each 200 μl incubation. One set of aliquots was filtered without washing and the second set of aliquots was filtered and then the filters washed with 500 μl of TMK buffer without BSA. The association constants determined by the two procedures were virtually identical. This result indicates that once

bound to a nitrocellulose filter, TF IIIA-RNA complexes are extemely stable to further washing of the filter and that dissociation of bound complexes is unlikely to occur during the normal filtration of aliquots. The ability of bound protein to trap free RNA was tested by first filtering a TF IIIA solution and then filtering a solution of free RNA through the same filter. At TF IIIA concentrations of less than 100 nM, less than 5% of the RNA was trapped by protein pre-bound on the filter.

Incomplete retention of protein-nucleic acid complexes on nitrocellulose filters is a general phenomenon [23-25]. For the TF IIIA-5S RNA binding assay, the fraction of RNA bound at the plateau was 65-100% of input RNA. The retention efficiency obtained varied with the particular preparation of labelled RNA, suggesting that the purification methods may affect the activity of the labelled RNA. Careful renaturation of the RNA did not increase the fractional retention efficiency obtained. The variability of retention efficiency with the preparation of labelled RNA has been observed before [25]. Assuming that the percent RNA bound at the plateau represents complete binding of active RNA [23-25], the K_d value for a simple bimolecular equilibrium can be expressed as the TF IIIA concentration at which 50% saturation is achieved, provided that 100% of the protein is active. A Scatchard analysis indicated that this level of activity was obtained with the TF IIIA preparations (see below).

Analysis of data from a number of experiments similar to that shown in Figure 1 indicate that TF IIIA binds Xenopus 5S RNA with an apparent association constant (K_a) of $1.0 \pm 0.5 \times 10^9\ M^{-1}$, wheat germ 5S RNA with a K_a of $2.7 \pm 0.5 \times 10^9\ M^{-1}$, E. coli 5S RNA with a K_a of $2.5 \pm 1 \times 10^8\ M^{-1}$, and yeast $tRNA^{phe}$ with a K_a of less than $1.0 \times 10^7\ M^{-1}$. Apparently TF IIIA has a strong affinity for 5S RNA in general, but not for $tRNA^{phe}$.

The specificity of the RNA binding activity of TF IIIA was further tested by a competition assay (Figure 2). In these experiments, labelled Xenopus 5S RNA (ca. 0.5 nM) was mixed with unlabelled competitor RNAs at the indicated concentrations, the assay was begun by the addition of TF IIIA to a final concentration of 6 nM and after 15 min incubation at 20°C, aliquots were withdrawn and filtered. The results indicate that both wheat germ and Xenopus 5S RNA are strong competitors with labelled Xenopus 5S RNA for binding to TF IIIA, whereas E. coli 5S RNA competes poorly and yeast $tRNA^{phe}$ does not compete at all. The weak competition strength of E. coli 5S RNA was surprising, given its relatively strong affinity for TF IIIA in the direct binding assay. To test whether E. coli and Xenopus 5S RNAs bind to separate sites

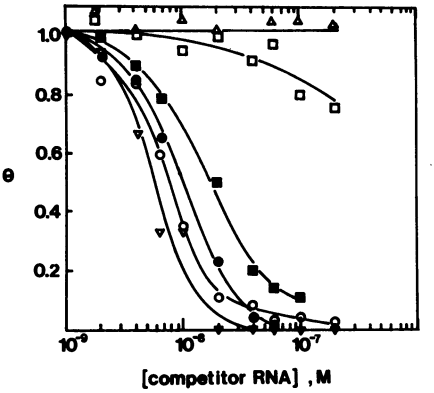


Figure 2. Competition between labelled Xenopus 5S RNA (open symbols) or labelled E. coli 5S RNA (closed symbols) and various unlabelled RNAs for binding to TF IIIA: wheat germ 5S RNA (▽), Xenopus 5S RNA (○, ●) E. coli 5S RNA (□, ■) and yeast $tRNA^{phe}$ (△). θ is the ratio of RNA bound in the presence and absence of competitor RNA.

on the protein, competition assays were repeated using ^{32}P-labelled E. coli 5S RNA, unlabelled E. coli and Xenopus 5S RNAs as competitors, and adding TF IIIA to a final concentration of 10 nM. The results indicate that Xenopus 5S RNA competes with labelled E. coli 5S RNA for the same binding site on TF IIIA (Figure 2).

