

## Alu RNA Transcribed *in Vitro* Binds the 68-kDa Subunit of the Signal Recognition Particle\*

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**Antibodies directed against the 68-kDa subunit of signal recognition particle (SRP) precipitate an Alu RNA-protein complex formed during *in vitro* transcription of a plasmid containing an Alu family sequence. The same Alu RNA-protein complex is precipitated by anti-Alu sera from certain patients with systemic lupus erythematosus and related autoimmune diseases (Kole, R., Fresco, L. D., Keene, J. D., Cohen, P. L., Eisenberg, R. A., and Andrews, P. G. (1985) *J. Biol. Chem.* 260, 11781-11786). Similarly to anti-SRP antibodies human, anti-Alu sera precipitate SRP from HeLa cell extract and detect a 68-kDa SRP subunit on immunoblots. These results indicate that the Alu antigen and the 68-kDa SRP subunit are the same polypeptide.**

Antibodies from patients with systemic lupus erythematosus and related autoimmune diseases have been very useful in elucidating the structure and the role of small nuclear ribonucleoprotein particles (RNPs)<sup>1</sup> in processing (1, 2) and in polyadenylation of mRNA (3). In addition to small nuclear RNP proteins, there are other antigens recognized by autoantibodies whose functions are unknown (for review, see Ref. 4), and it is likely that other, as yet undefined, antibody specificities are also present in the sera. Since the antibodies may be used as tools for studying the functions of the antigens, it is important to define new specificities of the autoimmune sera. In addition, characterization of the autoantibodies may provide clues as to the causes of the autoimmune disease.

Kole *et al.* (5) previously found an Alu antigen with a molecular mass of approximately 68 kDa that binds to *in vitro*-transcribed Alu RNA but not to adenovirus VAI RNA. Subsequent work<sup>2</sup> showed that, under similar conditions, the Alu antigen does not bind to other RNA polymerase III transcripts such as *Xenopus* 5 S RNA or human tRNA, excluding the possibility that the antigen is an unspecific factor binding to all RNA polymerase III transcripts. The function of the antigen was not established.

We had two reasons to believe that the Alu antigen may be

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<sup>1</sup> The abbreviations used are: RNP, ribonucleoprotein particle; SRP, signal recognition particle; Alu family sequence, the repetitive DNA sequence that is cut by Alu restriction enzyme; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

<sup>2</sup> P. G. Andrews and R. Kole, unpublished data.

a subunit of signal recognition particle (SRP), a complex of six polypeptides and 7 SL RNA (see Ref. 6 for review) that takes part in transport of secretory proteins through the endoplasmic reticulum. First, Alu sequences comprise about half of 7 SL RNA (7), and second, SRP contains a subunit with the same molecular mass as the Alu antigen (68 kDa; Refs. 5 and 6). However, the 68-kDa protein in the fully assembled and functional SRP has been reported to be associated with the portion of the 7 SL RNA that has no homology to Alu sequences (8, 9). Results presented below indicate that the 68-kDa SRP subunit does bind directly to Alu sequences and that this protein is also recognized by anti-Alu autoantibodies.

### EXPERIMENTAL PROCEDURES

Plasmid A 36 carrying an Alu sequence (10) and plasmid 7L 30.1 carrying the human 7 SL RNA gene (11, a kind gift from Dr. Elisabetta Ullu, Yale University) were used for *in vitro* transcription of Alu RNA and 7 SL RNA, respectively. Human autoimmune sera were from the bank of frozen samples from rheumatology patients of the North Carolina Memorial Hospital. Sera were used either without further treatment or, in some instances, were fractionated by precipitation with 40% ammonium sulfate and dialysis against 10 mM Tris-buffered saline. Antibody specificity of the autoimmune sera was characterized in detail in a previous report (5). In addition to anti-Alu specificity, these sera have anti-U1-RNP/anti-Sm (patient A. L.), anti-U1/U2 (McC.), and anti-U1-RNP (G. M.) antibodies. Preparation of rabbit sera raised against purified subunits of canine SRP was described in detail elsewhere (12). SRP was purified as described (13). Anti-SRP sera and purified SRP were kind gifts from Drs. P. Walter and P. Hoben (University of California, San Francisco).

