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Association of higher molecular weight ribonucleoproteins of 7 S particle preparations with multimers of transcription factor IIIA

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High molecular weight (HMW) fractions of *Xenopus laevis* 7 S ribonucleoprotein (RNP) particle preparations were analyzed for RNA and protein content. RNA/protein ratios, amino acid analyses and Western blots reveal that the major HMW fraction from a non-denaturing polyacrylamide gel (band *b*) contains two molecules of transcription factor protein IIIA (TFIIIA) to one 5 S RNA. Another HMW band appears to contain 4 molecules of TFIIIA to one 5 S RNA. Yet another RNP band (band *a*) contains 5 S RNA and a protein unrelated to TFIIIA. Thus, native 7 S particle preparations contain 5 S RNA bound to multimeric forms of TFIIIA as well as to an unrelated protein. The presence of additional TFIIIA molecules associated with 7 S particles may have significance in the sequestering of TFIIIA during transcriptional regulation of the 5 S gene.

Protein dimer; Nucleic acid-binding protein; TFIIIA; RNP, 7 S

1. INTRODUCTION

Previtellogenic oocytes of *Xenopus laevis* (Xlo) accumulate two characterized ribonucleoprotein (RNP) particles in the cytoplasm, one sedimenting at 7 S [1] and the other at 42 S [2–4]. Each of the two RNP particles contains about half of the early oocytes' total amount of stored 5 S ribosomal RNA [5].

The 7 S RNP is a complex of 5 S RNA and transcription factor protein IIIA (TFIIIA) [1,6,7]. TFIIIA is a DNA binding protein necessary for the accurate transcription of the 5 S RNA gene. Structural models for TFIIIA, a Zn^{2+} finger protein, have been proposed [8,9]. The 7 S particle may function in feedback regulation of 5 S RNA gene expression and/or in stabilization of 5 S RNA during cytoplasmic storage prior to incorporation into the large subunit of the ribosome [1,6,7]. In vitro, it has been shown (by competition studies, chase of labeled 5 S RNA from the 7 S RNP) that ^{32}P-labeled 5 S RNA can specifically compete and exchange with the unlabeled 5 S RNA in intact 7 S RNP particles under physiological conditions [10,11]. This exchange reaction has been used to investigate the nature of the interaction of 5 S RNA with TFIIIA [11,12].

Native 7 S particle preparations contain several high molecular weight (HMW) RNP particles besides the 7 S RNP [11,12]. In the study of 7 S particle formation and function, it is of interest to determine the nature of the HMW RNPs. The present study characterizes the HMW RNPs found in 7 S particle preparations and indicates: (i) dimers and higher multimers of TFIIIA bind to one 5 S RNA molecule; and (ii) a 60–63 kDa protein-5 S RNA RNP unrelated to the TFIIIA-5 S RNA RNP is present in native 7 S RNP preparations. 5 S RNA-multimer TFIIIA RNPs may play a role in feedback regulation of 5 S RNA synthesis, similar to the proposed function of 7 S RNPs.

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2. MATERIALS AND METHODS

7 S RNPs were extracted from oocytes (stages I, II) of young *X. laevis* frogs (Xenopus I, Ann Arbor, MI) and purified by glycerol gradient centrifugation for 20 h with subsequent DE52 (Whatman) column chromatography [7]. The concentration of protein in extracts was determined using Lowry analysis. Xlo 5 S RNA was extracted by phenol/chloroform extraction of the 0.5 M KCl eluate from the DE52 column and further purified by polyacrylamide gel electrophoresis.

5 S RNA was 3′ end-labeled with 5′,3′-pCp-[5′-^{32}P](NEN) and RNA ligase, then gel-purified, extracted, and renatured as previously described [11].

For exchange reactions, a trace amount of 5 S [^{32}P]RNA was incubated with native 7 S RNP preparations for 20 min at 24°C in 10 μl buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM dithiothreitol, 10 mM $MgCl_2$ and bovine serum albumin at 15 μl/ml. The concentration of 7 S RNP (10–52 mM) was estimated from the protein concentration of the 7 S RNP preparation. *E. coli* tRNA was also present in the incubation mixtures, as it was added as carrier for precipitation of labeled 5 S RNA.