Stoichiometry of the homologous complex

A Scatchard analysis [33] of the interaction between Xenopus 5S RNA and TF IIIA was used to determine the stoichiometry of the complex. The analysis carried out using the standard binding assay, except that the TF IIIA concentration was held constant at 3 nM, and the RNA concentration was varied from 0.6 nM to 30 nM. As the results in Figure 3 indicate the complex is formed between one molecule of 5S RNA and one molecule of TF IIIA. The association constant measured by the slope of the line is $1.3 \times 10^9\ M^{-1}$, in close agreement with that measured by the standard binding assay. This result also indicates that the TF IIIA preparation is 100% active in the RNA binding assay.

Comparison of isolated 7S RNP and the reconstituted homologous complex

To further investigate the nature of the reconstituted complex, the mobility of complexes formed between purified RNA and protein were compared to isolated 7S RNP on non-denaturing polyacrylamide gels. The results of autoradiography of such a gel are shown in Figure 4. At approximate protein excesses of 10 to 30-fold, Xenopus 5S RNA primarily forms a complex with TF

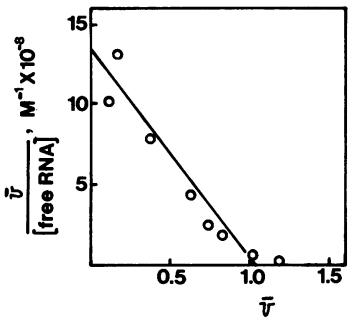


Figure 3. Scatchard analysis of the interaction between TF IIIA and Xenopus 5S RNA.

IIIA which has a mobility identical to 7S RNP. By contrast, complexes formed with E. coli 5S RNA have significantly different mobilities compared to the homologous complexes.

Bands migrating slower than 7S RNP in the exchange reaction (lanes i-l, Figure 4) are the result of aggregation of the 7S particles [17]. Either the labelled 5S RNA exchanges directly into the aggregates, or the aggregates are in equilibrium with monomers which have already been labelled by exchange. Aggregates are also observed in the reconstitution experiment (lanes e-h), containing approximately 20% of the total RNA (lane h) as determined by densitometry. The degree of aggregation observed in native 7S RNP or reconstituted particles varied anywhere from 0-50% in different preparations. Identification of the faster migrating band as 7S RNP was made by a similar experiment using end labelled 7S RNP that was not aggregated.

A further comparison of the reconstituted complex with native 7S RNP was made by probing with RNases T_1 and T_2. Figure 5A shows the results obtained with complexes reconstituted from 1 nM of 3' end labelled Xenopus 5S RNA and 3 nM TF IIIA. Some bands appear as doublets because of length heterogeneity in the labelled 5S RNA [16]. Band compression has obscured the sequence between nucleotides 91 to 96. There is a striking difference in the conformation of free 5S RNA and RNA bound to TF IIIA. Once bound to TF IIIA, the RNA has a reduced reactivity to RNase T_2 at residues A_{54}, A_{56}, A_{74}, A_{100}, A_{101} and also shows an enhanced cleavage at A_{88} (lanes 3-6, Figure 5A). The RNA bound to TF IIIA in reconstituted complexes also has a reduced reactivity to RNase T_1 at residues G_{53}, G_{75}, G_{82}, G_{98}, G_{99} and an enhanced cleavage at G_{89} (lanes 10-13, Figure 5A). The results of similar experiments with 3' end labelled 7S RNP are shown in Figure 5B. The patterns obtained are complicated by the presence of spontaneous cleavages in the undigested control (lane 2, Figure 5B), but a comparison of the respective lanes in Figures 5A and 5B