*In vitro* transcriptions were carried out with  $\alpha$ -<sup>32</sup>P]GTP as a label in the HeLa whole cell extract according to the protocol of Manley *et al.* (14). To obtain an *in vivo*-labeled whole cell extract, HeLa cells ( $5 \times 10^6$  cells/ml) grown in suspension were incubated overnight in low phosphate medium (low phosphate minimal Eagles medium, 5% fetal calf serum) with carrier free [<sup>32</sup>P]orthophosphate at 20  $\mu$ Ci/ml and sonicated as described (15). To prepare a cytoplasmic extract, which was used for immunoprecipitations of 7 SL RNA by human sera, labeled cells were harvested, resuspended at  $5 \times 10^7$  cells/ml in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, and swollen on ice for 10 min. Swollen cells were homogenized with 10 strokes in a Dounce homogenizer with tight-fitting pestle, nuclei and cell debris pelleted by centrifugation at 15,000  $\times g$  for 10 min, and the cytoplasmic supernatant collected.

Immunoprecipitations were carried out according to the protocol of Lerner and Steitz (16). In a typical experiment, 50  $\mu$ l of the cell extract (*in vivo*-labeled or after *in vitro* transcription) was diluted with 100  $\mu$ l of NT-2 buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.05% Nonidet P-40) and incubated with 5  $\mu$ l of the serum for 30 min on ice. The antibody-antigen complex was then precipitated with 200  $\mu$ l of Pansorbin (Behring Diagnostics). Immunoprecipitations of *in vivo*-labeled SRP (see Fig. 5) with human autoantibodies (ammonium sulfate fraction) were carried out overnight. The labeled RNA was isolated from the immunoprecipitates by phenol extraction and analyzed on an 8% polyacrylamide-sequencing gel (17). Immunoprecipitated RNAs were identified by their mobility on the gel compared to

*in vitro* transcripts. The identities of Alu RNA and 7 SL RNA transcripts were previously determined by RNA fingerprinting (10, 11, 18).

For RNA-protein cross-linking (19), the transcription reaction volume was increased 4-fold, to 200  $\mu$ l, and the concentration of the label ( $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  and/or UTP) to 1 mCi/ml. After a 1-h incubation, the reaction was diluted 10-fold with 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , and approximately a 50-fold excess of unlabeled nucleotide, and UV-irradiated in a thin layer on a weigh boat for 15 min at 4  $^\circ\text{C}$  (UV dose,  $1.8 \times 10^5$  ergs/ $\text{mm}^2$ ). This mixture was digested with 400 units/ml of micrococcal nuclease and 25  $\mu\text{g}/\text{ml}$  pancreatic RNase for 60 min at 37  $^\circ\text{C}$  and then shaken for 2 h at 4  $^\circ\text{C}$  with antibody-protein A-Sepharose conjugate (to prepare the conjugate, 20  $\mu$ l of serum was incubated for 2 h with 5 mg of preswollen protein A-Sepharose (Pharmacia) and washed three times with NT-2 buffer). The beads were washed five times with 1 ml of NT-2 buffer and the bound antibody-antigen complex dissolved by boiling for 5 min in 125  $\mu$ l of gel sample buffer (125 mM Tris/Cl, pH 6.8, 10% glycerol, 1 mM EDTA, 10 mM dithiothreitol, 6% SDS, and bromophenol blue). The samples were analyzed on an 8% SDS-polyacrylamide gel (20) which was autoradiographed for 2 weeks at  $-70^\circ\text{C}$  with Fuji RX film and intensifying screen (Lightning Plus, Du Pont Cronex). The weak signal is to be expected since only a few labeled nucleotides remain cross-linked to the protein after nuclease treatment.

