Human signal recognition particle (SRP) RNA is transcribed by RNA polymerase III and terminates with $-\text{GUCUCUUUOH}_9$ on its 3′ end. Our previous studies showed that the three terminal uridylic acid residues of human SRP RNA are post-transcriptionally removed and a single adenylic acid residue is added, resulting in a 3′ end sequence of $-\text{GUCUCUAOH}_9$ (Sinha, K. M., Gu, J., Chen, Y., and Reddy, R. (1998) $J$. Biol. Chem. 273, 6853–6859). In this study we show that the Alu RNA, corresponding to the 5′ and 3′ ends of SRP RNA, is also accurately processed and adenylated in vitro. Alu RNAs containing 7 or 11 additional nucleotides on the 3′ end were accurately processed and then adenylated. Deletion analysis showed that an 87-nucleotide-long motif comprising of the 5′ and 3′ ends, including stem IV of the Alu RNA, is sufficient and necessary for the 3′ end processing and adenylation. A 73-nucleotide-long construct with deletion of stem IV, required for the binding of SRP 9/14-kDa proteins, was neither processed nor adenylated. The adenylated Alu RNA as well as adenylated SRP RNA were bound to the SRP 9/14-kDa heterodimer and were immunoprecipitated by specific antibodies. A significant fraction of SRP RNA in the nucleoli was found to be processed and adenylated. These data are consistent with nascent SRP and/or Alu RNAs first binding to SRP 9/14-kDa protein heterodimer, followed by the removal of extra sequence on the 3′ end and then the addition of one adenylic acid residue in the nucleus, before transport into the cytoplasm.

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The signal recognition particle plays an important role in translocation of membrane proteins and secretory proteins (Refs. 11–16; reviewed in Refs. 13–15). Human SRP is composed of a 300-nucleotide-long RNA component and six proteins (Ref. 17; reviewed in Refs. 13 and 14). Human SRP RNA is transcribed by RNA polymerase III (18), and the 5′ and 3′ end portions of SRP RNA are over 80% homologous to the highly repeated Alu sequences in the primate genomes (19–24). Studies have shown that the Alu sequence of the SRP RNA is the progenitor of the highly repeated Alu interspersed elements (21, 25). It appears that Alu sequences arose from the removal of the middle portion (S fragment) of the SRP RNA, reverse transcription, and integration of the DNA into the genome (22, 25).

The SRP consists of two distinct functional domains. The first one is the Alu domain consisting of the 5′ and 3′ end portions of SRP RNA associated with two proteins, the 9- and 14-kDa protein heterodimer (26, 27). The 5′ end of the Alu domain has a tRNA-like structure and plays an important role in arresting the elongation of nascent peptide in the ribosome (28, 29). The minimal domain necessary for binding with 9/14-kDa protein heterodimer is an 86-nucleotide-long domain including 5′ end, 3′ end, and stems III and IV (see Fig. 3, A and B) in the Alu portion of SRP RNA (30). The second functional domain consists of the SRP RNA-specific S fragment and four SRP proteins. This domain is responsible for targeting the ribosome-nascent peptide chain complex to the surface of rough endoplasmic reticulum by interacting with SRP receptor (reviewed in Refs. 13 and 31). Recently, Jacobson and Pederson (32) showed that SRP RNA, when injected into the nucleoplasm, first migrates to the nucleolus and then to the cytoplasm. Deletion analysis showed that the minimal domain necessary for migration from the nucleoplasm to the nucleolus is an 86-nucleotide-long domain including 5′ end, 3′ end, and stems III and IV in the Alu portion of SRP RNA (32). These studies show that the SRP motif involved in binding 9/14-kDa protein heterodimer and migration from nucleoplasm via the nucleolus on its way to cytoplasm is the same (30, 32).

Maraia and colleagues (33) carried out studies on the biogenesis of Alu RNA in primates including humans. In humans, the primary transcript of Alu RNA is dimeric with left and right monomers. In some of the dimeric Alu RNAs, only the left monomer but not the right monomer can bind the SRP 9/14-kDa protein heterodimer with high affinity. The loss of Alu right monomer affinity for SRP9/14 binding is associated with the accumulation of Alu RNA in the cytoplasm containing only the left monomer. Export of SRP RNA from the nucleus to the cytoplasm involves Alu domain and Alu domain competes with SRP RNA in the nucleus for limiting transport factors (34). These and other studies show that common proteins bind to SRP and Alu RNAs, and it is likely that there is a common maturation pathway for both Alu and SRP RNAs.

