

A 5S rRNA/L5 Complex Is a Precursor to Ribosome Assembly in Mammalian Cells

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Abstract. A novel 5S RNA-protein (RNP) complex in human and mouse cells has been analyzed using patient autoantibodies. The RNP is small ($\sim 7S$) and contains most of the nonribosome-associated 5S RNA molecules in HeLa cells. The 5S RNA in the particle is matured at its 3' end, consistent with the results of in vivo pulse-chase experiments which indicate that this RNP represents a later step in 5S biogenesis than a previously described 5S*/La protein complex. The

protein moiety of the 5S RNP has been identified as ribosomal protein L5, which is known to be released from ribosomes in a complex with 5S after various treatments of the 60S subunit. Indirect immunofluorescence indicates that the L5/5S complex is concentrated in the nucleolus. L5 may therefore play a role in delivering 5S rRNA to the nucleolus for assembly into ribosomes.

THE biosynthesis of ribosomes in mammalian cells involves the assembly of rRNAs synthesized by RNA polymerase I (18S, 28S, 5.8S) and by RNA polymerase III (5S) together with proteins translated from mRNAs synthesized by RNA polymerase II. Not only must the appearance of these components be coordinated on the transcriptional level, but those that are not synthesized in the nucleolus (5S and ribosomal proteins) must become specifically concentrated there. In higher eukaryotes the genes for 5S rRNA exist in tandemly repeating units which are not closely linked to the large blocks of rDNA that comprise the nucleolus organizer regions (25). 5S rRNA must therefore travel from the nucleoplasm to its nucleolar site of assembly into ribosomes.

In 1967, Knight and Darnell (16) discovered that only about half of the 5S rRNA molecules in mammalian cells are associated with 60S ribosomes. The remainder sediment in a range that could represent either free 5S RNA or 5S bound by one or a few protein molecules. One mammalian protein that is known to bind 5S RNA is the 50-kD La antigen. The La-bound 5S represents only a very small fraction ($\sim 1\%$) of the total and is slightly elongated at its 3' end (called 5S*) (38). Another well-characterized 5S-binding protein is tran-

scription factor TF IIIA (10, 33). In the *Xenopus* oocyte, 5S RNA is stored for later assembly into ribosomes as a 7S RNP (37) containing TF IIIA; TF IIIA is not present in a larger (42S) oocyte particle that contains several other proteins and tRNAs as well as 5S RNA (15, 28, 36). Mammalian TF IIIA has not yet been identified. A third known 5S-binding protein is ribosomal protein L5 (34 kD) (6). 5S RNA/L5 complexes are released from 60S ribosomes by EDTA (2, 19, 42) or a variety of other treatments (27, 34), and the isolated L5/5S particle exchanges 5S rRNA specifically (13, 32). However, it has not been determined whether L5 initially associates with 5S before or only upon ribosome assembly.

Here we have used autoantibody probes to characterize a 5S RNP that represents a substantial fraction of the nonribosomal 5S rRNA in mammalian cells. The particle appears to contain a single protein component, which is ribosomal protein L5. Its cellular location, its kinetics of appearance, and previously reported data on protein L5 suggest that this RNP is a precursor to ribosome assembly in mammalian cells.

Materials and Methods

Cells: Cultures, Labeling, Extracts

HeLa cells were derived from standard laboratory stocks. Friend erythroleukemia cells were provided by A. Sartorelli, Yale University (New Haven, CT). Cells were maintained in RPMI 1640 medium supplemented with 5% bobby calf serum (Gibco, Grand Island, NY), 60 $\mu\text{g/ml}$ penicillin, and 100 $\mu\text{g/ml}$ streptomycin, in 5% CO_2 at 37°C. For labeling with ^{32}P , 2×10^7 cells were suspended in 100 ml phosphate-minus MEM supplemented with dialyzed calf serum (Gibco), [^{32}P]orthophosphoric acid was added at either 20 $\mu\text{Ci/ml}$ or 100 $\mu\text{Ci/ml}$, and the cells were grown for 16 h. For [^{35}S]

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methionine-labeling experiments, cells were resuspended in methionine-minus MEM supplemented with dialyzed calf serum and grown for either 3 or 14 h before harvesting. Extracts of labeled cells were prepared by sonication as described (22). The protocol for the pulse-chase experiment was exactly as described by Rinke and Steitz (38).

Preparation and Analysis of RNAs

Patient sera were the kind gifts of Drs. J. Hardin and J. Craft (Yale University) and of Dr. T. Greco (Waterbury, CT). Mouse monoclonal anti-ribosome antibody (Y28) (21) was from Dr. E. Lerner. Immunoprecipitation was performed using Pansorbin (Calbiochem-Behring Corporation, San Diego, CA) or Protein A-Sepharose (Pharmacia Fine Products, Piscataway, NJ). Because of the low titer of the anti-5SRNP₁ serum, it was routinely used in 3–5 × higher amounts than other sera; therefore, with this serum, 50 µg carrier tRNA per 10 µl serum was added to reduce RNase degradation of the RNAs to be analyzed. The protocol used to treat sonicates with proteinase K before immunoprecipitation was as described by Rosa et al. (39).

RNAs were precipitated with ethanol from the aqueous phase of phenol chloroform-isoamyl alcohol extracts of Pansorbin or Protein A-Sepharose pellets and were fractionated on 10% polyacrylamide gels in 7 M urea, 90 mM Tris-borate (pH 8.0), 1 mM EDTA as described (22). For fingerprint analysis of gel-purified RNAs, bands located by autoradiography were excised, eluted by soaking overnight in 0.5 M NaAc, 2.5 mM EDTA plus 20 µg carrier RNA from yeast, and collected by ethanol precipitation of the supernatant. T1 digests of gel purified RNAs were fractionated by electrophoresis on Kalex cellophane strips, at pH 3.5, followed by homochromatography on PEI Cel 300 (Brinkmann) according to Barrell (1).

Gradient Fractionation of Extracts

A cell extract was layered over an 11 ml 15–30% sucrose gradient in 20 mM Tris (pH 7.5), 130 mM NaCl, 10 mM MgCl₂ and centrifuged for 16 h at 18,000 rpm in an SW41 rotor at 4°C. Fractions were collected by puncturing the tube with a needle and collecting drops. For the quantitative immunoprecipitation experiment, a 5 ml 5–20% gradient was prepared, and the extract fractionated for 80 min at 47,000 rpm in an SW50 rotor at 4°C. This gradient gave the same profile as the longer 15–30% gradient. Portions of gradient fractions were either immunoprecipitated or extracted directly with phenol-chloroform-isoamyl alcohol. RNAs from the immunoprecipitates and from total extracts were displayed on 10% polyacrylamide gels as described above.

Immunofluorescence

Indirect immunofluorescence was performed using either patient sera followed by FITC-conjugated goat anti-human-IgG (Miles Laboratories Inc., Naperville, IL) or mouse monoclonal anti-ribosome antibodies followed by FITC-conjugated rabbit anti-mouse-IgG (the last three the gift of Dr. E. Lerner). Prepared HEP-2 cell substrate was purchased from Immunococepts, Inc. (Sacramento, CA). The immunofluorescence procedure was exactly as described (12).

Preparation of 80S Ribosomes, 60S Subunits, and the 5S/L5 Complex

The cytoplasmic fraction from a HeLa nuclear extract (8) was clarified at 13,000 rpm for 30 min at 4°C in a Sorvall SS34 rotor. 5–6 ml portions of the supernatant were loaded over 2 ml 1.35 M sucrose on top of 2 ml 2 M sucrose, both in 50 mM Tris (pH 7.0), 25 mM KCl, and 5 mM MgCl₂, and the 80S ribosomes were pelleted overnight at 4°C at 48K rpm in a 50Ti rotor. The pellets were rinsed gently with doubly distilled water and were resuspended on ice to a final concentration of 400 A_{260nm}/ml in low salt TKM (50 mM Tris [pH 7.0], 500 mM KCl, 5 mM MgCl₂). 100-µl aliquots of 80S ribosomes were frozen and stored at –80°C.