Immunoblots of purified SRP with human autoimmune and with rabbit sera were performed according to standard protocols (21). Horseradish peroxidase or alkaline phosphatase-conjugated (Cooper Biomedical) secondary antibodies were used with human sera. Binding of rabbit antibodies was detected by using a bridging antibody and a rabbit peroxidase-antiperoxidase complex (Cooper Biomedical) (22). Color detection was carried out using a Protoblot kit (Promega Biotec) for alkaline phosphatase and 3-amino-9-ethylcarbazole for horseradish peroxidase. Proteins on nitrocellulose filters were stained with 0.1% India ink (23) in 10 mM Tris, pH 7.4, 0.9% NaCl followed by 0.005% amido black in 0.5% acetic acid, 1% methanol (21).

## RESULTS

**Binding of 68-kDa Subunit to *in Vitro*-transcribed Alu RNA**—To test the hypothesis that the 68-kDa protein found in Alu-RNP and SRP are the same polypeptides, we have assayed two sera raised in rabbits against purified canine SRP subunits (12, 13) for immunoprecipitation of *in vitro*-transcribed Alu-RNA. An immunoblot (see Fig. 4, lanes 8–10) shows that one serum was active against the 72-kDa SRP subunit (anti-72 SRP) and the second serum against the 68-kDa SRP subunit (anti-68 SRP). Fig. 1, lane 2, shows that rabbit anti-68 SRP serum precipitates Alu RNA in this assay. To exclude the possibility that immunoprecipitation is due to some contaminating antibody in anti-68 SRP serum, we have used antibodies immunopurified on the 68-kDa SRP (12) and have shown that similar to human anti-Alu sera (Fig. 1, lane 6, see also Ref. 5) the immunoselected antibody precipitates Alu RNA (Fig. 1, lane 4). These results show that the 68-kDa subunit of SRP is associated with *in vitro*-transcribed Alu RNA. This was an unexpected finding since this protein was reported to remain with an SRP subparticle from which Alu sequences of 7 SL RNA have been removed (8, 9). Serum raised against the 72-kDa subunit of canine SRP (anti-72 SRP) was negative in this assay (Fig. 1, lane 1), in agreement with this subunit's reported binding to the S sequence of 7 SL RNA (8, 9), which has no homology to Alu RNA.

To ascertain whether *in vitro*-transcribed Alu RNA binds directly to 68-kDa SRP subunit, we have UV-cross-linked the RNA-protein complex, digested it with micrococcal and pancreatic ribonucleases, and immunoprecipitated with rabbit anti-68 SRP serum (see "Experimental Procedures"). In this procedure the proteins bound to RNA are labeled by  $^{32}\text{P}$ -labeled ribonucleotides which remain covalently cross-linked to the proteins after the nuclease treatment. Fig. 2, lane 2, shows that a labeled 68-kDa protein band (indicated by the

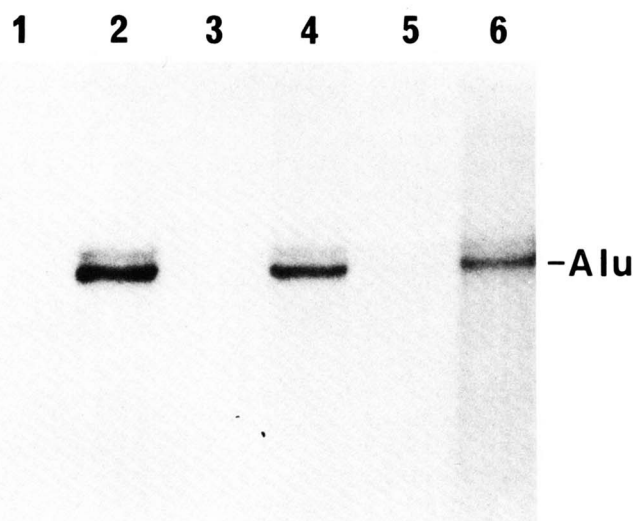


FIG. 1. Immunoprecipitation of Alu RNA transcribed *in vitro*. Alu RNA was transcribed *in vitro* and immunoprecipitated as described under "Experimental Procedures." Samples were analyzed on an 8% polyacrylamide-sequencing gel. Lane 1, anti-72 SRP rabbit serum. Lane 2, anti-68 SRP rabbit serum. Lane 3, nonimmune rabbit serum. Lane 4, immunoselected, rabbit anti-68 SRP antibody. Lane 5, normal human serum. Lane 6, serum AL, a human anti-Alu autoimmune serum.