In this study, we show that the minimal domain necessary
for 3’ end processing and adenylation is again an 87-nucleotide long domain including 5’ end, 3’ end, stem III, and stem IV in the Alu portion of SRP RNA. These data show that this tRNA-like domain of SRP RNA has multiple functions in the biogenesis of SRP RNA, including binding of 9/14-kDa protein heterodimer, 3’ end processing, adenylation, and migration to the nucleolus on its way to the cytoplasm.

MATERIALS AND METHODS

Chemicals and Isotopes—[α-32P]ATP and [α-32P]CTP were purchased from Amersham Pharmacia Biotech. All other chemicals including standard nucleoside triphosphates and dNTPs and radioactive labeling reagents were purchased from New England Biolabs. All RNA products were purified by fractionation on 10% polyacrylamide gel.

In Vitro Synthesis and Purification of Substrate RNAs—To prepare the Human SRP RNA, DNA template amplified by PCR was transcribed by T7 RNA polymerase. The SRP RNA obtained in this manner contained three uridylic acid residues on the 3’ end. Plasmid DNA containing Alu portion of canine SRP RNA (p7Alu) under T7 promoter was a gift from Dr. Katharina Strub (27). This Alu sequence was altered containing Alu portion of canine SRP RNA (designated Alu RNA) and cloned into pUC19 vector by insertion into EcoRI and HindIII sites. The Alu RNA was transcribed by T7 RNA polymerase from the DNA template linearized by DraI, and the transcribed RNA contained three uridylic acids on the 3’ end. Alu161+7 and Alu161+11 RNAs were transcribed from the plasmid DNA template linearized by HindIII and DNA obtained by PCR amplification, respectively. All the deletion mutants of Alu RNA were constructed by PCR-mediated mutagenesis and cloned into pUC19 vector (Alu Δ1–23, Alu Δ66–147) or TA cloning vector PCR™ 2.1 (Invitrogen) (Alu Δ68–141). Alu Δ1–23 and Alu Δ60–147 RNAs were transcribed by T7 RNA polymerase from the plasmid DNA template linearized by DraI. Alu Δ68–141+10 RNA was transcribed from the plasmid DNA template digested by EcoRI. Alu Δ151–159, Alu Δ68–141, and Alu Δ60–147+9 RNAs were transcribed from DNA prepared by PCR. All the Alu mutants with 3’ end variations were constructed by PCR amplification, respectively. All the deletion mutants of Alu RNA were constructed by PCR-mediated mutagenesis and cloned into pUC19 vector. Alu159 construct was cloned into pUC19 vector, and Alu159 RNA was made by T7 RNA polymerase transcribed of DNA plasmid linearized by DraI. The in vitro transcription with T7 RNA polymerase was performed according standard protocol (New England Biolabs). All RNA products were purified by fractionation on a 10% polyacrylamide gel, extracted from the gel, and purified by precipitation with ethanol. The concentration of the RNAs was determined by optical density measurements at 260 nm.

Preparation of HeLa Cell Nuclear Extract and in Vitro Labeling—Extracts were prepared from HeLa cells grown in suspension culture by the procedure of Dignam et al. (35). The final protein concentration of the extract was 5 mg/ml. In vitro labeling of RNAs, 5 μl of 10× in vitro labeling buffer (6 mM each GTP, UTP, and CTP, 250 μM ATP, 10 mM dithiothreitol, 200 mM KCl, 60 mM creatine phosphate, and 100 mM Tris-HCl, pH 8.0), 40 μl of nuclear extract, and 50 μCi of [α-32P]ATP were mixed in a total reaction volume of 50 μl and incubated at 30 °C for 3 h. The amount of in vitro synthesized Alu RNA used as substrate for adenylation assay was ~1 μg (20 pmol). In the case of other RNAs, correspondingly more or less amount of in vitro synthesized RNAs were added for adenylation assay to keep the amount of substrate RNAs at 20 pmol in each reaction. Labeled RNAs were extracted using phenol-chloroform procedure, purified, and fractionated on 10% polyacrylamide gel and/or size fractionation on 20% polyacrylamide gels containing 7 M urea.