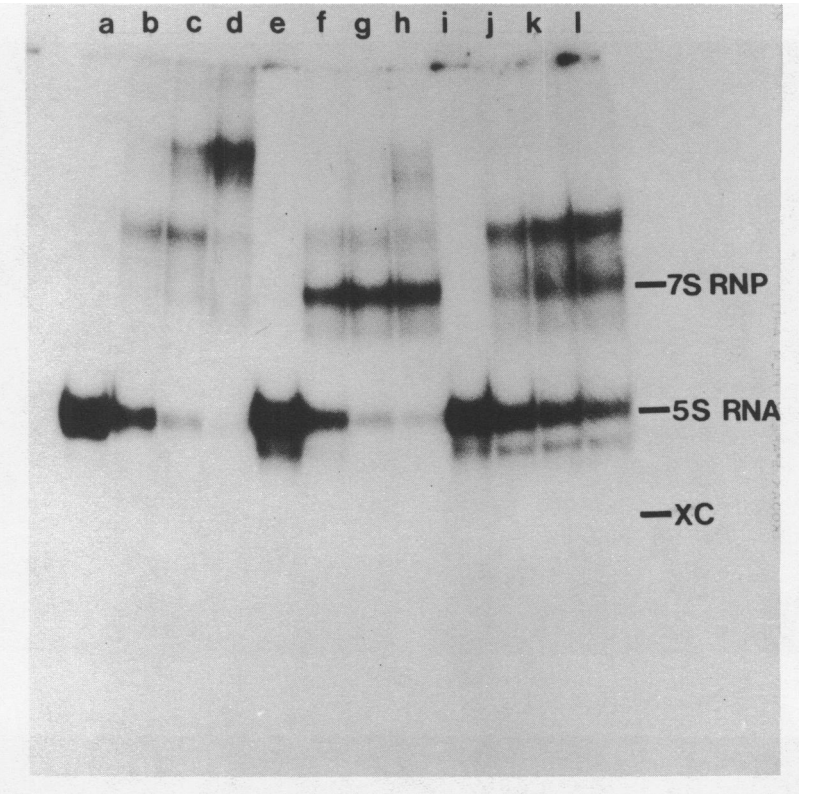
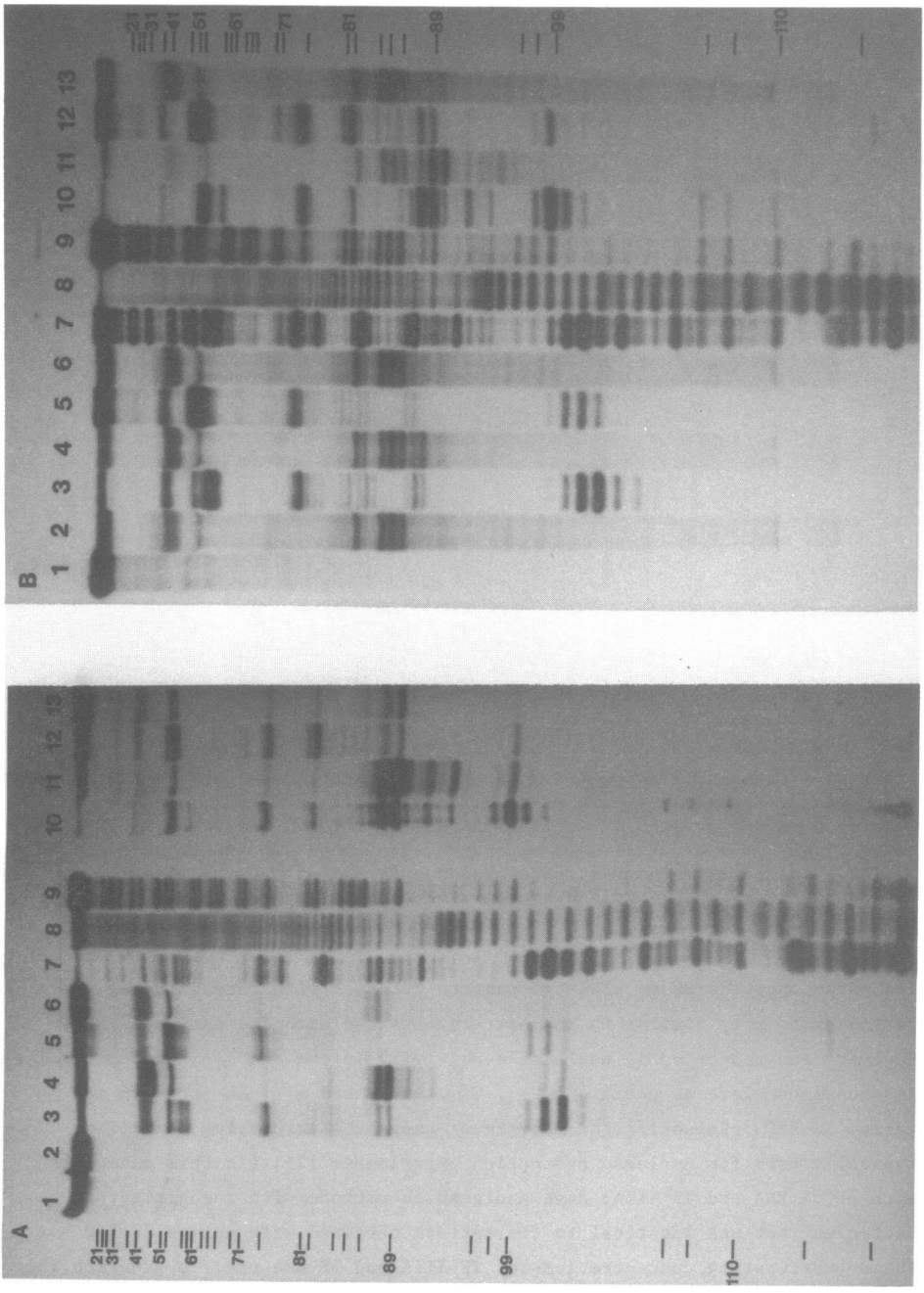