To prepare 60S ribosomal subunits (3), each 100-µl portion (40 A_{260nm}) of 80S ribosomes was diluted to a final concentration of 1.5 M KCl and 1 mM puromycin in a total volume of 0.5 ml. The ribosomes were incubated 15 min at 0°C, then 10 min at 37°C. 400 µl of high salt TKM (50 mM Tris [pH 7.0], 1 M KCl, 5 mM MgCl₂) was then added and 1.0 ml of dissociated ribosomes was loaded onto each 36 ml 5–20% sucrose gradient in low salt TKM. Gradients were centrifuged at 4°C at 28K rpm for 5.1 h in an SW28 rotor. The 60S ribosomal fractions were collected using an Isco density gradient fractionator, diluted 1:2 in low salt TKM, and centrifuged at 4°C for 16 h at 40,000 rpm in a 50Ti rotor. The 60S ribosomal pellets were either resuspended immediately or frozen and stored at –20°C for future use.

5S/L5 complexes were released from purified 60S ribosomes essentially as described by Blobel (2). Each pellet of 60S ribosomes was resuspended gently on ice in 200 µl of doubly distilled water. The ribosomes were then divided into 2 100-µl aliquots containing 5 A_{260nm} each and either 50 µl H₂O or 25 µl H₂O plus 25 µl 0.2 M EDTA was added. After incubation for 15 min at 0°C, samples were loaded onto 4.8 ml 5–20% sucrose gradients in 0.01 M Tris (pH 7–7.5), 0.01 M KCl and were centrifuged at 40,000 rpm for 2.5 h at 4°C in a SW50 rotor. After determination of their A_{260nm}, fractions were either immunoprecipitated immediately with Protein A-Sepharose-bound antibody or precipitated in 10% TCA for 1 h on ice, boiled in 1× Laemmli sample buffer (17), and were subjected to electrophoresis on 12% polyacrylamide gels. Immunoprecipitated fractions and total controls were phenol extracted and assayed by dot blot.

Dot Blots

RNA samples in 10 × SSC (0.15 M NaCl, 0.015 M Na citrate) were blotted onto 0.45 µm nitrocellulose using a Schleicher and Schuell minifold apparatus. The filters were washed in 20 × SSC, baked for 2 or more hours at 80°C and prehybridized overnight at 42°C in prehybridization mix (50% formamide, 5 × SSC, 5 × Denhardt's (26), 50 mM potassium phosphate (pH 7.3)) containing 2.5% freshly boiled sonicated salmon sperm DNA. The filters were then hybridized overnight at 42°C in hybridization mix (50% formamide, 5 × SSC, 1 × Denhardt's, 20 mM potassium phosphate [pH 7.3]) to which freshly boiled 1% sonicated salmon sperm DNA and nick-translated *Xenopus borealis* 5S somatic DNA (king gift of E. Gottlieb, Yale University) was added. The filters were washed three times for 10 min in 2 × SSC, 0.1% SDS at room temperature and two times for 20 min at 42°C in 0.1 × SSC, 0.1% SDS. The blot was wrapped in Saran Wrap (Dow Corning Corp., Midland, MI) and exposed to film at –70°C.

Protein Analysis by Partial Proteolysis

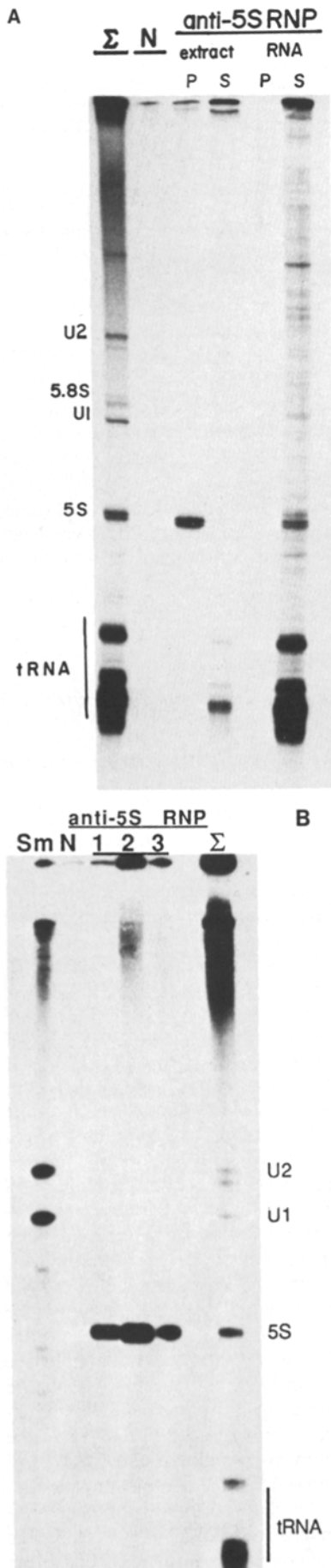
The L5 band from HeLa 60S ribosomes and the immunoprecipitated protein from [³⁵S]methionine-labeled HeLa cell extracts were compared by Cleveland mapping (digestion during electrophoresis) using *Staphylococcus aureus* V8 protease (7). Cleavage products were observed by silver staining (29) or autoradiography, respectively.

Results

Mammalian 5S RNA Is Found in a Ribonucleoprotein Particle Reactive with Patient Autoantibodies

Fig. 1 A shows HeLa cell ³²P-labeled RNAs immunoprecipitated by a previously described (11) patient serum designated anti-5SRNP₁ after fractionation on a 10% polyacrylamide gel. A single band, which comigrates exactly with the major 5S species of the total cellular RNA (lane labeled “Σ”), is seen (lane *extract P*). Two other patient sera (called anti-5SRNP₂ and anti-5SRNP₃) with the same specificity were subsequently identified; their immunoprecipitation patterns are shown in Fig. 1 B.

To ask whether protein is required for precipitation of 5S RNA by these antibodies, two types of experiments were performed. First, immunoprecipitations were attempted using naked RNAs purified by phenol-extraction/ethanol precipitation from the HeLa cell extract. Deproteinized 5S RNA did not react detectably (Fig. 1A, lane *RNA, P*); the RNA profile of the corresponding supernatant (lane *RNA, S*) demonstrated that the lack of 5S precipitation was not simply due to degradation by RNases present in the serum. Second, the requirement of protein for antigenicity was confirmed in experiments using proteinase K (39); upon proteolysis of the cell extract, 5S precipitability disappeared (not shown) whereas control experiments showed that the treatment neither destroyed the RNA nor interfered with the ability of other autoantibodies to recognize naked RNA antigens (11). Antigenic conservation could be demonstrated in at least one other mammalian species; the anti-5SRNP₁ serum also precipi-



tated a single 5S-sized band from extracts of mouse Ehrlich ascites cells (not shown).

The RNA Contained in the Immunoreactive RNP is Mature 5S RNA

To confirm that the immunoprecipitated RNA was 5S rather than some other comigrating species, it was subjected to T1 RNase fingerprint analysis. Fig. 2 shows the fingerprint of the 5S band excised from a gel-fractionated immunoprecipitate, compared with 5S ribosomal RNA obtained using the same type of gel to separate RNAs isolated from gradient-purified HeLa cell ribosomes. The pattern is exactly the same as that of ribosome-associated 5S RNA, including the mature 5' and 3' termini. This is in contrast to another 5S-like RNA called 5S*, previously identified in ribonucleoprotein complexes reactive with anti-La autoantibodies (38). 5S* molecules have additional uridylyte residues at their 3' ends and give a series of easily identifiable spots that migrate lower and to the left of the indicated 3' terminus in T1 RNase fingerprints.

The 5S RNP Is Small and Contains Most of the Nonribosomal 5S RNA in the Cell

From the results shown in Fig. 1, it was clear that the autoantibodies under study were distinct from anti-ribosome antibodies (11, 21); anti-ribosome autoantibodies precipitate from ³²P-labeled cell extracts equimolar amounts of 5S and 5.8S rRNAs as well as the large rRNAs (which barely enter the gel). However, it was conceivable that the antigenic RNP might be generated by ribosome disassembly during preparation and handling of the cell extract; for instance, the 5S/L5 complex can be released from ribosomes by various treatments (2, 19, 27, 34, 42).