Micrococcal Nuclease Treatment—The micrococcal nuclease treatment was done essentially as described by Parker and Steitz (36). HeLa cell nuclear extract was incubated with micrococcal nuclease at a concentration of 1 unit/μl in the presence of 0.75 mM CaCl2 at 30 °C for 30 min. The reaction was inactivated by the addition of 2.5 mM EGTA, and the extract was then used for adenylation reactions.

Immunoprecipitations—Immunoprecipitations were carried out as described by Steitz (37). Alu RNA was incubated with 15 μl of HeLa cell nuclear extract in the presence of [α-32P]ATP in a total volume of 50 μl. One microliter of the labeled extract was immunoprecipitated with different amounts (20 and 50 μl) of anti-SRP 9.6-kDa protein antibodies (kindly provided by Dr. Maraia, National Institutes of Health, Bethesda, MD) or anti-bovine serum albumin antibodies as negative control. RNAs from the starting material, supernatant, and immunoprecipitate were purified and separated on 10% polyacrylamide gel.

In Vitro Processing—Approximately 10 fmol of the internally labeled Alu161+1, Alu161+7, and Alu161+11 RNAs were added for adenylation assay to keep the amount of substrate RNAs at different amounts (20 and 50 pmol in each reaction. Labeled RNAs were extracted using phenol-chloroform procedure and purified by precipitation with ethanol. The purified RNAs were fractionated on 10% polyacrylamide gel at 7 M urea gels, dried, and subjected to autoradiography.

Determination of 3’ End Adenylic Acid of SRP RNA—An oligonucleotide (5′-GATCTGATAGTGTCACCTAAATGAATTCA*-3′) with 3’-cordycepin (A*) was ligated to RNAs purified from the HeLa cells or HeLa cell nuclei. The nucleotides were prepared as described by Rothblum et al. (39). A BglII restriction site would be created if the 3’ end nucleotide of the RNA is an adenylic acid. A human SRP RNA-specific oligonucleotide (SRP RNA220, 5′-ACTCCCGTGCTGATCAGTAG-3′) was used for PCR amplification. Internally labeled PCR products were subjected to BglII digestion and fractionated on a 10% polyacrylamide gel.

RESULTS

Adenylation of SRP and Alu RNA in Vitro—We showed previously that SRP RNA present in the HeLa cell extract gets adenylated when incubated in the presence of [α-32P]ATP (Ref. 10; also see Fig. 1, lane 1). In this study, we standardized an in vitro adenylation system that is dependent on the addition of exogenous SRP RNA or Alu RNA. First, the HeLa cell extract was treated with micrococcal nuclease and the nuclease was inactivated by the addition of EGTA. This HeLa cell extract digested with micrococcal nuclease was unable to adenylate any endogenous RNAs since the substrate RNAs were degraded by the nuclease (Fig. 1, lane 2). There is some residual tRNA resistant to micrococcal nuclease treatment. This may be due to secondary structure of tRNAs and/or association with protein(s). Addition of SRP RNA resulted in labeling of exogenous SRP RNA or Alu RNA. First, the Alu RNA extract was treated with micrococcal nuclease and the nuclease was inactivated by the addition of EGTA. This HeLa cell extract digested with micrococcal nuclease was unable to adenylate any endogenous RNAs since the substrate RNAs were degraded by the nuclease (Fig. 1, lane 2). There is some residual tRNA resistant to micrococcal nuclease treatment. This may be due to secondary structure of tRNAs and/or association with protein(s). Addition of SRP RNA resulted in labeling of exogenous SRP RNA or Alu RNA. First, the Alu RNA extract was treated with micrococcal nuclease and the nuclease was inactivated by the addition of EGTA. This HeLa cell extract digested with micrococcal nuclease was unable to adenylate any endogenous RNAs since the substrate RNAs were degraded by the nuclease (Fig. 1, lane 2). There is some residual tRNA resistant to micrococcal nuclease treatment. This may be due to secondary structure of tRNAs and/or association with protein(s). Addition of SRP RNA resulted in labeling of exogenous SRP RNA or Alu RNA. First, the Alu RNA extract was treated with micrococcal nuclease and the nuclease was inactivated by the addition of EGTA. This HeLa cell extract digested with micrococcal nuclease was unable to adenylate any endogenous RNAs since the substrate RNAs were degraded by the nuclease (Fig. 1, lane 2).
Adenylation of SRP and Alu RNA in Vitro

