Characterization of RNA-Protein Interactions in 7 S Ribonucleoprotein Particles from Xenopus laevis Oocytes

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(Received for publication, June 20, 1985)

5 S RNA interactions with transcription factor protein A (TFIIIA) in 7 S particles from Xenopus laevis oocytes (Xlo) have been characterized by the use of an in vitro RNA exchange assay. 32P-labeled Xlo 5 S RNA can rapidly be incorporated into 7 S particles by simple incubation of the RNA with intact particles. Incorporation of the labeled RNA during exchange reaches an equilibrium within 20 min at 20 °C. Labeled Xlo 5 S RNA already incorporated in 7 S particles can be chased out by an excess of unlabeled 5 S RNA. Non-denaturing gel electrophoresis of 7 S particle samples segregates several ribonucleoprotein particles containing TFIIIA and 5 S RNA. Time course experiments reveal incorporation of 32P-labeled 5 S RNA first in a higher molecular weight ribonucleoprotein particle before incorporation into the 7 S particle. In the exchange process, the integrity of the higher order structure of the RNA is essential for a recognition of the 5 S RNA by TFIIIA. Denatured Xlo 5 S RNA exchanges poorly in the presence of EDTA, but can exchange into the particle at a high level if sufficient divalent cations are present to allow the higher order structure of the RNA to reform. Xlo 5 S RNA fragments that have the 5' or 3' ends deleted past helix I markedly lose their ability to exchange. Heterolosylic eukaryotic and eubacterial 5 S RNAs can exchange into 7 S particles, although the eubacterial 5 S RNAs exchange at a low level.

In the cytoplasm of previtellogenic oocytes of Xenopus laevis, 5 S ribosomal RNA is found complexed with several proteins in two known ribonucleoprotein (RNP) particles and in the ribosome (1-4). One of these proteins has been identified as the transcription factor A (TFIIIA) protein which binds to an intragenomic transcriptional control region and in the ribosome interacts with 5 S RNA by RNA polymerase II. TFIIIA associates with the RNA transcript to form TFIIIA and 5 S RNA. The understanding of the interaction of 5 S RNA with TFIIIA in vitro transcription of Xlo 5 S RNA is the predominant feature recognized by TFIIIA.

5 S RNA interactions with transcription factor protein A (TFIIIA) in 7 S particles from Xenopus laevis oocytes (Xlo) have been characterized by the use of an in vitro RNA exchange assay. 32P-labeled Xlo 5 S RNA can rapidly be incorporated into 7 S particles by simple incubation of the RNA with intact particles. Incorporation of the labeled RNA during exchange reaches an equilibrium within 20 min at 20 °C. Labeled Xlo 5 S RNA already incorporated in 7 S particles can be chased out by an excess of unlabeled 5 S RNA. Non-denaturing gel electrophoresis of 7 S particle samples segregates several ribonucleoprotein particles containing TFIIIA and 5 S RNA. Time course experiments reveal incorporation of 32P-labeled 5 S RNA first in a higher molecular weight ribonucleoprotein particle before incorporation into the 7 S particle. In the exchange process, the integrity of the higher order structure of the RNA is essential for a recognition of the 5 S RNA by TFIIIA. Denatured Xlo 5 S RNA exchanges poorly in the presence of EDTA, but can exchange into the particle at a high level if sufficient divalent cations are present to allow the higher order structure of the RNA to reform. Xlo 5 S RNA fragments that have the 5' or 3' ends deleted past helix I markedly lose their ability to exchange. Heterologous eukaryotic and eubacterial 5 S RNAs can exchange into 7 S particles, although the eubacterial 5 S RNAs exchange at a low level.

Materials and Methods

Extraction of 7 S Particles, 5 S RNAs, and tRNAs—7 S particles were extracted from the oocytes of young X. laevis frogs (from Xenopus I, Ann Arbor, MI) and purified by glycerol gradient centrifugation for a period of 20 h and by DEAE-column chromatography (6, 16, 17). The concentration of protein in purified samples was determined using the Bio-Rad protein assay.

Xlo 5 S RNA and heterologous 5 S RNAs were extracted by phenol extraction of ribosomes and subjected to Sephadex column chromatography and gel purification as previously described (18). Xlo tRNA and wheat germ tRNA were also obtained in this manner. Escherichia coli tRNAs were purchased from Boehringer Mannheim.