To test this possibility, we first asked from what region of a sucrose gradient the 5S RNP can be immunoprecipitated. In the experiment shown in Fig. 3, we confirm the long-known fact (16) that only about half of the 5S RNA molecules in HeLa cells are ribosome associated; in *A*, where total RNAs are displayed, 5S RNA peaks both with 5.8S (in fraction 5) and again at the top of the gradient (fraction 10). Surprisingly, no immunoprecipitable 5S (*B*) is observed in the ribosome peak (fractions 5, 6). Rather, it appears exclusively at the top of the gradient (in fraction 10 and 11) moving slightly slower than U1 RNA, which is quantitatively contained in U1 snRNPs sedimenting at about 10S (23). *C* shows that the 5S at the top of the gradient is not nonspecifically precipitated by normal human serum.

Figure 1. Gel analysis of anti-5S RNP immunoprecipitates. (*A*) Anti-5S RNP₁ serum was incubated with either sonicated ³²P-labeled HeLa cells (lanes *extract*) or with deproteinized RNAs (lanes *RNA*) prepared from the sonicate. RNAs were recovered from Pan-sorbin immunoprecipitates (*P*) or supernatants (*S*) and fractionated on 10% polyacrylamide/7 M urea gels. Σ indicates total RNAs in the cell sonicate; *N* indicates precipitation with normal human serum. Whereas RNAs from equal amounts of sonicate were analyzed in the lanes *P* or *N*, 1/40 as much was used in the Σ lane, 1/45 as much in the lane *extract S*, and 1/10 as much in lane *RNA S*. (*B*) ³²P-labeled RNAs recovered from a Protein A-Sepharose immunoprecipitate using anti-5SRNP₁ were compared with those of anti-Sm, anti-5SRNP₂, anti-5SRNP₃ and a normal human serum (*N*) on a 10% polyacrylamide gel.

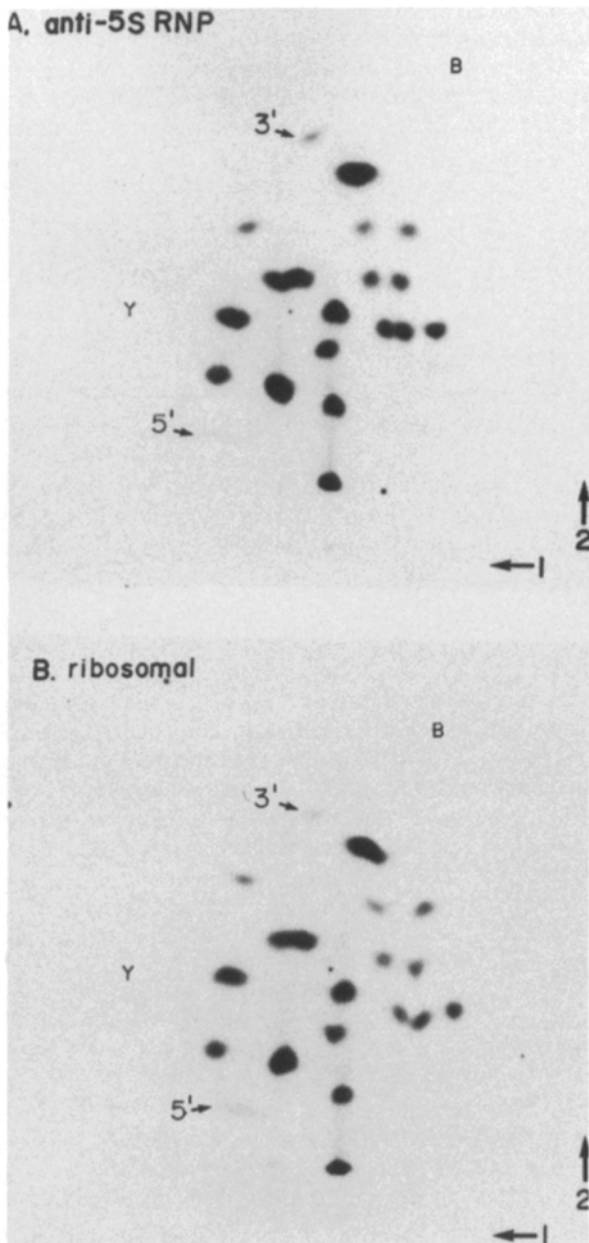


Figure 2. T1 RNase fingerprints of RNA. Gel-purified 5S RNA from an anti-5SRNP immunoprecipitate (*A*) or from ribosomes (*B*) (the 60S-80S peak of a gradient as in Fig. 3) were digested to completion with T1 RNase and subjected to electrophoresis at pH 3.5 followed by homochromatography as described by Barrell (1). The identity of all spots was confirmed by secondary digestion with RNase A. The positions of the blue (*B*) and yellow (*Y*) dyes and of the 5' end (pppGp) and 3' end (CUUUOH) of mature 5S rRNA are indicated.

To determine what fraction of the nonribosome-associated 5S is contained in antigenic complexes, we ran freshly prepared cell extracts in small sucrose gradients (which required minimal fractionation time). Immunoprecipitation of the top gradient fraction (*D*) revealed that over half of the 5S RNA could be immunoprecipitated when saturating amounts of antibody were used. (Compare the supernatant (*S*) with the immunoprecipitate (*P*) for the fraction comparable to

fraction II in *A-C*.) By contrast, precipitation of another aliquot of the same gradient fraction with saturating amounts of anti-La antibody (*D*) confirmed our previous conclusion (38) that only a very small amount ($\sim 1-2\%$) of the nonribosome-bound 5S RNA is the 5S* precursor associated with the La antigen.

Is the 5S RNP a Precursor to Ribosome-Associated 5S?

To ask whether the antigenic 5S RNP might be an intermediate in ribosome assembly, growing HeLa cells were exposed for 15 min to ^3H -uridine, followed by the addition of excess unlabeled uridine and cytidine. Portions of cell sonicates from various times up to 6 h were used for the preparation of total RNA and for immunoprecipitation with anti-La, anti-ribosome, and anti-5SRNP antibodies. The results are shown in Fig. 4. As the uridine label is chased out of anti-La precipitable 5S* precursor during the first hour (lanes 9-13), label accumulates in the RNA-protein complex recognized by anti-5SRNP antibodies (lanes 25-29). Only after 4 h of chase does 5S RNA become detectable in ribosomes (lanes 23-24). Meanwhile, the label in the anti-5SRNP-precipitated band declines (lanes 29-32). Since the total label in the 5S region remains approximately constant over the time course of the experiment (lanes 1-8), these observations are consistent with the following precursor product relationship: La 5S* \rightarrow 5S RNP \rightarrow ribosomes.