for adenylation was 161 nucleotides long with UCUCUUU\textsubscript{OH} on its 3’ end. We expected this RNA to yield UCUCUUU\textsubscript{OH} after adenylation reaction and subsequent digestion with T1 RNase. Instead, it was surprising that Alu RNA yielded UCUCU AU\textsubscript{OH} after adenylation and digestion with T1 RNase (Fig. 1B). These data showed that from the Alu161 RNA two uridylic acid residues, corresponding to positions 160 and 161, were removed prior to adenylation on the uridylic acid 159 of Alu RNA. Similarly, SRP RNA added to the adenylation reaction contained three uridylic acid residues on its 3’ end, and two of these uridylic acid residues were removed before the addition of adenyl acid on the 3’ end of SRP RNA.

To investigate this 3’ end processing further, Alu RNAs with longer 3’ end sequences were prepared and incubated with HeLa cell nuclear extract in the presence of [\textalpha-\textsuperscript{32}P]ATP. Since Alu RNA migrates differently from the endogenous SRP RNA, the HeLa cell extract used in this experiment was not treated with micrococcal nuclease. Three RNAs were used as substrates in this assay including Alu RNA with UCUCUUU\textsubscript{OH} on its 3’ end and two other RNAs with 7 (designated Alu+7 RNA) or 11 additional nucleotides (designated Alu+11 RNA) on the 3’ end of Alu RNA. As expected, these three RNAs migrated differently on a 10% polyacrylamide gel (Fig. 2A, lanes 1–3). However, when these three RNAs were incubated with the HeLa cell extract, only labeled RNAs with similar electrophoretic mobility were obtained (Fig. 2B). T1 RNase digestion of these RNAs yielded only one labeled fragment corresponding to UCUCUAOH (Fig. 2C). After 3 h of incubation with HeLa cell nuclear extract, significant quantities of Alu+7 and Alu+11 RNAs were still detectable by staining with methylene blue at the end of adenylation reaction (data not shown). There was no consistent difference in the efficiency of adenylation between Alu+7 and Alu+11 RNAs. Since longer adenylation products were not detected (see Fig. 2, B and C), it appears that only Alu RNA molecules that are completely processed on the 3’ end are used as substrates for adenylation.

The kinetics of the 3’ end processing was studied using Alu+11 RNA which was internally labeled (Fig. 2D). The processing in vitro was rapid, with 50% of the RNA processed within 5 min and was nearly complete in 1 h. These data indicate that Alu RNA and Alu RNA with longer 3’ ends are first processed and then adenylation in the in vitro system. In addition, it appears that 3’ end processing is rapid in the in vitro system.

**Determination of Minimal Cis-elements in Alu RNA Necessary for Processing and Adenylation**—Since 161-nucleotide-long Alu portion of SRP RNA was processed and adenylated, this RNA was used to carry out deletion experiments to determine the domains essential for processing and adenylation. Fig. 3 (A and B) shows the schematic representation of SRP RNA, Alu161 RNA, and some of the mutant Alu RNAs tested in this adenylation system. As expected, treatment of the HeLa cell nuclear extract with micrococcal nuclease degraded endogenous SRP and other RNA substrates and these RNAs were not labeled in the presence of [\textalpha-\textsuperscript{32}P]ATP (Fig. 3C, lane 2). Addition of Alu161 RNA resulted in adenylation RNA (Fig. 3C, lane 3); however, in the case of Alu RNA with a 5’-deletion (lane 4) or a 3’-deletion (lane 5), there was no detectable adenylation product. An 87-nucleotide-long Alu RNA (Alu168–141), where nucleotides 68–141 were removed, was adenylated (Fig. 3C, lane 6). Further deletion, resulting in a 73-nucleotide-long RNA (Alu160–147) where integrity of stem IV was disrupted, resulted in complete absence of adenylation (Fig. 3C, lane 7). These data show that a large central portion of Alu RNA is dispensable and both the 5’ and 3’ end sequences are necessary for adenylation. In addition, integrity of stem IV is also essen-