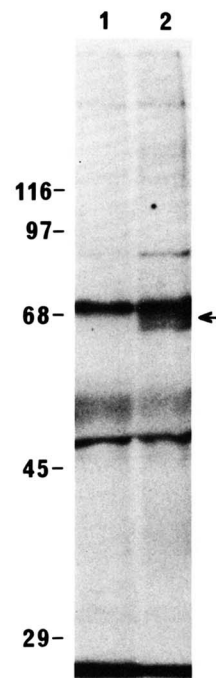


FIG. 2. Cross-linking of Alu RNA to 68-kDa antigen. Alu RNA-protein complex formed during *in vitro* transcription was cross-linked with UV radiation, digested with nucleases, and immunoprecipitated as described in detail under "Experimental Procedures." Immunoprecipitated proteins were analyzed on an 8% SDS-polyacrylamide gel (20) and the gel autoradiographed. Lane 1, immunoprecipitation with nonimmune rabbit serum. Lane 2, immunoprecipitation with anti-68 SRP rabbit serum.

arrow) is specifically precipitated by anti-68 SRP serum and not by nonimmune serum (Fig. 2, lane 1), indicating that this subunit can bind directly to Alu RNA sequences. All other bands visible on the gel were repeatedly precipitated by both sera apparently due to nonspecific interaction between RNA-protein complexes and IgGs.

**Immunoprecipitation of *in Vitro*-transcribed 7 SL RNA**—

*In vitro*-transcribed 7 SL RNA was immunoprecipitated by human anti-Alu sera (Fig. 3, lanes 2 and 3) indicating that the Alu-antigen binds to 7 SL RNA. Both anti-SRP rabbit sera (lanes 5 and 6) are also positive in this assay indicating that in this experiment the 68- and 72-kDa antigens bind to 7 SL RNA as could be expected. Quantitative analysis of the experiments presented in Fig. 1 shows that about 25% of the input, transcribed Alu RNA was immunoprecipitated (not shown, see also Ref. 5), whereas only a small fraction of *in vitro*-transcribed 7 SL RNA was immunoprecipitated by human and rabbit sera. Since the whole cell extract used for *in vitro* transcriptions contains significant amounts of 7 SL RNA (cf. Ref. 2),<sup>2</sup> it is likely that the low level of precipitation of labeled 7 SL RNA is due to competition for the antibody by the excess of the unlabeled SRP present in the extract.

**Immunoblots of SRP Proteins**—Identity of the Alu antigen with the SRP 68-kDa subunit was tested in immunoblots of purified dog SRP. Four  $\mu$ g/lane of SRP protein was separated on an SDS gel, blotted, and probed with human and rabbit antibodies as described under "Experimental Procedures." Three anti-Alu human sera reacted with the 68-kDa subunit of SRP, albeit very weakly (Fig. 4, lanes 4, 5, and 7). Another anti-Alu serum, which was somewhat weaker in immunoprecipitations, could not be detected in this assay (not shown). As can be seen in Fig. 4, rabbit antibodies (lanes 9 and 10) react with their respective antigens more strongly than all three human sera. This difference in reactivity can be ascribed either to species differences between human and dog antigens or to loss of antigenicity of the 68-kDa SRP subunit due to SDS denaturation. Since rabbit anti-SRP sera were raised against SDS-denatured proteins, they, in contrast to human sera, may react equally well with native and denatured proteins. It should also be noted that the method of detection of rabbit antibodies (see "Experimental Procedures") may be somewhat more sensitive than that for human sera.