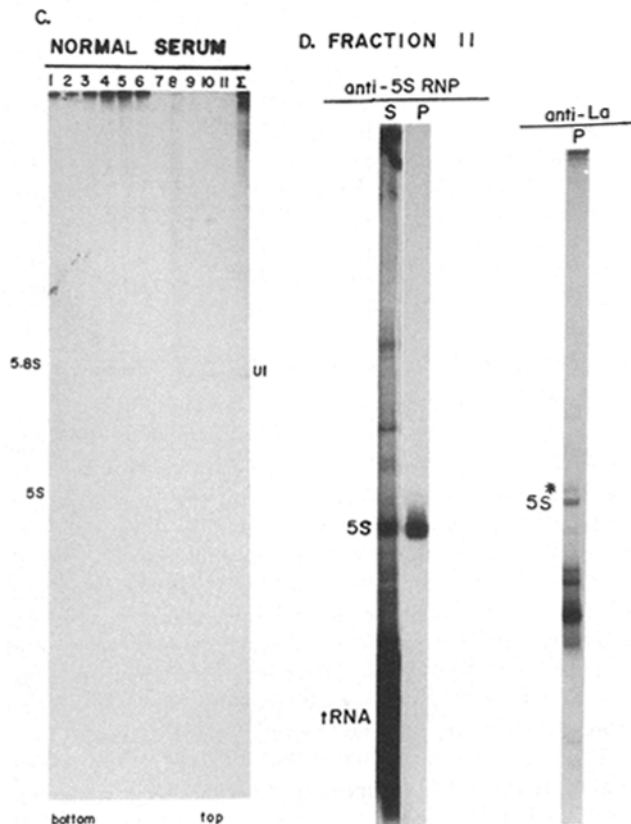
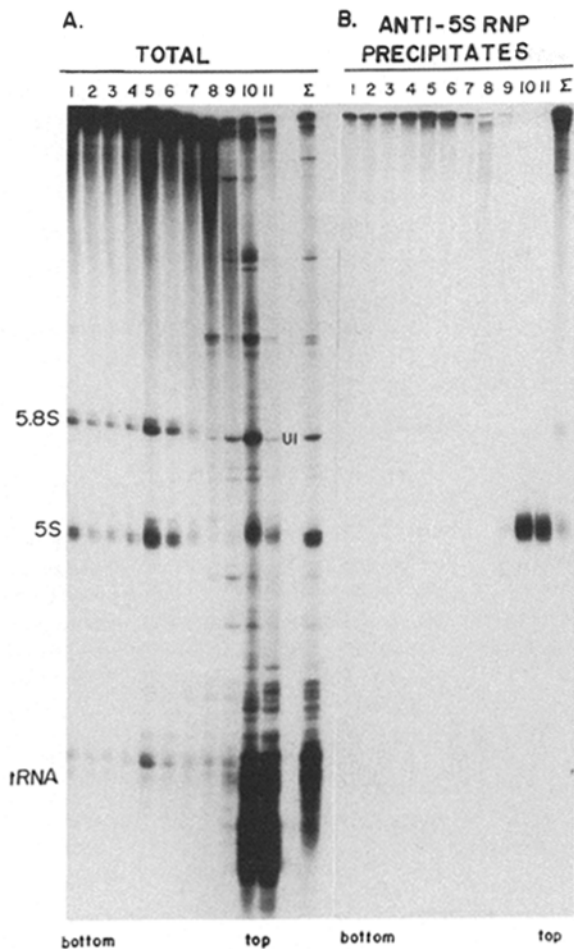
Immunofluorescence Localization of the 5S RNP in the Nucleolus

To determine the cellular location of the antigenic 5S RNP, indirect immunofluorescence studies were performed using monkey (Vero) cells and human (HEp-2) cells (Fig. 5). The pattern obtained with anti-5SRNP₁ (*A*) shows bright nucleoli and some cytoplasmic staining relative to normal serum (*C*). It is therefore similar to that given by an anti-ribosome monoclonal antibody (*B*; also see reference 21), except that the nucleoli are relatively brighter with anti-5SRNP. As a control, we show the nuclear, non-nucleolar location (*D*) observed for the Sm snRNPs (containing U1, U2, U4, U5, and U6 RNAs). Caution should always be exercised when interpreting immunofluorescence results since many autoimmune sera contain mixtures of antibodies; however, anti-5SRNP₂ and anti-5SRNP₃ gave similar staining patterns (not shown) to that in Fig. 5 *A*.

The Protein Component of the 5S RNP is Ribosomal Protein L5

To identify the protein(s) associated with 5S RNA in the RNP, we subjected [^{35}S]methionine-labeled HeLa cell extracts to immunoprecipitation using the three anti-5SRNP sera available. As shown in Fig. 6, all precipitates included a common protein band of ~ 35 kD. While anti-5SRNP₁ and anti-5SRNP₂ showed no additional bands compared with the lane containing normal human serum (*N*), the strongest serum, anti-5SRNP₃, precipitated a number of other proteins, presumably because of second specificities. The 35-kD band comigrates with the 60S ribosomal subunit protein that has been identified as L5 (lane 60S) (6).

Although the data shown in Fig. 6 made it seem highly likely that the protein component of the 5S RNP is ribosomal protein L5, more rigorous identification was necessary. In



particular, since *Xenopus* TF IIIA is ~35 kD, this molecular mass might also be expected for mammalian TF IIIA, which has not yet been identified and characterized. Our first approach was to use Western blots in the hope of demonstrating anti-5SRNP reactivity with a protein component of purified 60S ribosomal subunits. Unfortunately, after many trials, we were unable to obtain a positive signal with any of the three sera available. Lack of reactivity in Western blots is often a problem with patient autoantibodies (45), even those that exhibit extremely high titers in recognizing their corresponding native autoantigens.

We then asked whether it might be possible to immunoprecipitate the well-studied 5S/L5 complex that is released from 60S ribosomes. Fig. 7 confirms the early findings of Blobel (2) and shows that exposure to EDTA (bottom gel) releases 5S rRNA nearly quantitatively (see dot blot) along with a single protein previously identified as L5 (42). Both migrate in the top three fractions of the gradient analyzed. When fractions from a gradient containing EDTA-treated 60S ribosomes were subjected to immunoprecipitation by anti-5SRNP₂ versus normal serum (Fig. 8), 5S RNA was precipitated by the autoantibody only from the top of the gradient (fractions 8–10) where the 5S/L5 complex sediments. This argues strongly that the protein recognized by the anti-5SRNP antibodies is L5.

Finally, we compared the partial proteolysis profile of the ³⁵S-labeled immunoprecipitated protein with that of L5 excised from an SDS gel fractionating purified 60S ribosomes. Fig. 9 shows that upon exposure to V8 protease, the labeled and unlabeled proteins yield similar patterns of peptide products; some differences are to be expected since the methionine content of the various silver-stained peptides will vary. By contrast, the 60S ribosomal protein migrating just above L5 (designated U) gives a distinctly different pattern. This further argues that the 5S RNP protein is L5 rather than another ribosomal or nonribosomal polypeptide.

Discussion

We have studied a novel 5S RNA-containing RNP from

Figure 3. Sedimentation of the 5S RNP in a sucrose gradient. A ³²P-labeled HeLa cell sonicate was layered onto a 15–30% sucrose gradient and sedimented as described in Materials and Methods. Eleven fractions were collected and divided into three portions with relative volumes of 1:10:10. (A) Total RNAs extracted from the small portion of each gradient fraction. (B) RNAs immunoprecipitated from one of the large portions using anti-5SRNP₁ serum. (C and D) (C) RNAs immunoprecipitated from the other large portion using normal human serum. The Σ lanes display fractions of the original cell sonicate that were treated like the remaining samples in panels A, B, and C, respectively. Markers run in a parallel gradient indicated that fraction 5 corresponded to about 70S. (D) Two aliquots from the top fraction of a smaller gradient (see Materials and Methods) were subjected to precipitation with saturating amounts (5× the usual ratio of serum to extract) of anti-5SRNP₁ antibody or of anti-La antibody. P = RNAs from the immunoprecipitated pellets. S = RNAs in the supernatant from the anti-5SRNP immunoprecipitation. The anti-La lane was run on another gel, but represents an equal amount of the same gradient fraction and was exposed for the same period of time as the anti-5SRNP gel lanes. 5S* is a 3' extended precursor to 5S RNA which is bound by the antigenic La protein (38).