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**Fig. 1.** A, adenylation of SRP RNA and Alu RNA. HeLa cell nuclear extract treated with micrococcal nuclease was prepared as described under “Materials and Methods.” The SRP and Alu RNAs were prepared by transcription with T7 RNA polymerase of appropriate DNA templates. The adenylation in vitro was carried out in the presence of [\textalpha-\textsuperscript{32}P]ATP as described under “Materials and Methods.” The labeled RNAs were purified, separated on a 10% polyacrylamide gel, dried, and subjected to autoradiography. B, analysis of 3’ end sequences of SRP and Alu RNAs. Labeled SRP RNA from lane 1 of panel A and Alu RNA from lane 4 of panel A were purified, digested with T1 RNase, fractionated on a 20% polyacrylamide gel, dried, and subjected to autoradiography. Lane M, size markers. C, analysis of 3’ end nucleotide of SRP and Alu RNAs. Labeled SRP RNA from lane 1 of panel A and Alu RNA from lane 4 of panel A were purified, digested with T2 RNase, and subjected to two-dimensional chromatography. The first dimension was isobutyric acid/water/ammonium hydroxide (66/33/1, v/v/v), and the second dimension was 0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/n-propanol (100/60/2, v/v/v).

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**Cell extracts and adenylation of SRP RNA that occurs in vivo** (10). These data also show that RNAs synthesized in vitro by T7 RNA polymerase are accurately adenylated in this in vitro adenylation system.

**Alu RNAs with Longer 3’ Ends Are Accurately Processed in Vitro**—The Alu RNA synthesized in vitro and used as substrate...
above each lane) were incubated with HeLa nuclear extract with 9 RNAs with extra nucleotides on the 3' end were fractionated on a 10% polyacrylamide gel and visualized by staining with methylene blue. B, adenylation of Alu RNA and Alu RNAs with extra nucleotides on the 3' end. Different RNAs (shown above each lane) were incubated with HeLa nuclear extract with [α-32P]ATP for 3 h. Purified RNAs were separated on a 10% polyacrylamide gel, dried, and subjected to autoradiography. C, analysis of 3' end sequence of adenylated Alu RNAs. Labeled SRP RNA from panel B (lane 1) and Alu RNAs from panel B (lanes 2, 3, and 4) were purified, digested with T1 RNase, separated on a 20% polyacrylamide gel, dried, and subjected to autoradiography. D, processing of Alu+11 RNA in vitro. Uniformly labeled Alu+11 RNA was incubated with HeLa cell nuclear extract for different time periods, as indicated on the top of each lane. RNAs were purified, fractionated on a 10% polyacrylamide gel, dried, and subjected to autoradiography. Lanes 1 and 2 are uniformly labeled Alu and Alu+11 RNAs used as size markers.

Stem IV of Alu RNA Is Required for 3' End Processing—As shown earlier, Alu161 RNA was first processed by removal of two uridylic acid residues and then adenylated (see Fig. 2). We tested whether 87-nucleotide-long RNA that gets adenylated and 73-nucleotide-long RNA that does not get adenylated can be processed or not. The AluΔ68–141 and Alu60–147 RNAs with 10 and 9 additional nucleotides, respectively (designated AluΔ68–141 +10 RNA and Alu60–147 +9 RNA), were injected into frog oocytes, and the 3' end processing was studied (Fig. 4). The AluΔ68–141 +10 RNA was processed in a time-dependent manner to yield an RNA that migrates similar to 87-nucleotide-long RNA (Fig. 4, lanes 1–4). The Alu60–147 +9 RNA was not processed since there was no detectable 73-nucleotide-long RNA even 18 h after injection (Fig. 4, lanes 1–4). Similar results were obtained when the experiment was performed using HeLa cell nuclear extract (data not shown). Since the only difference between these two constructs is the presence of stem IV in AluΔ68–141 RNA and absence of intact stem IV in Alu60–147 RNA, it is concluded that stem IV of Alu RNA is required both for 3' end processing and adenylation.