5 S Labeling of 7 S Particles and of RNAs—The 5 S RNA associated with 7 S particles was 32P-labeled directly with 5'-32P-pCp-5'-32P and RNA ligase (19) as previously described (13). 5 S RNAs and tRNAs used in exchange incubation were labeled at their 3' ends in the same manner as the 7 S RNPs or were 5'-labeled with [γ-32P]ATP and polynucleotide kinase after treatment with alkali phosphatase (20). The labeled RNAs were gel-purified in 7 M urea and extracted in a salt solution containing 0.1% sodium dodecyl sulfate and tRNA carrier. Labeled RNAs were renatured as previously described (21).

Non-denaturing Polyacrylamide Gel Electrophoresis—All exchange incubations were subsequently electrophoresed on non-denaturing 6% polyacrylamide preparative gels containing 50 mM Tris/borate, pH 8.3, and 1 mM EDTA (TBE) at 30 mA and 4 °C. Gels were routinely fixed in 7.5% acetic acid, stained in 0.02% Coomassie Blue, 7% acetic acid, 50% methanol, and destained in 7.5% acetic acid before being autoradiogrammed. Autoradiograms of gels gave qualitative visual data of the distribution of the competitive labeled RNA used in each experiment. The autoradiograms provided a template for the excision of radioactive bands from gels. Excised bands were placed in vials containing a toluene/1,4-bis[2-(5-phenoxazolyl)]benzene/2,5-di-phenyloxazole scintillation mixture and counted with a liquid scintillation counter. Samples were usually counted with a minimum of 10,000 counts. The reproducibility of measured exchange levels was within ±10% when repeated measurements of exchanges were made under identical conditions and using the same sample extracts.

Exchange Reactions in General—Unless stated otherwise, all exchange incubations were for 2 h at 20 °C and in 10-μl volumes. A concentration greater than 2.5 μM 7 S particle is necessary in order to obtain appreciable exchange. The exchange reactions shown in this paper were done using 7.7 μM 7 S particle unless otherwise.
specified. The 7 S particle concentrations were estimated from the protein concentrations assayed for each purified sample.

Exchange buffers contained 50 mM Tris-HCl, pH 7.5, 3 mM dithiothreitol, and bovine serum albumin at 15 µg/ml. In each exchange incubation, the 7 S protein storage buffer (13) was diluted out so that the concentration of KC1 was 135 mM and the concentration of MgCl2 was less than 1 mM. Additional MgCl2, or dithiotreitol, or EDTA was added to the incubation mixture to obtain various concentrations (1–10 mM).

Only trace molar amounts of labeled RNA were used in each exchange incubation. We estimate that the unlabeled RNA in the particle exceeds the labeled RNA added to the incubation mixture by the labeled end RNA fragments were determined by polyethyleneimine-cellulose chromatography (22, 23). Labeled 5' end RNA fragments were obtained by partial digestions of labeled intact 5 S RNA with ribonucleases T1 (G-specific) and U3 (A-specific) in 7 M urea (20). Samples were electrophoresed on 8% polyacrylamide sequencing gels containing 7 M urea and TBE, and extracted from the gel (13). The identities of the labeled end nucleotides were determined by polyethyleneimine-cellulose chromatography (22, 23). Labeled 5' end RNA fragments were obtained by partial digestions of labeled intact 5 S RNA with ribonucleases T1 (G-specific) and U3 (A-specific) in 7 M urea (20). Samples were electrophoresed on 8% polyacrylamide sequencing gels containing 7 M urea and TBE. Bands were cut from gels using an autoradiogram as a template and the RNA fragments from each band were eluted from the gel in a salt solution containing 0.1% sodium dodecyl sulfate and tRNA carrier. The fragments were identified by the positions of their bands in the sequencing gel.

RESULTS

Gel Electrophoresis of 7 S RNPs Particle—Experiments were designed around the ability of unbound 5 S RNA to incorporate into 7 S particles by RNA exchange. Nondenaturing gel electrophoresis was used to segregate the RNPs from the unbound RNA. Fig. 1 shows the characteristic Coomassie Blue patterns seen on such gels: a dark staining band which migrates ahead of lightly staining bands. The RNA found associated with the protein staining bands containing TFIIB had been analyzed previously and found to be only 5 S RNA (13). The RNPs do not appear to be significantly disrupted during electrophoresis since discrete bands are seen and are smear-free.