Figure 4. Autoradiogram of non-denaturing polyacrylamide gel electrophoresis of complexes formed between RNA and either TF IIIA or 7S RNP. Lanes a-d: complexes formed between E. coli 5S RNA and 0, 0.15, 0.3 and 0.6 μM TF IIIA respectively. Lanes e-h: same as a-d except Xenopus 5S RNA replaced E. coli 5S RNA. Lanes i-l: interaction of Xenopus 5S RNA with 0, 6, 15 and 30 μM 7S RNP respectively. XC = xylene cyanol.

indicates that the RNase cleavage pattern obtained with reconstituted particles is very similar to the pattern obtained with the native 7S RNP. However, the reduction in nuclease sensitivity in the reconstituted particles is not as complete at positions G_{53}, G_{99}, A_{100}, and A_{101} as it is in the native 7S RNP. Repeating the experiment under the saturating conditions typically used for nuclease protection experiments [25] (in this case 1 μM each of 5S RNA and TF IIIA) gave a digestion pattern with reconstituted particles that was identical to the pattern obtained with native 7S RNP. At the sub-saturating concentrations of TF IIIA and 5S RNA used to generate the data in Figure 5A, free 5S RNA which has been nicked by nuclease may still


B
1 2 3 4 5 6 7 8 9 10 11 12 13
21 31 41 51 61 71 81 89 99 110
A
1 2 3 4 5 6 7 8 9 10 11 12 13
21 31 41 51 61 71 81 89 99 110

exchange with bound 5S RNA. The data from the nuclease digestions experiments are summarized in Figure 6 using the general eukaryotic 5S RNA structural model [34]. The results indicate that authentic 7S RNP particles are formed in the conditions of the standard binding assay and are retained on nitrocellulose filters.

Dissociation of the homologous complex

The dissociation of the homologous complex was measured by pre-forming complexes between ca. 0.5 nM ^{32}P-labelled Xenopus 5S RNA and 20 nM TF IIIA at 20°C in standard TMK buffer, supplemented with the addition of 200 U/ml RNasin. After a 15 min incubation, an aliquot was removed, filtered and the filter washed with 400 µl TMK lacking BSA. Unlabelled Xenopus 5S RNA was added to a final concentration of 0.4 µM and aliquots were removed at the indicated times and filtered with washing (Figure 7A). In the control experiment, buffer was added at time zero rather than unlabelled 5S RNA. As the results in Figure 7A indicate, 29% of the complexes dissociate very rapidly and the remainder of the complexes appear to be quite stable. Although it has not been possible to accurately measure the rate constant for the rapidly dissociating species, a semilog plot of the initial time points (Figure 7B) indicates that the slow dissociating component has an estimated rate constant of ca. 4.5×10^{-4} s^{-1}.

The dissociation of the complex was also measured by simply diluting the complex 100-fold rather than adding unlabelled RNA as competitor. The kinetic pattern measured in this way is indistinguishable from that measured by the first method (data not shown). This result ruled out the possibility that the incomplete dissociation observed was the result of an inability of the unlabelled RNA to bind to free TF IIIA. To establish whether an equilibrium existed between fast and slow dissociating complexes, the time of incubation of labelled RNA and TF IIIA was extended by up to 4 h. This extended time of complex formation did not significantly reduce the population of quickly dissociating complexes (data not shown).