Exchange incubations were electrophoresed on non-denaturing 6% polyacrylamide gels containing 50 mM Tris/borate, pH 8.3, and 1 mM EDTA at room temperature. After autoradiography, gels with ^{32}P-labeled 5 S RNA were fixed in 7.5% acetic acid, stained in 0.02% Coomassie blue R-250, 7% acetic acid, 50% methanol, destained in 7.5% acetic acid, and dried. Autoradiograms and dried Coomassie

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blue-stained gels were quantified with a laser densitometer (Ultroscan XL; LKB) and software (2400 Gel Scan; LKB).

A standard Laemmli SDS-polyacrylamide gel system was used for analysis of proteins from native 7 S RNP preparations. Samples were dissolved in 2 × sample buffer (0.16 mM Tris-HCl, pH 6.8, 20% glycerol, 3% 2-mercaptoethanol, 4% SDS) boiled for 5 min, and electrophoresed at 70 V for 1.5 h (5% stacking gel) and 150 V for 3.5 h (7% separating gel).

For Western blots, native 7 S RNP preparations were separated by a 6% non-denaturing gel. In addition, RNP bands *a* and *b* (from 6% non-denaturing gel) were excised and proteins separated by 7% denaturing SDS-PAGE. Separated RNPs or proteins were electrotranferred to 0.05 μm nitrocellulose (S&S) for 35 min at 2.5 mA/cm^2 (Polyblot, ABN) as previously described [13], then incubated with 1:250 dilution of polyclonal anti-TFIIIA antiserum (gift from Drs M. Darby and D. Brown, Carnegie Institution of Washington) and 1.0 μCi/ml anti-rabbit Ig (^{125}I-labeled species-specific F(ab^1)$_2$ fragment from donkey (Amersham). Controls using labeled anti-rabbit antibody without anti-TFIIIA showed no reaction.

Amino acid analysis of TFIIIA and 95 kDa protein was according to LeGendre and Matsudairo [14]. Proteins were separated by 7% SDS-PAGE, electroblotted to Immobilon-P transfer (PVDF) membrane (Millipore), and visualized with aqueous Coomassie blue-staining. Protein bands were subsequently excised for hydrolysis and extraction using the Waters Pico-Tag protocol. Amino acid analyses were performed by The Center for Macromolecular Synthesis and Analysis, SUNY Stony Brook, NY. Values shown in table 2 were normalized to Typ using amino acid sequences from the TFIIIA gene [15], amino acid analysis (picomoles recovered, (this study), and residues/molecule [1]). Cys and Trp were destroyed by acid hydrolysis; His values are frequently artificially low because of Erdman degradation [16], and Ile values were discarded as the Ile value for the blank was higher than for the samples.