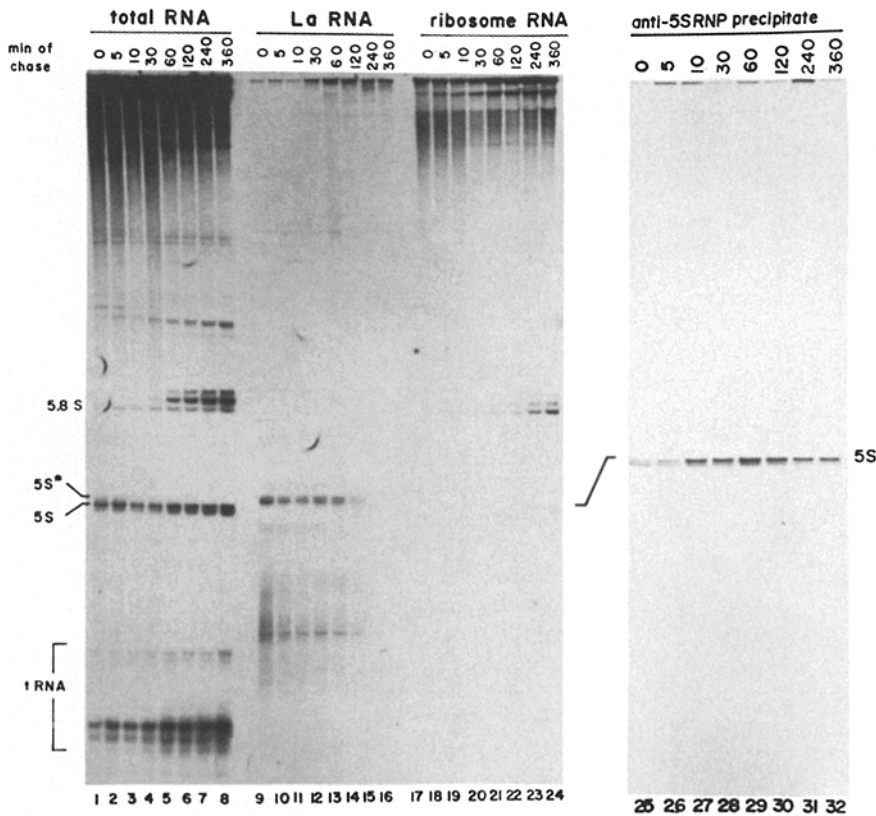


Figure 4. Immunoprecipitates of HeLa cell sonicates after pulse-chase labeling. HeLa cells, labeled for 15 min with [5,6-³H]-uridine, were chased with unlabeled uridine and cytidine for various times (0, 5, 10, 30, 60, 120, 240, 360 min). Total small RNAs from each time point are shown together with RNAs precipitated by anti-La antibody, by anti-ribosome antibody, and by anti-5SRNP₁ antibody, after fractionation on a 10% polyacrylamide gel. The relative amounts of cell sonicate used for total RNA (lanes 1-8) compared with anti-La (lanes 9-16), anti-ribosome (lanes 17-24) and anti-5SRNP immunoprecipitation (lanes 25-32) were 1:5:25:5, but precipitation by the anti-ribosome antibody was far from quantitative in the experiment presented.

mammalian cells that is recognized by rather rarely occurring autoantibodies. The RNP contains 5S RNA that has been matured at its 3' end and comprises at least 50% of the nonribosome-associated 5S RNA in cell extracts. The RNP appears to contain a single protein component, which has been identified as the 60S ribosomal protein L5. Pulse-chase studies and the nucleolar location of the 5S/L5 RNP suggest that this particle is a precursor to ribosomes in somatic mammalian cells.

The Protein in the 5S RNP is L5

From the start, it seemed most likely that the protein component of the antigenic 5S RNP might be ribosomal protein L5. This is the protein that is bound tightly to released 5S when 60S ribosomes are exposed to EDTA (2, 19, 42), formamide (34), or citrate (27). Also, HeLa cell L5 exhibits an unusually large free pool size (18, 35), making it a very attractive candidate for interacting with 5S RNA before its incorporation into ribosomes. However, for two reasons, identification of this protein proved difficult. (a) We never succeeded in obtaining a positive signal in Western blots with any of the three sera available. This could be due to a labile determinant or to a requirement for the presence of RNA to maintain the reactive autoepitope. Lack of reactivity in blots has been observed with other RNP autoantigens (45). (b) Mammalian L5 is an exceedingly insoluble polypeptide (6, 13). This property accounts for the fact that L5 was not initially identified as a 5S binding protein (44, but see 30) and could explain recurrent problems we had "losing" the protein during analysis of the 5S RNP.

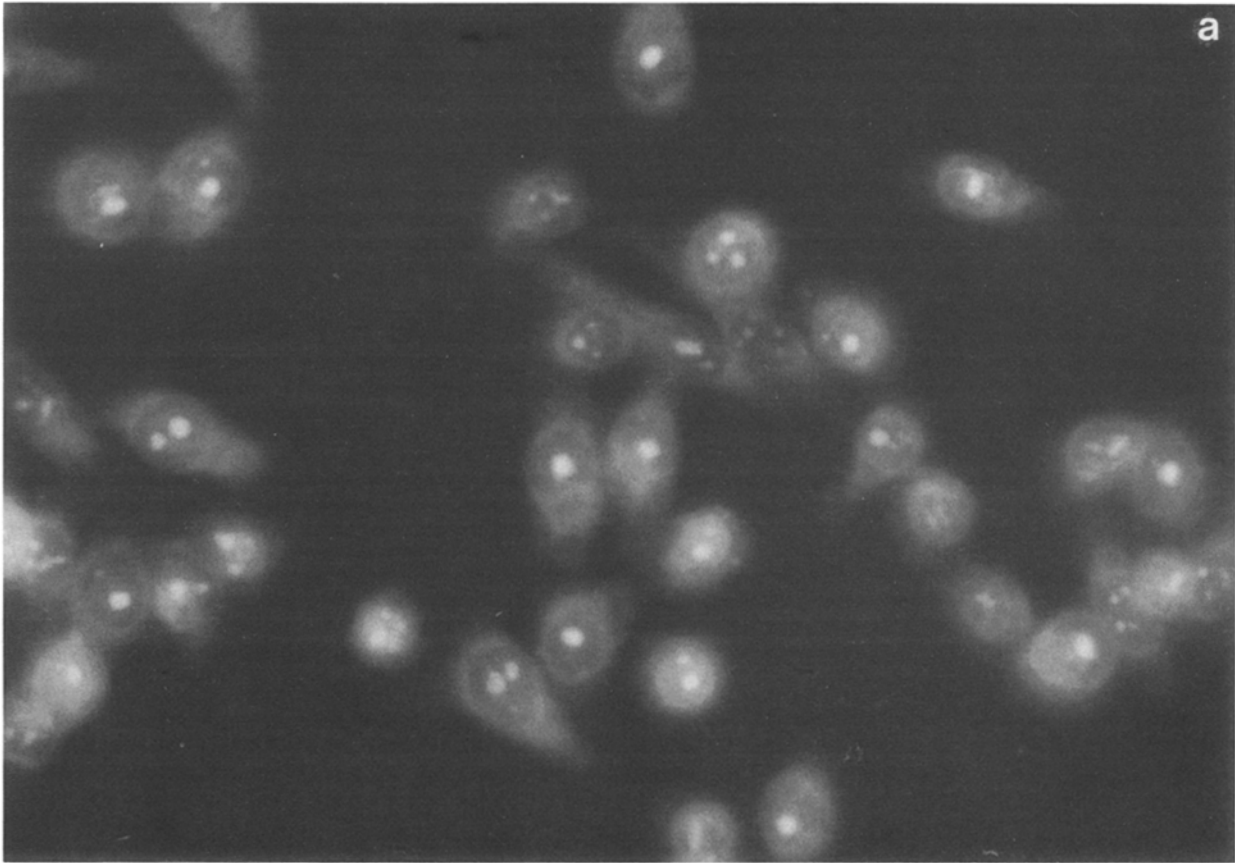
However, by subjecting the newly-released 5S/L5 complex

directly to immunoprecipitation, we were able to demonstrate its reactivity with the antisera (Fig. 8). Because L5 is the only protein comigrating with 5S rRNA in gradients of EDTA-dissociated ribosomes (Fig. 7), we conclude that L5 must be the antigenic protein.