Chase of Label from Radioactive 7 S Particles—The 7 S RNP particle containing labeled 5 S RNA was incubated with various times with an 8-fold excess of unlabeled wheat germ 5 S RNA (Fig. 2, lanes 2A–2D). Approximately 80% of the labeled Xlo 5 S RNA is chased from the particle after incubation for 20 min. This demonstrates that unbound 5 S RNA can replace 7 S particle-bound 5 S RNA, i.e. RNA exchange. Incubations with unlabeled E. coli tRNA (Fig. 2, lanes 1A–1D) do not show the same displacement of label from the 7 S particle, thus confirming that the RNA exchange interaction of TFIIB has a specificity for 5 S RNA.

Time Course of Incorporation of Labeled 5 S RNA—Trace amounts of labeled Xlo 5 S RNA were incubated with unlabeled intact 7 S particle for various times at 0 °C or at 20 °C and immediately electrophoresed on nondenaturing polyacrylamide gels (Fig. 3, A and B). Autoradiograms of the gels reveal that exchange begins instantly and that an equilibrium of distribution of labeled RNA is reached within 20 min at 20 °C and within 40 min at 0 °C (Fig. 3B). At equilibrium, approximately 75% of the total counts/min co-migrates with the dark Coomassie Blue band of the 7 S particle.

Of importance is that the radioactive RNA does not appear in the 7 S particle band directly during the time course incubation, but appears first in a slower moving RNP band. This component can be seen in the lane of the shortest incubation time at 0 °C (see the arrow at lane 2 of Fig. 3A) and is observed before a second slow moving band subsequently appears (see arrow at lane 3 of Fig. 3A). This second slow moving band co-migrates with the Coomassie Blue-stained band that migrates above the major 7 S RNP band during electrophoresis (Fig. 1). The higher molecular weight RNP forms may represent the addition of 5 S RNA to the 7 S particle, possibly at a secondary binding site on the protein or, alternatively, exchange may occur first in aggregates of the 7 S particle. Since the 5 S RNA exchanges first in a higher molecular weight form that is transient (arrow at the band in lane 2, Fig. 3A), we favor the concept that exchange initially occurs by the addition of the 5 S RNA to the 7 S particle.

Exchange of Denatured and Renatured Xlo 5 S RNA—

![Fig. 1. A dried, Coomassie Blue-stained 6% polyacrylamide gel where 7 S particle samples had been electrophoresed after a 1-h incubation at 20 °C in an incubation buffer with various concentrations of Mg++, EDTA, or Zn++. The incubation buffer is as described under "Materials and Methods" and also contained: 1, 10 mM Mg++, 2, 5 mM Mg++, 3, 3 mM Mg++, 4, 2 mM Mg++, 5, less than 1 mM Mg++, 6, 1 mM EDTA; 7, 2 mM EDTA; 8, 1 mM Zn++. The protein stained in the gel constitutes protein bound by RNA.](image1)

![Fig. 2. Autoradiogram of 6% polyacrylamide gel showing electrophoresis of 32P-labeled 7 S particle after incubation at 20 °C with an 8-fold excess of E. coli tRNA (1), wheat germ 5 S RNA (2), and no RNA (3). Incubation times are as follows: A, 0 min; B, 20 min; C, 40 min; D, 60 min.](image2)
Renatured Xlo 5 S RNA readily exchanges into 7 S particles under various buffer conditions (Fig. 4A). The level of exchange is greatest in 3–10 mM Mg2+ (65–70%) and falls off slightly in the presence of EDTA (50%). The intensity of Coomassie Blue staining (Fig. 1) appears to correlate with the levels of exchange that occur in the various buffers. The lack of exchange in the presence of 1 mM Zn2+ (lane 9 of Fig. 4A) is probably due to a disassociation of the 7 S particle (see lane 8 of Fig. 1). The levels of exchange observed with renatured labeled Xlo 5 S RNA appear to reflect the levels of exchange that occur with native unlabeled Xlo 5 S RNA endogenous to the 7 S particle preparations. There are variations in the measured levels of exchange in different preparations of 7 S particle. This may be due, in part, to variations in the amount of protein-free 5 S RNA present in each sample.