**Immunoprecipitation of *in Vivo*-labeled 7 SL RNA**—If the Alu antigen and the 68-kDa SRP subunit are identical, one would expect that signal recognition particle will be immunoprecipitated from cellular extracts by anti-Alu lupus sera

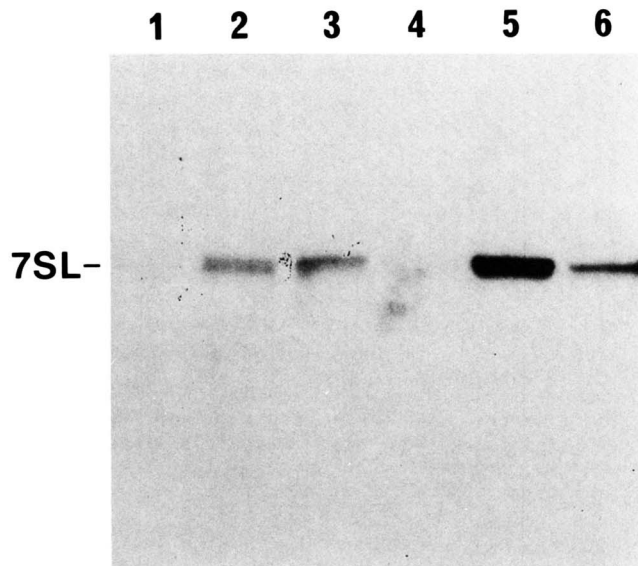


FIG. 3. **Immunoprecipitation of 7 SL RNA transcribed *in vitro*.** 7 SL RNA was transcribed, immunoprecipitated, and analyzed as in Fig. 1. Lane 1, normal human serum. Lane 2, serum AL. Lane 3, serum McC, human anti-Alu serum. Lane 4, nonimmune rabbit serum. Lane 5, anti-68 SRP rabbit serum. Lane 6, anti-72 SRP rabbit serum.

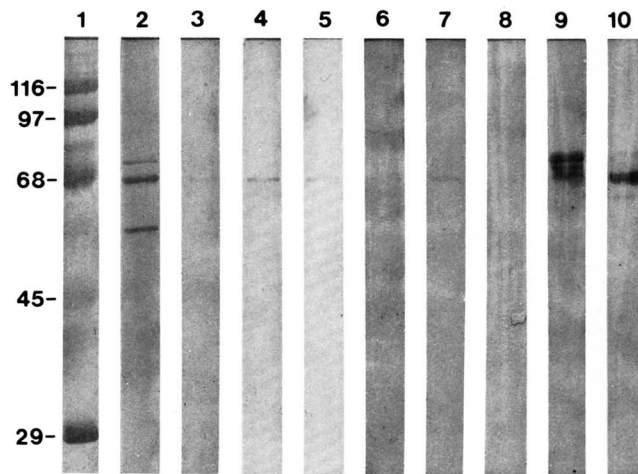


FIG. 4. **Immunoblots of purified dog SRP.** 4  $\mu$ g/lane of SRP proteins were separated on an 8% SDS-polyacrylamide gel (20) and blotted on nitrocellulose. Strips were incubated with the sera and developed using either alkaline phosphatase-conjugated (lanes 3-5) or horseradish peroxidase-conjugated (lanes 6-10) secondary antibodies. In lanes 3-5 primary antibody was diluted 50-fold, in lanes 6-10, 100-fold. Lane 1, molecular weight standards. The molecular mass in kilodaltons is indicated. Lane 2, proteins of SRP. Blots in lanes 1 and 2 are stained with India ink followed by amido black as described under "Experimental Procedures." Lane 3, normal human serum. Lane 4, serum GM, human anti-Alu serum. Lane 5, serum AL. Lane 6, normal human serum. Lane 7, serum McC. Lane 8, nonimmune rabbit serum. Lane 9, anti-72 SRP rabbit serum. Note that the 72-kDa protein is partially degraded. Lane 10, immunoselected anti-68 SRP antibody.