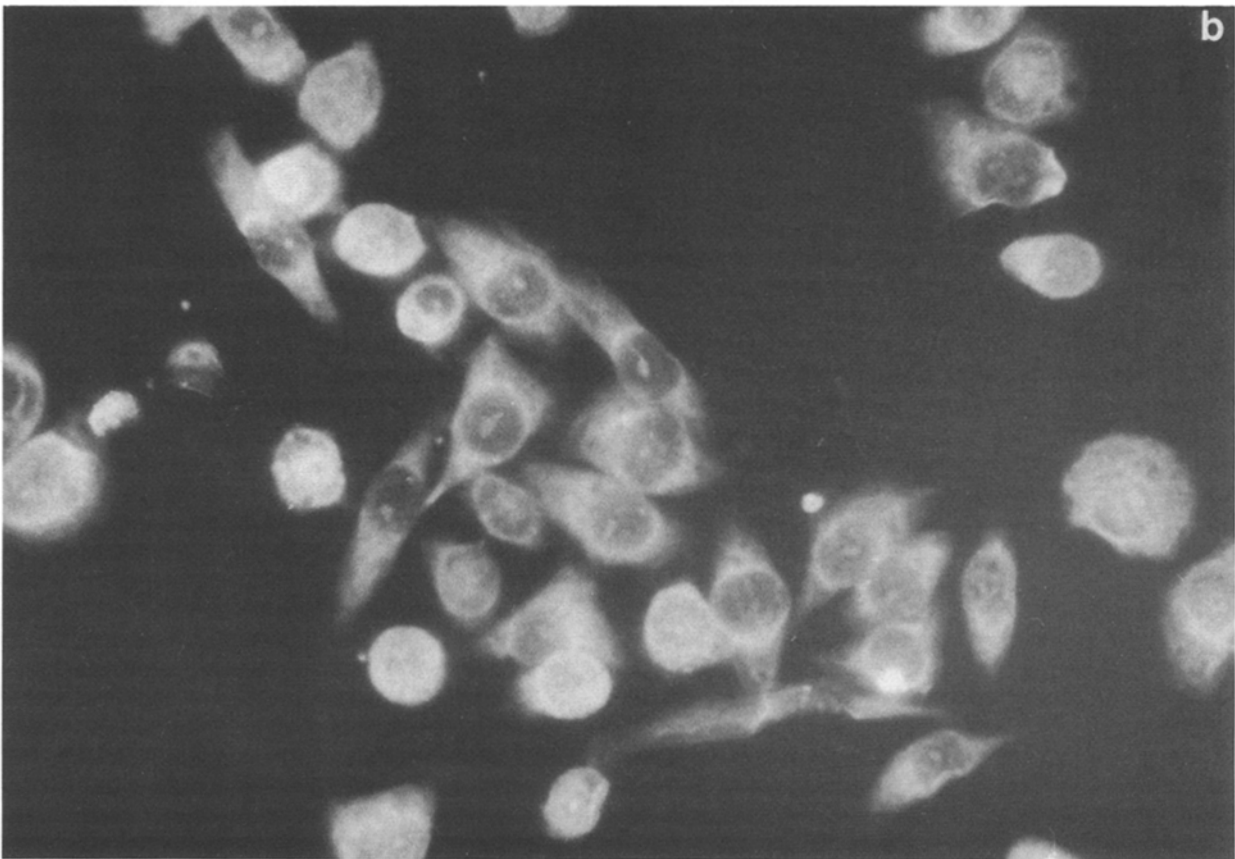
Since L5 is a ribosome component, it may seem curious that the anti-5SRNP antisera do not immunoprecipitate intact ribosomes from cell extracts or brightly stain the cytoplasm in immunofluorescence studies. However, 5S rRNA has been shown to be largely unavailable to the exterior once in place on the ribosome (24), making it likely that L5 is also buried. Some reexposure of the determinant upon cell fixation might explain the slight cytoplasmic fluorescence that is observed (Fig. 5) with anti-5SRNP antibodies.

A Pathway for 5S Biogenesis in Mammalian Cells

The identification of autoantibodies directed against three different ribonucleoprotein states of mammalian 5S RNA has provided a unique opportunity to order the corresponding steps in the lifetime of this RNA molecule. Previously, we demonstrated (38) that newly synthesized HeLa cell 5S* RNA, the majority of which has one or two additional U residues at its 3' end, is transiently associated with the 50 kD La antigen, which binds its U-rich terminus (41). Here we have shown that once matured, the 5S RNA binds L5 to enter a second small RNP state, whose labeling profile in pulse-chase experiments (Fig. 4) suggests that it too is a precursor to ribosome-bound 5S. As previously observed (16, 20, 43), the assembly of 5S RNA into ribosomes (a large RNP state) in HeLa cells is very slow; even after 4 h of chase the 5S band contains less than 1/10 as much label as 5.8S (see Fig. 4, lanes



a



b

Figure 5. (cont.)

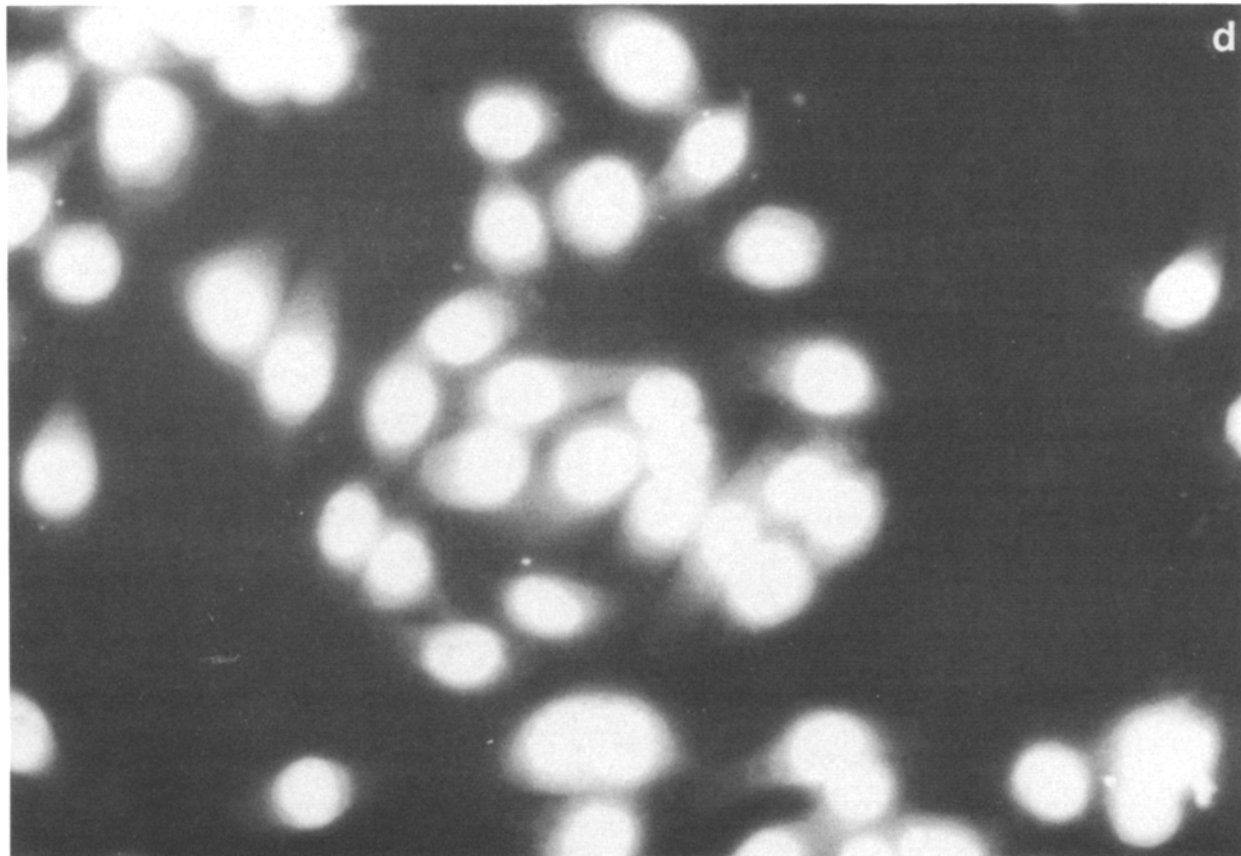
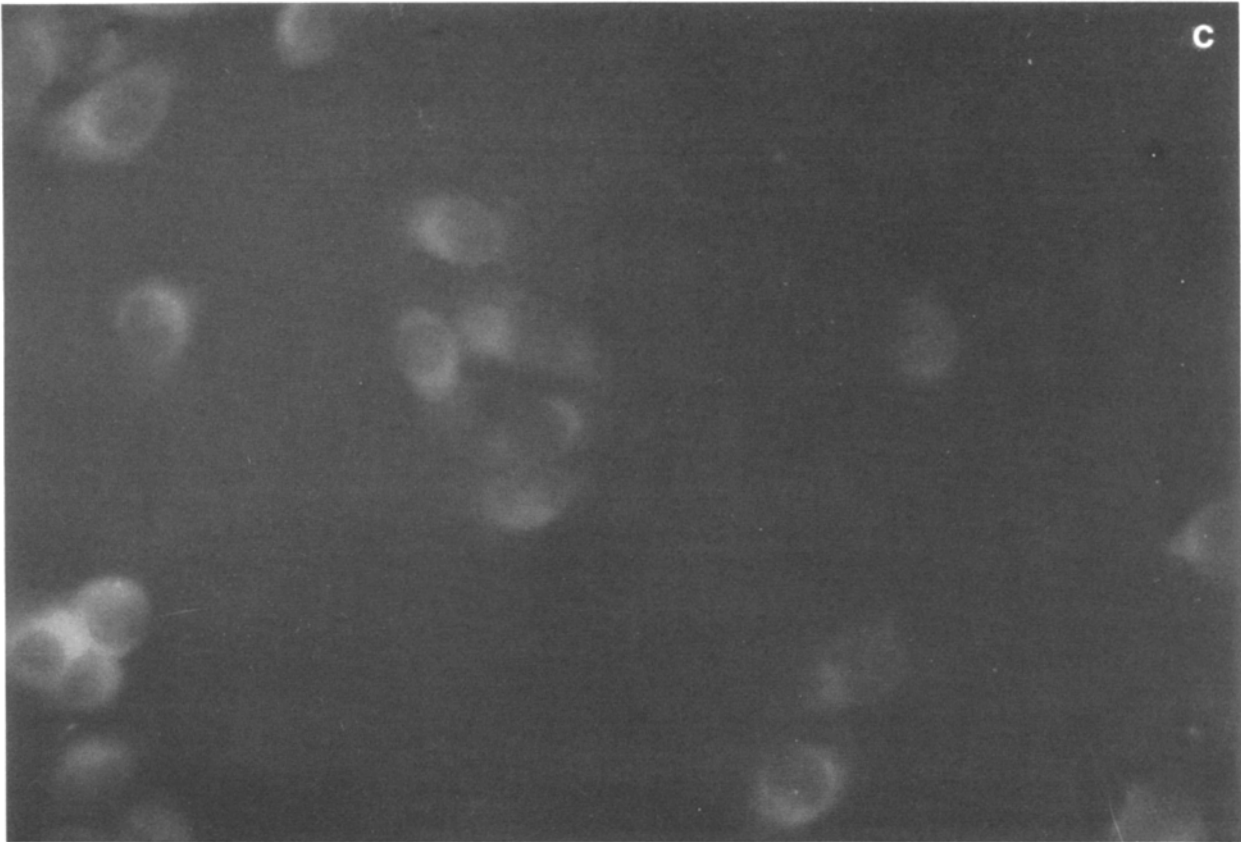