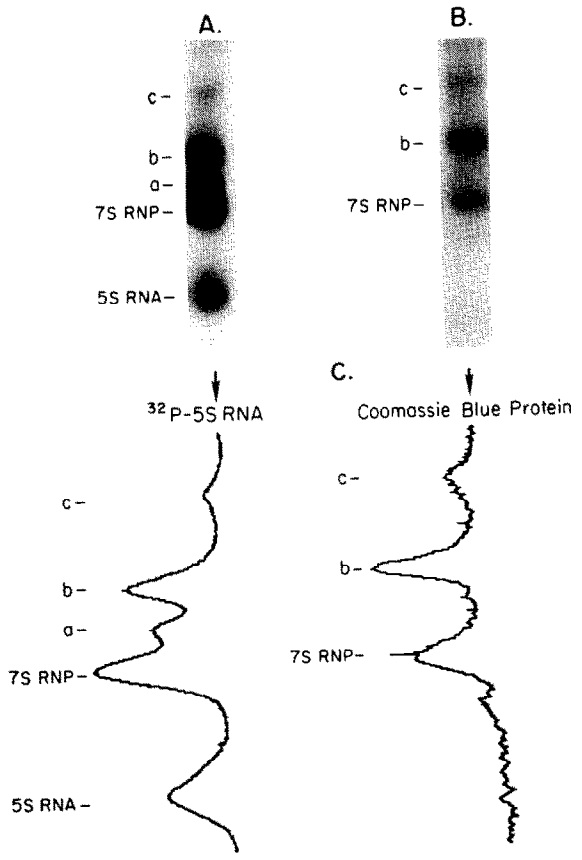

Fig.1. Incorporation of ^{32}P-labeled 5 S RNA into 7 S RNP particles by RNA exchange. (A) Autoradiogram of 40 mM 7 S particle incubated with trace amounts of ^{32}P-labeled Xlo 5 S RNA at 24°C and analyzed by 6% non-denaturing PAGE. (B) Same gel stained with Coomassie blue. (C) Laser densitometer traces of autoradiogram (A) and stained gel (B).

Table 1

Relative amounts of 5 S RNA and protein in 7 S and HMW RNPs

Band	Autoradiogram	Coomassie blue	Ratio[a] (5 S RNA:protein)
c	0.69	0.57	1:3.9
b	2.91	1.27	1:2.1
a	1.78	–	–
7 S RNP	4.17	0.88	1:1
5 S RNA	2.26	–	–

[a] Scanned by laser densitometer (mm^2 for fig.1A and 1B; traces in fig.1C). To determine ratios, all Coomassie blue values were multiplied by 4.7 to create a 1:1 (5 S RNA:TFIIIA) ratio for the 7 S RNP

3. RESULTS AND DISCUSSION

3.1. *Gel electrophoresis of native 7 S RNP*

Several HMW RNPs (*a,b,c*) can be visualized by autoradiography after exchange of ^{32}P-labeled 5 S RNA into native 7 S RNP extracts (fig.1A) or after Coomassie blue staining of the same gel (fig.1B). Relative to the [^{32}P]RNA label and Coomassie blue protein stain of the 7 S particle, comparisons showed a 5 S RNA:protein ratio of 1:2.1 for band *b* and 1:3.9 for band *c* (fig.1C, table 1). Additionally, band *a* appeared on autoradiography, but stained poorly with Coomassie blue. However, it appeared on Coomassie blue-stained gels when 7 S RNP concentrations were >40 mM.

3.2. *Stability and composition of HMW RNPs from native 7 S RNP*

After incubation of 7 S particle with 5 S [^{32}P]RNA and non-denaturing gel electrophoresis, RNP bands were excised and subsequently electrophoresed on a second non-denaturing gel. The *a, b* and *c* bands contain stable RNP particles during gel electrophoresis as their position on the second gel was similar to their position on the first gel (fig.2).

Stability of the HMW RNPs allowed for additional assays which resolved their protein components. RNP bands *a, b* and *c* from the non-denaturing gel were excised and subsequently separated by SDS-PAGE (fig.3). With Coomassie blue staining, band *a* showed a poorly staining doublet 60–63 kDa protein and a small amount of TFIIIA (fig.3, lane 2). Presence of TFIIIA in band *a*, as well as the 60–63 kDa protein in the 7 S RNP band (fig.3, lanes 2 and 3), is probably due to cross-contamination. Protein extracted from band *b*