The level of exchange of labeled urea-denatured Xlo 5 S RNA is more sensitive to buffer conditions (Fig. 4B). The extent of incorporation of denatured 5 S RNA in 5–10 mM Mg2+ (65–75%) is comparable to that observed for renatured Xlo 5 S RNA; however, the level of exchange of the denatured RNA without the addition of Mg2+ or in EDTA falls off considerably (20–25% for the samples shown in Fig. 4B). The difference in the levels of exchange of denatured 5 S RNA in various buffers when compared to the exchange of renatured 5 S RNA in those same buffers shows that the ability of the RNA to exchange is dependent on the integrity of its higher order structure. Sufficient concentrations of Mg2+ appear to allow for the reformation of a conformation of the RNA that is recognizable by TFIIIA. If 5 mM Ca2+ or 5 mM Mn2+ is added to the incubation mixture, the level of exchange of denatured Xlo 5 S RNA approximates the level that occurs in 5 mM Mg2+ (data not shown). Ca2+ and Mn2+ may function to balance the divalent ionic concentration in the exchange buffer and thus, also, allow for reformation of the RNA structure. If sufficient cations are present for the denatured 5 S RNA to reform a higher order structure, or if a higher order structure is already present, as is in the case of renatured 5 S RNA, then the labeled RNA can compete well with the endogenous native Xlo 5 S RNA for the primary binding site on the protein during RNA exchange.

Helix V (nomenclature is that of Delihas and Andersen (24), i.e. positions 78–98) shows characteristic band compres-
sion associated with the presence of secondary structure when partially digested Xlo 5 S RNA is run on 7 M urea sequencing gels (13, 21). This suggests that urea-treated 5 S RNA maintains secondary structure in helix V. The presence of helix V may be sufficient for the denatured 5 S RNA to interact with TFIIIA in a specific manner. Under the right ionic conditions, the RNA could reform the overall conformation necessary for TFIIIA recognition at the primary site. Several discrete minor bands that migrate slower than the 7 S particle appear on overexposed autoradiograms of gels containing labeled 5 S RNA 3' end fragments which had been incubated with 7 S particles but not of the gels containing samples with radioactive 5' end fragments (data not shown). Pieler et al. (11) have shown that a 5 S RNA 3' end fragment (40-120) but not the 5' end fragment (1-40) was able to inhibit in vitro transcription of 5 S RNA. They propose that the stem containing helices IV and V is essential to the interaction of the RNA with TFIIIA. The interaction (albeit at a low level) of the RNA 3' end fragments as reported here is consistent with the conclusion of these authors but may suggest that the interaction of these 3' end fragments with TFIIIA involves an interaction with higher molecular weight RNP forms or secondary binding sites on the protein.

**Exchange of Xlo 5 S RNA Fragments**—A series of labeled Xlo 5 S RNA fragments were incubated with 7 S particles to test for exchange properties. The ability of the RNA fragments to exchange is lost if either the 5' or 3' ends are deleted so that helix I is totally disrupted (Fig. 5). However, significant levels of exchange take place if part of the helix remains intact. Deletions up to position 5 from the 5' side (lane 3, Fig. 5A) or to position 117 from the 3' side (lane 2, Fig. 5B) still allow for appreciable exchange of the RNA fragment. These results may point to helix I as a domain of protein interaction at the primary binding site or may point to the role of helix I in the stabilization of the higher order structure of the RNA which is necessary for TFIIIA recognition at the primary site.

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**Exchange of Labeled Heterologous 5 S RNAs**—Several 5' labeled heterologous 5 S RNAs have been incorporated into 7 S particles by means of RNA exchange. Table I gives the amount of exchange that occurs at equilibrium and the per cent sequence homology of the exchanged 5 S RNAs to Xlo 5 S RNA. Fig. 6 shows two autoradiograms of several of these exchanges.