Figure 5. Nuclease probing of the RNA conformation in reconstituted complexes and native 7S RNP. A: probing of 1 nM 3' end labelled 5S RNA and particles reconstituted from 1 nM 5S RNA and 3 nM TF IIIA. 1, 5S RNA (no nuclease); 2, complex (no nuclease); 3, 5S RNA + 100 U/ml T_2; 4, complex + 100 U/ml T_2; 5, 5S RNA + 10 U/ml T_2; 6, complex + 10 U/ml T_2; 7, RNase U_2 sequencing reaction; 8, base ladder; 9, RNase T_1 sequencing reaction; 10, 5S RNA + 1000 U/ml T_1; 11, complex + 1000 U/ml T_1; 12, 5S RNA + 100 U/ml T_1; 13, complex + 100 U/ml T_1, B: probing of 1 µM 5S RNA and 1 µM 3' end labelled 7S RNP. Conditions same as for panel A. The lines to the side indicate the positions of guanosines in the sequence.

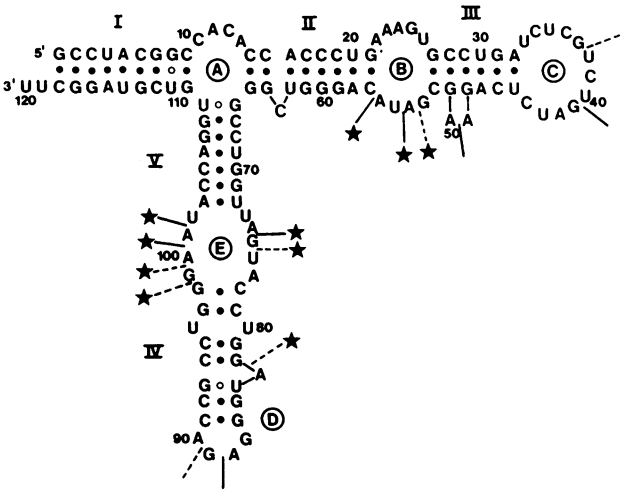


Figure 6. Xenopus 5S RNA drawn according to the general eukaryotic secondary structure model [34]. Solid lines indicate RNase T_2 cleavage sites, and broken lines indicate RNase T_1 cleavage sites. An asterisk indicates that cleavage is reduced in reconstituted complexes and native 7S RNP.

Attempts have been made to further investigate the origin of the rapidly dissociating complexes. After incubation with TF IIIA, the labelled RNA has been analysed on denaturing polyacrylamide gels to determine whether rapid dissociation results from fragmentation of the RNA. Under the conditions used

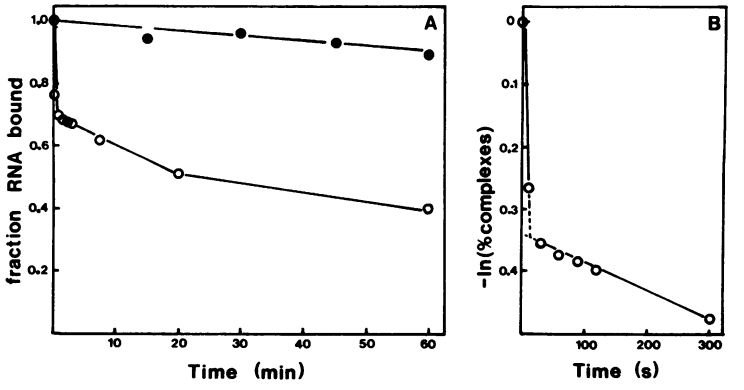


Figure 7. A. Dissociation of the complex formed between 0.5 nM Xenopus 5S RNA and 20 nM TF IIIA after addition of unlabelled Xenopus 5S RNA to a final concentration of 400 nM (○), or addition of buffer alone (●). B. Semilogarithmic plot of data in A.

to measure the dissociation rate, there is no detectable fragmentation of the RNA (for example, see lane 2 Figure 5A). There is some evidence to suggest that rapid dissociation is related to the preparation of TF IIIA. A series of independent experiments that have spanned several preparations of TF IIIA have shown that G-50 purified TF IIIA forms an average of 46% rapidly dissociating complexes, while BioRex-70 purified TF IIIA forms an average of 20% rapidly dissociating complexes. The experiment shown in Figure 7 was carried out with BioRex-70 purified TF IIIA. A potential candidate for a rapidly dissociating complex would be the aggregates of 7S RNP observed in the native gel (Figure 4), however there does not appear to be a correlation between aggregation and the amount of rapidly dissociating species. Varying the concentration of components used to pre-form complexes, and in particular increasing the ratio of 5S RNA to TF IIIA, does reduce the amount of rapidly dissociating complexes, but never below a level of 10%.