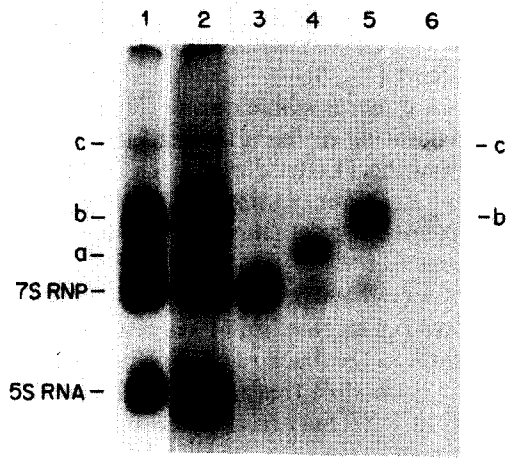

Fig.2. Stability of HMW RNPs from 7 S RNP extracts. Autoradiogram of ^{32}P-labeled Xlo 5 S RNA exchanged into the 7 S RNP and then analyzed by 6% non-denaturing PAGE (lane 1). The resulting 7 S and HMW bands were excised from the non-fixed gel and analyzed by a second 6% non-denaturing PAGE. Lane 2, marker (7 S RNP extract); lanes 3–6 represent excised bands 7 S, *a*, *b*, and *c*, respectively.

contained mostly a 95 kDa protein, some TFIIIA, and a small amount of a 160 kDa protein. The appearance of the 160 kDa protein on gels was variable and may have arisen from cross-contamination from band *c* which contains a 160 kDa protein. The monomer TFIIIA seen in band *b* may result from an equilibrium between the monomer and the putative TFIIIA dimer (the 95 kDa protein).

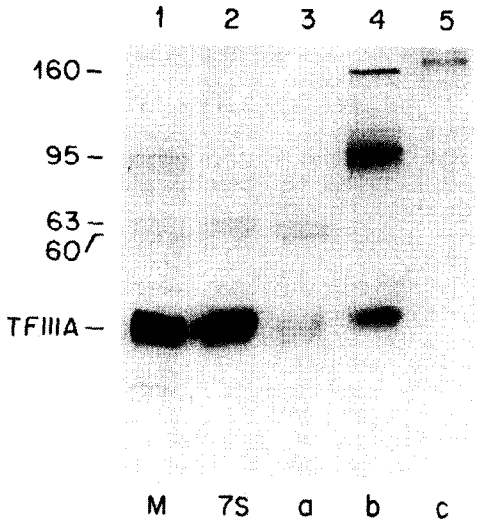

Fig.3. Characterization of proteins in 7 S RNP extract by 7% SDS-PAGE (Coomassie blue stained). Denatured 7 S RNP extract as marker (lane 1). Lanes 2–5 represent bands 7 S, *a*, *b*, and *c* excised from a 6% non-denaturing gel and electrophoresed on a 7% SDS-polyacrylamide gel. Values are molecular masses (kDa) estimated from protein standards. 95 kDa fraction migrated as a broad band which may be due to the dimerization of the two species of TFIIIA, 38 kDa and 40 kDa [17].

Table 2

Amino acid composition of TFIIIA and 95 kDa protein

	TFIIIA[a] [15]	TFIIIA [1]	TFIIIA (this study)	95 kDa
Met	0.2	0.2	<0.1	0.1
Glx	2.6	3.7	3.4	5.4
Lys	3.6	3.9	4.3	3.0
Ala	1.2	1.8	1.1	1.9
Leu	2.2	2.3	2.3	2.7
Pro	1.2	2.0	1.4	1.3
Val	1.3	1.3	1.5	1.7
Tyr	1.0	1.0	1.0	1.0
Arg	1.6	2.0	2.3	1.7
Ser	1.8	2.2	2.3	2.3
Phe	1.4	1.8	1.6	1.1
Asx	2.5	3.1	3.3	3.5
Gly	1.4	1.8	1.8	2.7
Thr	1.8	1.8	2.2	2.1
His	2.1	2.3	2.4	0.6

[a] From DNA sequence

When purified TFIIIA was reconstituted with high concentrations of protein to 5 S RNA in an in vitro reconstitution assay [18], subsequent electrophoresis on a non-denaturing gel revealed the formation of the 7 S particle and band *b*, but the absence of band *a* (unpublished results). This was an indication that the protein component of band *a* was eliminated during the purification of TFIIIA. In addition, the formation of band *b* by incubation of purified TFIIIA with 5 S RNA suggests that the 95 kDa protein is a TFIIIA dimer.