Eukaryotic 5 S RNAs from *Euglena gracilis* and *Triticum aestivum* (wheat germ) show exchanges comparable to that of Xlo 5 S RNA. However, exchange of the 5 S RNA from *Phycomyces blakesleeanus* results in most of the radioactivity being incorporated into the slower moving bands. Thus, this 5 S RNA appears to be able to interact at a putative secondary binding site but is a poor competitor of Xlo 5 S RNA for the primary site. Sequence homology alone cannot be used to explain why *P. blakesleeanus* 5 S RNA is a poor competitor for the primary binding site since it has a similar percentage of sequence homology to Xlo 5 S RNA that wheat germ 5 S RNA has. The 5 S RNA of *P. blakesleeanus* has a disrupted helix II (18) and may have a variant conformation. A presumed variant conformation may decrease the ability of *P. blakesleeanus* 5 S RNA to compete with Xlo 5 S RNA for the primary interaction but not for a putative secondary binding since the latter interactions do not appear to be dependent on the overall conformation of the 5 S RNA.

It has been previously shown that eubacterial 5 S RNAs can exchange into Xlo 7 S particles (13). As with renatured Xlo 5 S RNA (described above), the incorporation of renatured eubacterial 5 S RNA into 7 S particles is not sensitive to buffer conditions. The radioactivity pattern appears similar to that of the exchange of *E. coli* 5 S RNA in Mg\(^{2+}\) (Fig. 6B). Interestingly, the fastest moving 7 S RNP containing eubacterial 5 S RNA moves slightly ahead of the native Xlo 7 S RNP band. This may reflect conformational differences in the eubacterial hybrid 7 S RNP particle.

If radioactive, urea-denatured eubacterial 5 S RNA is incubated with 7 S particles in Mg\(^{2+}\) or in EDTA, the distribution of label that appears after electrophoresis differs from

**Table 1**

<table>
<thead>
<tr>
<th>Source of 5 S RNA</th>
<th>% sequence homology to Xlo 5 S RNA</th>
<th>% exchange</th>
<th>Reference to sequence</th>
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</thead>
<tbody>
<tr>
<td>X. laevis</td>
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<td>57</td>
<td>23</td>
</tr>
<tr>
<td>Wheat germ</td>
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<td>46</td>
<td>22</td>
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<tr>
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<td>52</td>
<td>16</td>
</tr>
<tr>
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<td>18</td>
<td>59</td>
</tr>
<tr>
<td><em>E. coli</em></td>
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<td>8</td>
<td>21</td>
</tr>
<tr>
<td><em>Rb. sphaeroides</em></td>
<td>57</td>
<td>12</td>
<td>34</td>
</tr>
</tbody>
</table>

a Determined by the % counts/min in the major 7 S RNP band.
b Determined by the % counts/min in the higher molecular weight RNP bands.

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**Fig. 5.** Autoradiograms of polyacrylamide gels showing electrophoresis of labeled Xlo 5 S RNA or 5 S RNA fragments after incubation with or without the 7 S particle. A, incubated with 7 S particle; --, without 7 S particle. A, the RNA fragments are from the 3' side of the 5 S RNA: 1, 3' labeled Xlo 5 S RNA; 2, RNA fragment U101-U136; 3, RNA fragment A38-U136. 4, RNA fragment G21-G110 and 5, RNA fragment G1-G110. B, the RNA fragments are from the 5' side of the 5 S RNA: 1, RNA fragment G1-G110; 2, RNA fragment G1-G117; 3, 5' labeled Xlo 5 S RNA.
that seen with denatured Xlo 5 S RNA. The radioactive pattern following exchange of *Rhodobacter sphaeroides* (formerly *Rhodopseudomonas sphaeroides*) 5 S RNA (Fig. 7) is typical of the pattern seen for exchanges with other denatured eubacterial 5 S RNAs. These 5 S RNAs exchange better in the presence of EDTA than in Mg2+ (13). This was attributed to a decrease in the specificity of TFIIIA in the presence of EDTA. However, the radioactivity concentrates more in the slower moving RNP band (45%) (i.e. putative secondary interaction) than in the major 7 S RNP band (21%) (i.e. primary interaction) (see Fig. 7, lane 4). So the loss of specificity of TFIIIA in the presence of EDTA may involve a putative secondary binding site to a greater degree than its primary binding site.