and by rabbit anti-SRP sera. This is indeed the case, as can be seen in Fig. 5. 7 SL RNA was immunoprecipitated overnight from a <sup>32</sup>P-labeled HeLa cell cytoplasmic extract by an anti-Alu lupus antibody (Fig. 5, lane 8). Additional bands visible in this lane are most likely cytoplasmic precursors to small nuclear RNAs (24) immunoprecipitated by anti-Sm antibody also present in this serum. (Sm antibody precipitates mature and/or precursors to U1, U2, U4, U5, and U6 small nuclear RNAs). Under the conditions of the experiment, only a small fraction, less than 5-10% of 7 SL RNA, was immunoprecipitated. (The intensities of 7 SL RNA bands in lane 6 and 8 appear to be comparable; however, only 1/10 of the amount used in immunoprecipitation in lane 8 was loaded on the gel in lane 6). The addition of more antibody did not increase the amount of precipitated 7 SL RNA, apparently due to increased degradation of RNA. As could be expected normal human serum (lane 7) and nonimmune rabbit serum (lane 3) are negative, whereas rabbit anti-68 SRP and anti-72 SRP immunoprecipitate <sup>32</sup>P-labeled 7 SL RNA in this assay (Fig. 5, lanes 1 and 2). We have also carried out immunoprecipitation experiments using *in vivo*-labeled sonicates from Raji cells, which were reported to have higher levels of 7 SL RNA (25). The efficiency of the precipitation in these experiments was no better than that for HeLa cell extracts (results not shown).

#### DISCUSSION

The experiments presented above show that both the Alu antigen and the 68-kDa SRP subunit bind to Alu RNA and 7 SL RNA transcribed *in vitro*, and both bind to 7 SL RNA in HeLa cells. These experiments as well as the similar molecular mass of the Alu antigen (5) and the 68-kDa SRP subunit, point to the identity of these proteins. It remains formally possible that two different 68-kDa proteins bind to both 7 SL RNA and to Alu RNA and that the sera react with their