Figure 5. Immunofluorescent staining of HEp-2 human cells with autoantibodies. (*a*) Anti-5SRNP₁ serum; (*b*) anti-ribosome monoclonal antibody; (*c*) normal human serum; (*d*) anti-Sm serum. Note that the exposure time in (*c*) is much longer than for the other antibodies, thereby exaggerating the background staining.

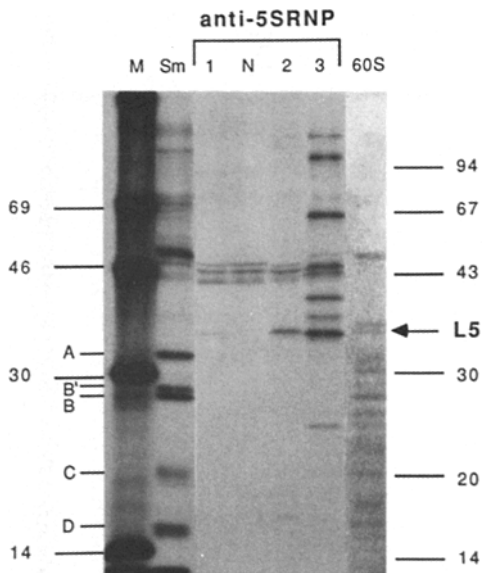


Figure 6. Gel analysis of [³⁵S]methionine-labeled anti-5SRNP immunoprecipitates. Antibodies preabsorbed to Protein A-Sepharose were incubated with ³⁵S-labeled HeLa cell sonicates. The washed immunoprecipitates were boiled in 1× Laemmli gel sample buffer and fractionated on 10% SDS gels. M indicates ¹⁴C-labeled high MW protein markers (Amersham Corp., Arlington Heights, IL). Sm and N indicate precipitation with anti-Sm serum and normal human serum, respectively. Proteins from purified 60S ribosomal subunits were fractionated in an adjacent lane of the same gel and visualized by Coomassie staining. The L5 band was identified by comparison with the known pattern of 60S ribosomal proteins (6).

23 and 24), and several more hours are required for the two RNAs to appear equimolar in anti-ribosome immunoprecipitates (not shown).

A three-step pathway, consistent with the above data can be proposed for mammalian 5S RNA assembly into ribosomes: La 5S* RNP → 5S/L5 RNP → ribosomes. Because it was not possible to use saturating amounts of each type of antibody in our pulse-chase studies, we cannot claim that this pathway is the only pathway of 5S rRNA biogenesis. Likewise, additional RNP intermediates may exist, and we do not know to what extent the various RNP states might overlap. It is even conceivable that 5S RNA complexed with L5 might be destined for degradation (20), but its nucleolar location makes this seem unlikely.

Indirect immunofluorescence studies using the same three autoantibodies have further suggested where the various intermediates in 5S biogenesis might be localized in the mammalian cell. Anti-La stains the nucleus most brightly, but the nucleoli and cytoplasm also show some La antigen (12). Since there are numerous RNA polymerase III transcripts in the cell, all of which bind the La protein at least initially (12), definitive conclusions concerning the precise location of only one of these, 5S*, cannot be made. However, since mammalian 5S genes are not associated with the rDNA at nucleolus organizer regions (25), it seems most likely that newly synthesized 5S* RNA appears first in the nucleoplasm, bound by La.

In contrast to anti-La, the anti-5S RNP antibody gives primarily nucleolar fluorescence. It is known that 5S assem-

bles into ribosomes in the mammalian cell nucleolus (43). Either of the two following scenarios are therefore possible: (a) after (or during) its 3' end maturation 5S RNA makes a short excursion into the cytoplasm (where it picks up L5) before entering the nucleolus, as first suggested by Leibowitz et al. (20); or (b) newly synthesized 5S RNA binds L5 already present in the nucleoplasm and quickly travels to the nucleolus where it assembles as a bimolecular complex into nascent ribosomes. The observation that 5S RNA synthesized in isolated nuclei in vitro can be chased from the anti-La precipitable to the anti-5S RNP precipitable form (Rinke, J., data not shown) is more consistent with the second possibility, for it strongly argues that the L5 protein must be present in isolated nuclei.

The pathway proposed for 5S biogenesis in mammalian cells begins to resemble the multiple protein-bound states that have been elucidated for 5S RNA in another well-studied vertebrate system, the amphibian oocyte. A 7S particle containing 5S RNA and transcription factor TF IIIA (10, 33) serves as a storage particle in previtellogenic oocytes (37). Another 5S storage form, a 42S RNP, contains 2 different proteins, 5S and tRNA molecules (15, 28, 36). A third 5S RNP can be released from mature amphibian oocyte ribosomes by EDTA treatment (15). Also, a 55-kD protein which cross-reacts with human anti-La autoantibodies binds to newly synthesized 5S RNA made in *Xenopus* oocyte extracts (Gottlieb, E., and H. Pelham, unpublished observations). The order of these various states and how they might relate to 5S biogenesis in *Xenopus* somatic cells are still unclear.

Likewise in the yeast, *S. cerevisiae*, 5S rRNA is released from ribosomes complexed with a protein which is the yeast L5 equivalent, YL3 (31). Very recent studies by Brow and Geiduschek (5) have detected formation of a 5S/YL3 complex in an active yeast cell-free system programmed with a 5S rRNA gene. Their data further indicate the existence of a TFIIIA/5S complex, and suggest that binding of the two proteins to 5S is mutually exclusive. Although the order of these states has not been determined, the yeast complex involving 5S and L5 strongly resembles that described here for HeLa (and presumably other mammalian somatic) cells.

Is L5 a Nucleolar Targeting Protein?

Previous studies have identified polypeptide sequences that target proteins to the nucleus (9). Whether analogous signals specify protein delivery to the nucleolus is not known. Alternative explanations for subcellular localization suggest that the mechanism is not an active process, but that cellular components simply concentrate wherever specific binding sites exist. In the case of the 5S/L5 RNP, its high abundance (approximately equal to that of cytoplasmic ribosomes [10⁷/cell] far greater than the number of assembling ribosomes) argues against the idea that this complex becomes concentrated in the nucleolus simply because of its binding to nascent ribosomes.