Neither wheat germ tRNA (Fig. 6A), E. coli tRNA-Protein Recognition in 7 S Particles

**FIG. 6.** Autoradiograms of polyacrylamide gels showing electrophoresis of 3' labeled Xlo and heterologous 5 S RNAs after incubation with or without 7 S particles. +, incubation with 7 S particle; −, incubation without 7 S particle. A. RNAs used in the exchange were: Xlo, *X. laevis* oocyte 5 S RNA; P.b., *P. blakesleeanus* 5 S RNA; W.G., wheat germ 5 S RNA; and tRNA, wheat germ tRNA. B. RNAs used in the exchange were: Xlo, *X. laevis* oocyte 5 S RNA; E.g., *E. gracilis* 5 S RNA; E.c., *E. coli* 5 S RNA; and tRNA, E. coli tMet-tRNA.

**FIG. 7.** Autoradiogram of a 6% polyacrylamide gel showing electrophoresis of the 7 S particle after incubation with labeled, denatured Xlo or *Rb. sphaeroides* 5 S RNA. The incubation buffer is as described under “Materials and Methods” and also contains either 5 mM Mg2+ or 1 mM EDTA as indicated: 1, Xlo 5 S RNA in 5 mM Mg2+; 2, *Rb. sphaeroides* 5 S RNA in 5 mM Mg2+; 3, Xlo 5 S RNA in 1 mM EDTA; 4, *Rb. sphaeroides* 5 S RNA in 1 mM EDTA.

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**DISCUSSION**

The addition of exogenous 5 S RNA to *Xenopus* in vitro transcription systems inhibits the transcription of 5 S RNA (6). Several investigators have found that heterologous as well as homologous 5 S RNAs can be used in this type of transcription competition assay (10–12). A large amount of RNA is needed for successful transcriptional inhibition. The presence of multiple RNA-binding sites on TFIIIA may, in part, account for the excess amounts of 5 S RNA needed in these analyses.

Pielar et al. (11) find that a wide variety of 5 S RNAs from eukaryotes, eubacteria, and archaeabacteria can interfere with the function of TFIIIA in the in vitro transcription system and conclude that universal 5 S RNA structural elements and not sequence homology are important in the interaction of 5 S RNA with TFIIIA. Their study agrees with the findings reported previously on heterologous 5 S RNA exchange (13) and with studies reported here.

The importance of RNA higher order structure has long been stressed in 5 S RNA-protein interactions involving ribosomal proteins (29–35) and RNA processing proteins (36–39). Spieler and Zimmermann (30) discussed the dependence of Mg2+ concentration on the binding of *E. coli* ribosomal proteins to 5 S RNA in terms of the effect of Mg2+ on the higher order structure of the RNA. Stiekema et al. (37) demonstrate that only 5 S RNAs with an intact helix I can interact with the processing enzyme RNase M5. Also, while particular nucleotides of 5 S RNA have been implicated in...
the interaction of the RNA with RNase M5 (36), the position of these nucleotides in relationship to helix I correlates with the ability of the RNA to interact with the protein (37). 5 S RNA-protein interactions as described by these authors appear to conform to the "lock and key" analogy that is used to describe substrate-enzyme interactions.

The conformation of 5 S RNA is the essential factor involved in the recognition of the RNA by TFIIIA. This is demonstrated by the fact that denatured 5 S RNA exchanges poorly into 7 S particles unless sufficient divalent cations are present for the RNA to reform its higher order structure while renatured 5 S RNA exchanges well independent of the concentration of divalent cations. The ability of heterologous 5 S RNAs to interact with TFIIIA confirms that the "lock and key" analogy may be extended to include 5 S RNA-TFIIIA interactions in the 7 S particle. The fact that eubacterial 5 S RNAs can exchange into 7 S particles suggests that TFIIIA is capable of recognizing a generalized 5 S RNA conformation and is not recognizing contiguous sequences in the RNA.

Intrinsic to the interactions that occur between 5 S RNA and TFIIIA is RNA exchange. Chase experiments confirm that substitution of 5 S RNAs in 7 S particles occurs during RNA exchange reactions. Time course experiments show that exchange reaches equilibrium very rapidly and that the exchanging RNA is incorporated into a higher molecular RNP form before appearing in 7 S particles. This suggests a possible mechanism of exchange whereby the binding of one RNA to a secondary site on TFIIIA in the 7 S particle allows for RNA competition for the primary binding site.

Acknowledgments—We thank Dr. Daniel F. Bogenhagen for aid in extraction of the 7 S particle. We also thank Dr. Laura Liss for the unique gel drying techniques and Edward Villanueva for discussions concerning RNA exchange.

REFERENCES