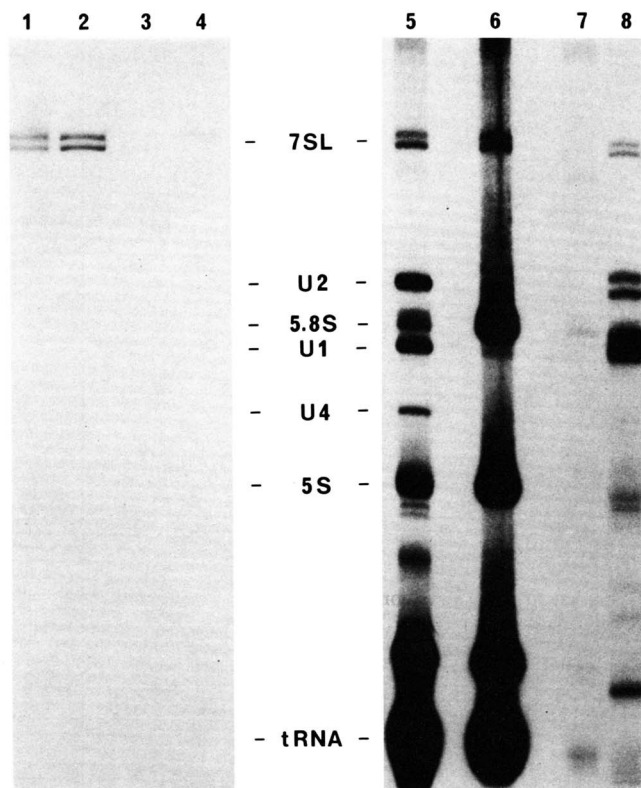


FIG. 5. Immunoprecipitation of *in vivo*  $^{32}\text{P}$ -labeled HeLa cell extracts. Labeled extracts were prepared and immunoprecipitated with the sera as described under "Experimental Procedures." Lane 1, anti-68 SRP rabbit serum. Lane 2, anti-72 SRP rabbit serum. Lane 3, normal rabbit serum. Lane 4, *in vitro*-transcribed 7 SL RNA, as size marker. Lane 5, whole cell extract from HeLa cells, position of different small RNAs is indicated. Lane 6, cytoplasmic HeLa cell extract, which was used for immunoprecipitations with human sera. Lane 7, normal human serum. Lane 8, serum AL, anti-Alu human serum. Due to partial degradation, 7 SL RNA appears on a gel as a doublet (29).

respective, different antigens. However, Walter and Blobel (12) showed that most, if not all, cellular 7 SL RNA can be precipitated by anti-SRP sera which indicates that, in the cell, 7 SL RNA is present exclusively in the signal recognition particle. Therefore, precipitation of 7 SL RNA by anti-Alu serum must be due to recognition of an SRP subunit by the antibody. In view of the fact that the composition of SRP is very well established, the possibility that there are two different 68-kDa antigens in this particle seems unlikely.

It was previously reported that anti-Alu sera do not precipitate 7 SL RNA from HeLa whole cell extract and that the Alu-antigen could not be detected on immunoblots of HeLa whole cell or nuclear extracts (5). Several changes in the experimental protocols explain these differences. Immunoblots in this report were carried out with purified SRP preparation which provided higher concentrations of antigen on the filter than the crude extracts used previously. Since the reaction with the autoimmune sera is weak under present conditions, it is not surprising that the antigen could not be detected in crude extracts. All the immunoprecipitations reported previously were carried out for 10–30 min at 0 °C, following established protocols (15). These conditions were sufficient for precipitation of SRP by the rabbit sera but human anti-Alu sera required prolonged, overnight incubation with the antigen. The difference in reactivity between the human and the rabbit antisera is probably due to binding to different sites on the 68-kDa subunit. As noted above, the

latter antibodies were raised against denatured SRP subunits and may recognize different epitopes than the autoimmune sera. In functional, fully assembled SRP, the 68-kDa subunit interacts with the 72-kDa protein and possibly with other subunits or subcellular components (26). These interactions may hinder access of anti-Alu antibodies to a greater degree than the rabbit antibodies. This explanation is supported by the fact that *in vitro*-transcribed Alu RNA, which does not bind to the 72-kDa protein, is immunoprecipitated by anti-Alu and anti-68 SRP rabbit serum equally well (Fig. 1, compare lanes 6 and 4).

The fact that immunoprecipitation and cross-linking experiments show binding of 68-kDa SRP protein to Alu RNA was somewhat surprising since this protein had been reported to be associated with the S region and not the Alu sequences of 7 SL RNA (8, 9). These conclusions were based on sedimentation analysis of proteins and RNA fragments after partial nuclease digestion of SRP (8) and on the functional assay of the partially reconstituted particle (9). Our data indicate that, at least immediately after *in vitro* transcription, this protein associates with Alu RNA. These results are not necessarily contradictory. It is possible that the 68-kDa subunit's association with the S sequence is due primarily to interactions with other subunits, most likely 72 kDa, and not directly with the RNA. In fact the 68- and 72-kDa protein copurify through several steps during purification and are considered to behave as a heterodimer (26). It is also possible that the 68-kDa protein binds initially to the Alu portion of 7 SL RNA and then moves to the S fragment during the assembly of the functional signal recognition particle or that it binds to both the Alu and the S regions.

It is not clear if Alu RNAs are transcribed *in vivo* as separate transcription units, but they are known to transcribe as part of some pre-mRNAs (27). In some cases, *e.g.* mRNA for low density lipoprotein receptor (28), the Alu sequences are not removed from the precursor during splicing and remain in cytoplasmic mRNA. It would be of interest to test if these sequences bind the SRP subunits which are associated with the Alu portion of 7 SL RNA.

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