pH dependence of K_a

The pH dependence of K_a was determined by measuring association constants using the standard filter binding assay, in which the Tris buffer was substituted by a buffer with an appropriate pKa. The interaction has a broad pH optimum from pH 6.0 to 8.0 and shows a modest decrease in K_a at pH values higher than 8.0 (data not shown). This decrease could result from deprotonation of an amino acid on the protein, or by deprotonation of a guanine or uracil on the RNA.

Temperature dependence of K_a

The temperature dependence of K_a was measured using the standard binding assay, in which the pH of the binding buffer was carefully adjusted at each temperature. In all cases, filtration was also carried out at the incubation temperature and the filters were washed with 400 µl of buffer. It has been shown for some DNA-protein interactions that enthalpy changes are associated with protonation of functional groups on the protein [26]. The temperature dependence of K_a was measured at several pH values to determine whether ΔH is independent of pH for the TF IIIA-5S RNA interaction. A van't Hoff plot of the data is shown in Figure 8. The ΔH given by the slope of the line is -8.3 kcal/mole of complex. Clearly the observed enthalpy change is not the result of protonation of groups on the transcription factor.

Ionic strength dependence of K_a

The contribution of ionic interactions to the binding energy of the interaction between Xenopus 5S RNA and TF IIIA was evaluated by measuring the ionic strength dependence of K_a [28,29]. At K^+ concentrations above 0.1 M, a

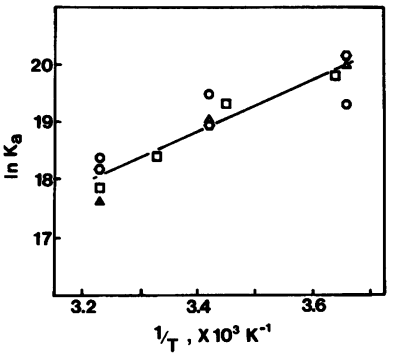


Figure 8. Temperature dependence of K_a measured at pH 6.0 (⬡), 7.0 (○), 7.5 (□), and 8.0 (△). The enthalpy is determined from the slope of the line using the equation:

$$\frac{d \ln K_a}{d (1/T)} = \frac{-\Delta H}{R}$$

decrease in K_a is observed, presumably as the increasing salt titrates electrostatic contacts formed between the protein and RNA (Figure 9). An analysis of the salt dependence of K_a based on ion displacement has been developed for DNA-protein interactions [28] and has been applied by others to RNA-protein

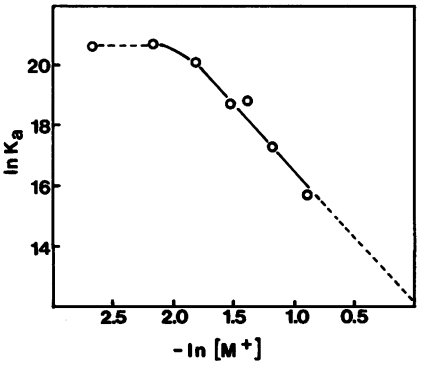


Figure 9. Variation of K_a as a function of ionic strength. Calculation of the number of ion pairs (m') formed in the TF IIIA-5S RNA interaction was made using the equation of Record et al. [29]:

$$\frac{d \ln K_a}{d \ln [M^+]} = -m' \psi$$

where ψ is the fractional counterion bound per phosphate in the RNA (taken to be 0.89 for double helical RNA).

interactions [27,30]. Because application of the theory to RNA-protein interactions requires several untested assumptions [27], the number of ion pairs that is determined from the analysis must be regarded as an upper limit. By assuming that the fractional counterion bound per phosphate for 5S RNA is adequately described by homopolymer data, and that anion effects can be ignored [27,30], analysis of the data in Figure 9 reveals that approximately 5 nucleotide phosphates on the RNA are involved in ion pairs with cationic groups on the protein.