3.3. *Amino acid analysis of protein extracts from band b HMW RNP*

TFIIIA and the 95 kDa fraction were analyzed for amino acid composition. Unlabeled native 7 S RNP preparation was separated by SDS-PAGE, electrotransferred to a PVDF membrane, and stained with Coomassie blue. The TFIIIA and 95 kDa bands were excised and subjected to on-membrane amino acid

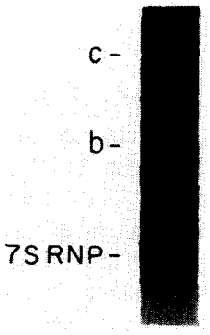

Fig.4. Western blot of 7 S RNP. Autoradiogram of 7 S RNP extract separated by 6% non-denaturing PAGE, electrotransferred to nitrocellulose, and incubated with anti-TFIIIA and ^{125}I-anti-rabbit antibody.

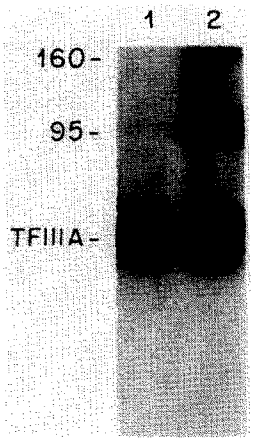

Fig.5. Western blot of protein fractions from 7 S RNP extracts (autoradiogram). Bands were excised from a 6% non-denaturing gel using adjacent ^{32}P-labeled bands as a template, placed in the wells of a 7% SDS gel, and overlaid with 5–10 μl 2 × sample buffer for 10–15 min prior to electrophoresis (lane 1, band *a*; lane 2, band *b*). Transfer and incubation as in fig.4. Distortions are due to electrophoresis from a gel slice.

analysis. Results show that TFIIIA and 95 kDa protein have a similar amino acid composition (table 2). The appearance of band *c* was variable and thus it was difficult to obtain amino acid analysis of this fraction. Amino acid analysis for the protein from band *a* was not obtained.

3.4. *Characterization of HMW RNPs with anti-TFIIIA antibody*

In addition to the amino acid analysis, polyclonal rabbit antibody generated against purified TFIIIA was used to analyze native 7 S particle extracts separated on a non-denaturing gel. Fig.4 shows that antibody reacted with RNPs of 7 S, *b,* and *c* bands but not with band *a* RNP. In addition, unlabeled *a* and *b* bands were excised from a non-denaturing gel, separated by SDS-PAGE, electrotransferred to nitrocellulose, and probed with anti-TFIIIA antibody (fig.5). The antibody reacted with TFIIIA, 95 kDa protein and 160 kDa protein. No reaction was seen with 60–63 kDa protein.

4. CONCLUSIONS

The 1:2 ratio of RNA/protein in band *b*, the TFIIIA-like amino acid composition of the 95 kDa protein fraction, and the anti-TFIIIA reactivity against the 95 kDa protein, as well as the formation of 95 kDa-containing RNP by reconstitution with purified TFIIIA, all indicate that band *b* is a dimer of TFIIIA bound to 5 S RNA. Band *c* was more difficult to analyze but may contain a tetramer of TFIIIA bound to one 5 S RNA molecule. No dimer of the 7 S particle was detected, i.e. two 5 S RNAs bound to two TFIIIAs. The 60–63 kDa-5 S RNA RNP appears to be unrelated to the 7 S particle and the protein needs to be further characterized. The molecular weight of the protein does not correspond to any of the known 5 S RNA binding proteins [19,20].

The TFIIIA dimer is stable during electrophoresis in both non-denaturing (in band *b*) and denaturing gels. The stability of the TFIIIA dimer, even in the presence of SDS, implies a tight binding of TFIIIA to itself by strong hydrophobic interactions and may indicate a specific interaction.

Previous studies have proposed that 7 S RNP formation in oocytes is a mechanism of sequestering TFIIIA, which functions in feedback regulation of the 5 S RNA gene [6,7]. It is possible that the formation of TFIIIA dimer-5 S RNA RNP may be an additional mechanism of sequestering TFIIIA in oocytes, resulting in further inhibition of 5 S RNA gene transcription.

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