If L5 is a nucleolar targeting protein, it may explain delivery of another important small RNA molecule to the nucleolus. Viroids, which are important plant pathogens, replicate in plant cell nucleoli (40). Within the highly conserved central domain of nearly all viroid RNAs (14) is a bulge-loop structure which exhibits primary sequence homology to eukaryotic 5S rRNA. These regions are apparently also struc-

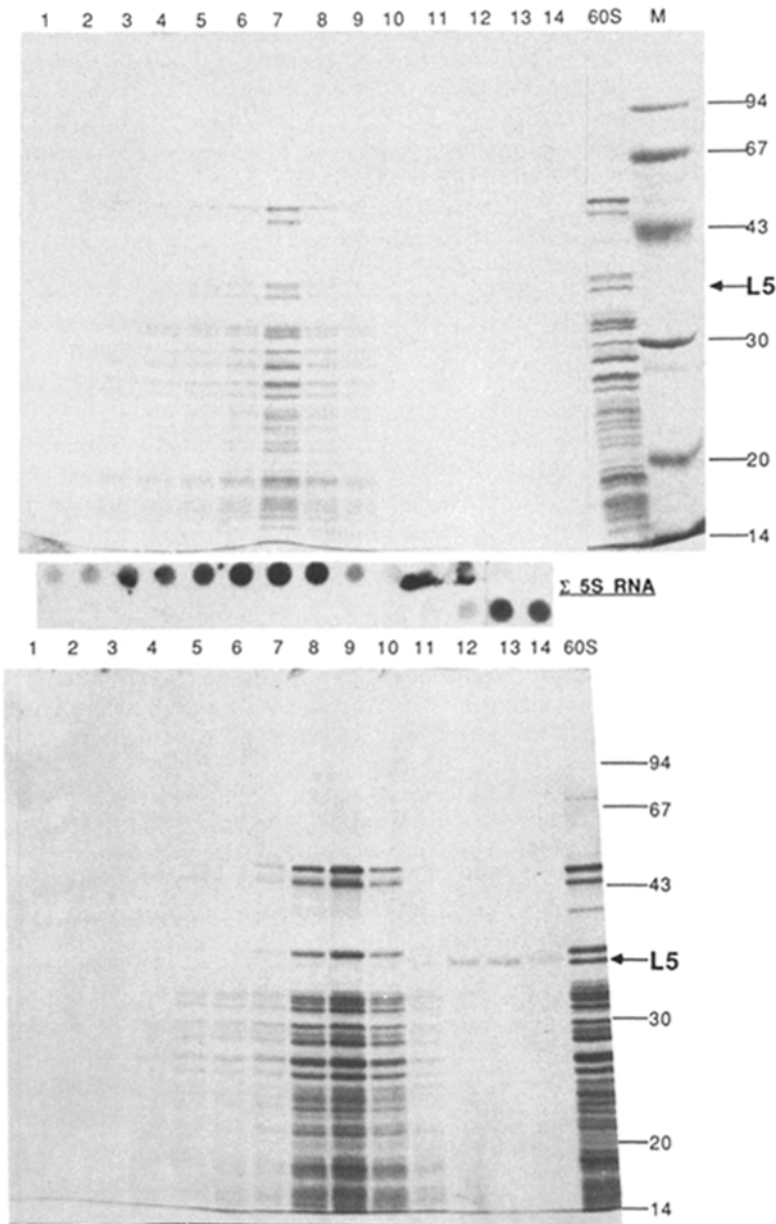


Figure 7. Release of the 5S/L5 complex from 60S subunits by EDTA. 60S ribosomal subunits, in the absence (top gel) or presence (bottom gel) of EDTA, were fractionated on 5–20% sucrose gradients. An aliquot of each fraction (1–14) was blotted onto nitrocellulose and probed with nick-translated *Xenopus borealis* 5S somatic DNA (Σ 5S RNA). Gradient fractions were then TCA precipitated and analyzed on 10% Laemmli gels along with 60S ribosomal subunits and low molecular weight protein markers (Pharmacia Fine Chemicals) as in Fig. 6.

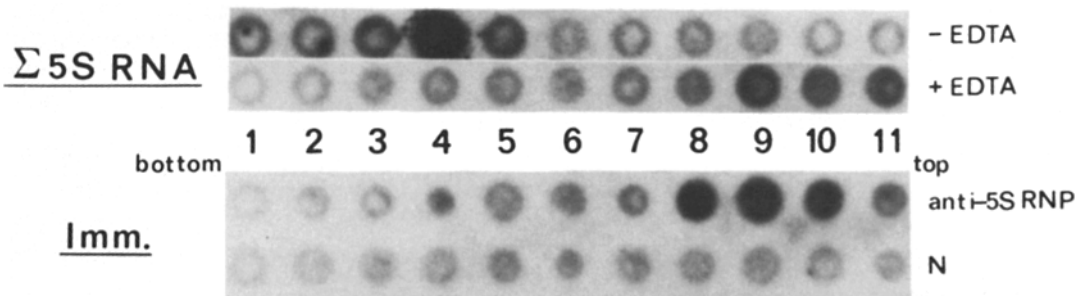


Figure 8. Immunoprecipitation of gradient fractions containing EDTA-treated 60S ribosomes with anti-5SRNP₂ or normal human serum. Small portions of each fraction from two 5–20% sucrose gradients, one containing untreated (–EDTA) and one containing EDTA-treated (+EDTA) 60S ribosomes, were phenol extracted, blotted onto nitrocellulose, and probed with ³²P-labeled 5S RNA (Σ 5S RNA). The remainder of each fraction from the gradient containing EDTA-treated 60S ribosomes and from a parallel identical gradient were immunoprecipitated with anti-5SRNP₂ or normal human serum. The immunoprecipitates were phenol-extracted and analyzed by dot blot as above (Imm.).

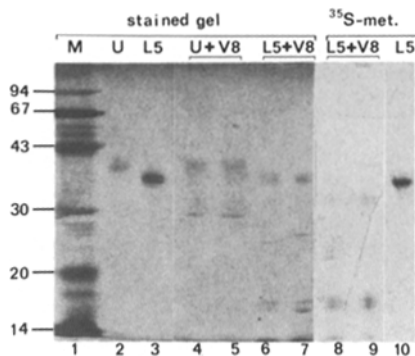


Figure 9. Comparison of the ^{35}S -labeled immunoprecipitated protein with 60S ribosomal proteins by partial proteolysis. 60S ribosomal subunits and a 1:1 mixture of an equal amount of 60S subunits and a ^{35}S -labeled immunoprecipitate were fractionated on a 15% Laemmli gel. The L5 band was excised from the gel as was a second band running just above it (U, lane 2). These proteins were then subjected to proteolysis with *Staphylococcus aureus* V8 protease (V8) during electrophoresis on a second 15% Laemmli gel. Lane 1 contains low molecular mass protein markers while lanes 2 and 3 contain the excised proteins, U and L5, in the absence of V8 protease. In lanes 4 and 5, the upper protein (U), excised from pure 60S ribosomes, was digested with 0.1 μg or 0.2 μg of V8 protease, respectively. Similarly, in lanes 6 and 7, L5 was treated with 0.1 μg or 0.2 μg V8 protease. The resulting partial proteolysis profiles (lanes 1-7) were visualized by silver stain. In lanes 8 and 9, the L5 band excised from a mixture of purified 60S ribosomes (nonradioactive L5) and ^{35}S -labeled immunoprecipitate (^{35}S -labeled L5) was digested with 0.05 μg or 0.025 μg V8 protease. Lane 10 contains the same mixture of ^{35}S -labeled and nonradioactive L5 in the absence of V8 protease. Patterns of proteolysis in lanes 8-10 were visualized by autoradiography.

naturally homologous, since both 5S rRNAs and viroids form a high efficiency UV-crosslink within this sequence (4). Intriguingly, this sequence occurs within that portion of the 5S rRNA molecule that is protected from α -sarcin by binding to ribosomal protein L5 (13). The possibility that L5 selectively binds to viroid RNAs and delivers them to the nucleolus is currently under investigation.

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