The non-electrostatic component of the free energy of binding can be calculated from the extrapolated value of K_a at 1 M salt using the equation $\Delta G_{NE} = \Delta G_{1M} - N\Delta G_{lys}$ [28,29]. From the data in Figure 9, the ΔG at 1 M KCl is -7.2 kcal/mole. Using a value of ΔG of +0.2 kcal/mole of lysine-phosphate ion pair, and an estimate of 5 such ion pairs in the Xenopus 5S RNA-TF IIIA complex, the non-electrostatic component of the free energy of interaction is -8.2 kcal/mole. This represents approximately 68% of the total free energy of interaction in standard TMK buffer at 20°C.

DISCUSSION

The RNA binding activity of TF IIIA has been studied previously using indirect assays which measure the ability of RNA molecules to compete with the oocyte 5S RNA gene for the binding of the transcription factor [7,18]. The results from these studies have lead to the conclusion that an RNA molecule must have part or all of the general 5S RNA tertiary structure in order to bind to TF IIIA.

To provide a more complete description of the RNA binding activity of TF IIIA, a quantitative assay was developed that directly measures the binding equilibrium between the transcription factor and RNA. The use of a nitrocellulose filter binding assay requires preparations of TF IIIA which are highly purified and fully active. It was discovered that the mild urea treatment of 7S RNP required by some purification procedures [2,9] resulted in the loss of 70-80% of the RNA binding activity of TF IIIA. The procedure of Hanas et al. [7] which uses RNase A digested 7S RNP avoids the use of urea, although it is difficult to remove all traces of ribonuclease by the gel filtration column. This problem was overcome by reducing the amount of ribonuclease used, and extending the digestion time. The TF IIIA prepared by this method was typically 85-90% pure, but was not always 100% active in the RNA binding assay. More highly purified TF IIIA was prepared by chromatography of RNase A digested 7S RNP on BioRex 70, as described by Smith et al. [9]. Again, by

extending the incubation time of the digestion, it was possible to quantitatively bind the transcription factor to the column without the need for urea treatment. Transcription factor prepared by this method was 98-99% pure [9] and fully active in the RNA binding assays.

TF IIIA prepared by both methods has been compared in RNA binding experiments, and found to give identical results. Evidently the impurities present in TF IIIA prepared by gel filtration chromatography (which are absent in the BioRex 70 purified protein) do not interfere or contribute to the RNA binding activity measured. Slight traces of contaminating RNase A were found in some preparations of TF IIIA, and could be fully inhibited by the addition of 200 U/ml RNasin to the assay. RNasin had no other effect on the assay other than to inhibit RNase A.

The results of the nitrocellulose filter binding assay indicate that TF IIIA has a strong affinity for eukaryotic 5S RNA molecules, as demonstrated by K_a values of $1 \times 10^9\ M^{-1}$ and $2.7 \times 10^9\ M^{-1}$ for Xenopus and wheat germ 5S RNA respectively. A similar K_a has been determined for the specific interaction of TF IIIA with the 5S RNA gene [7,19]. The affinity of the protein for E. coli 5S RNA is lower by a factor of 4-fold and $tRNA^{phe}$ binds approximately two orders of magnitude less well. A Scatchard analysis indicates that under the binding conditions employed here, Xenopus 5S RNA and TF IIIA form a 1:1 complex. This stoichioimetry is identical with that of the naturally occurring 7S storage particle [1].

In competition assays, TF IIIA apparently is able to discriminate more rigorously between eukaryotic and prokaryotic 5S RNA. Based upon the direct binding results, the competition strength of E. coli 5S RNA was unexpectedly weak. However, repeating the experiment by following the competition of labelled E. coli 5S RNA with unlabelled Xenopus 5S RNA for binding TF IIIA demonstrated that both RNAs bind to the same site on the protein. E. coli 5S RNA also competes poorly with the Xenopus 5S gene for binding to the transcription factor [12].

The dissociation constant for a similar 7S particle from T. tinca has been determined recently [35] and found to be 300 nM at 37°C, which is an approximately 100-fold weaker interaction than that reported here for Xenopus. It is difficult to make a direct comparison of our data with that measured for T. tinca for several reasons. The protein bound to 5S RNA in T. tinca has an apparent molecular weight of 32 Kd, and is therefore a smaller protein than TF IIIA [35]. Once purified from the 7S particle by nuclease digestion, the T. tinca protein has only limited solubility in aqueous buffers [35], while in

comparison TF IIIA retains its solubility after purification from Xenopus 7S RNP. Because of the limited solubility of the 32 Kd protein, it was necessary to include 4M urea in the incubation buffer used to determine the dissociation constant [35]. The RNA binding activity of TF IIIA is inhibited by urea. Clearly the properties of the two proteins are quite different, and these differences may also extend to their interaction with 5S RNA.

The measurement of the dissociation kinetics for the reconstituted Xenopus 7S RNP indicated the presence of two populations of complexes: a rapidly dissociating species, and a species which dissociates with a rate constant of approximatley 4.5×10^{-4} s^{-1}. Rapid dissociation is not the result of fragmentation of the RNA, not does it appear to be the result of any aggregation of the reconstituted particles. In individual experiments, the rapidly dissociating species has varied between 10-50% of the total population. The proportion of rapidly dissociating complexes can be reduced when reconstitution is effected with the BioRex-70 purified TF IIIA, or by increasing the ratio of RNA to protein used in reconstitution. Under the binding conditions employed in this study, there does not appear to be an equilibrium between the slow and fast dissociating complexes. The varying proportion of rapidly dissociating complexes also has no measurable effect on the association constant measured either by the standard binding assay or by a Scatchard analysis. Although it is not possible to rigorously exclude the possibility that rapidly dissociating complexes result from non-specific interaction of TF IIIA with 5S RNA, under the conditions used to measure dissociation, the protein has a barely detectable interaction with $tRNA^{phe}$. Further investigation will be necessary to clarify whether the dissociation kinetics of reconstituted complexes has any biological significance.

The values of the thermodynamic parameters for the Xenopus 5S RNA-TF IIIA interaction can be compared to those determined for other 5S RNA-protein interactions. The 5S RNA-TF IIIA interaction at 24°C has a ΔG = -12.1 kcal/mole, ΔH = -8.3 kcal/mole, and a ΔS = +13.1 cal mol^{-1} deg^{-1}. Complex formation between 5S RNA and TF IIIA is favoured by both enthalpy and entropy. Thermodynamic parameters have been reported for the 5S RNA binding proteins of E. coli, ribosomal proteins L5, L18 and L25 [32]. The three 5S RNA binding proteins from E. coli also form complexes with a favourable enthalpy and entropy.

A theory developed to determine the number of ionic contacts formed in DNA-protein complexes from the salt dependence of K_a [28,29] has been applied to the TF IIIA-5S RNA complex with the result that up to 5 ionic bonds might

contribute to the free energy of complex formation. Chemical modification of phosphates in the 5S RNA gene was used to measure the potential number of ionic contacts formed in the specific TF IIIA-DNA complex, with the result that as many as 8 phosphates may be involved [10]. These values for the DNA-protein and RNA-protein interactions can be considered to be in reasonable agreement, given the limitations of the two methods used to determine the number of ionic contacts. Extrapolation of the data for the salt dependence of K_a to 1 M salt indicates that approximately 68% of the free energy of complex formation is contributed by non-electrostatic interactions in the TF IIIA-5S RNA complex. Large contributions to free energy from non-electrostatic interactions were also found for the R17 coat protein-RNA complex [27] and the BL16-5S RNA complex of B. subtilis [30].

The nature of the reconstituted complex was investigated further by comparing both its mobility on non-denaturing gels, and its RNA conformation, with 7S RNP. Complexes reconstituted from Xenopus 5S RNA and TF IIIA have a mobility which is indistinguishable from 7S RNP, whereas the conformation and aggregation state of complexes formed between E. coli 5S RNA and TF IIIA are markedly different. The conformation of the 5S RNA in reconstituted complexes and native 7S RNP was compared by limited nuclease digestion. RNA bound to TF IIIA in both complexes has a significantly different conformation from unbound 5S RNA, with the major differences being reduced nuclease sensitivity in bound RNA in loops B and E (Figure 6), and an enhanced reactivity in loop D. These results are in reasonable agreement with the data obtained for the diethylpyrocarbonate modification of native 7S RNP reported by Pieler and Erdmann [15]. We do not observe the enhanced nuclease susceptibility in native 7S RNP that has been reported by others [17]. The similarity of the RNA conformation in reconstituted and native particles, and their identical mobility on non-denaturing gels indicates that the equilibrium under study here leads to the formation of authentic 7S RNP. The quantitative assay should prove valuable in studies directed towards elucidating the specific contacts formed between TF IIIA and Xenopus 5S RNA.

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