Adenylated RNAs Are Associated with SRP Proteins—It is interesting to note that the minimal domain of Alu RNA required for 3' end processing and adenylation is very similar, if not identical, to that required for SRP 9/14 protein heterodimer binding (30). Deletion of stem IV, which is important for binding of SRP 9/14 protein heterodimer (33), completely abolished processing and adenylation. Therefore, we tested whether adenylated SRP and Alu RNAs are associated with SRP 9/14 protein heterodimer or not. After adenylation of SRP and Alu RNAs in vitro in the presence of [α-32P]ATP, immunoprecipitations were carried out with anti-SRP 9 antibodies. Both endogenous SRP RNA and exogenously added Alu RNA were adenylated and were found in the immunoprecipitates obtained with anti-SRP9 protein antibodies. Approximately 20% of the adenylated SRP and Alu RNAs were immunoprecipitated when 20 μl of the anti-SRP9 antibodies was used (Fig. 5, lanes 1 and 2), and this percentage increased to about 50% when the amount of antibody was increased to 50 μl (Fig. 5, lanes 3 and 4). Under conditions of antibody excess, virtually all the adenylated SRP and Alu RNAs were found in the immunoprecipitates obtained with anti-SRP9 protein antibodies (data not shown). Uniformly labeled Alu60–147 RNA, which cannot bind SRP9/14 heterodimer, was also used as a control, and no detectable radioactivity was found in immunoprecipitates obtained with anti-SRP9 antibodies (data not shown). These data show that adenylated SRP and Alu RNAs are associated with SRP 9/14 heterodimer.

Importance of 3' End Sequences for Adenylation—Since the adenylate for adenylation. It is notable that 87-nucleotide-long Alu RNA with intact stem IV binds SRP 9/14-kDa protein heterodimer, whereas 73-nucleotide-long RNA with deletion of stem IV does not bind SRP 9/14-kDa protein heterodimer (30). These data show that structural features necessary for the binding of SRP 9/14-kDa protein heterodimer are also important for adenylation of Alu RNA.
3' end sequence where the adenylation is occurring was found to be important, several mutant RNAs were made and tested in the in vitro adenylation system. A schematic representation of mutant Alu RNAs is shown in Fig. 6A. We already showed that deletion of 9 nucleotides near the 3' end (AluΔ151–159) completely abolished adenylation (Fig. 3C, lane 5). RNAs terminating with A or C instead of U at position 160 (Fig. 6B, lanes 4, 5, and 6, respectively), were recognized as substrates for adenylation, and in every case RNAs adenylated at position 160 were observed. Disruption of base pairing in stem III of Alu RNA,
were also digested with T2 RNase, and, in every case, only labeled Up was observed (data not shown). These data show that all longer RNAs were first processed and the adenylation occurred accurately at uridylic acid 159 of mutant Alu RNAs.

**Adenylation Is an Early Event**—In mammalian cells, SRP RNA is synthesized in the nucleoplasm by RNA polymerase III and is first seen in the nucleolus before migrating to the cytoplasm (32). We tested whether SRP RNA found in the nucleolus is adenylated or not. Total RNA was isolated from HeLa cells and also from nucleoli purified from HeLa cells. An oligonucleotide was ligated to these RNAs, and after a quantitative RT-PCR, which selectively amplifies the 3′ end portion of SRP RNA, the resulting DNA was analyzed before and after digestion with BglII restriction enzyme. The oligonucleotide ligated to the SRP RNA was designed in such a way that only SRP RNA containing adenyl acid on the 3′ end will be digested with BglII. Data presented in Fig. 7 show that approximately 35% of the nucleolar SRP RNA contained adenyl acid on the 3′ end (Fig. 7, lane 2) and 50% of the SRP RNA in HeLa cells contained adenyl acid on the 3′ end (Fig. 7, lane 4). These data show that the 3′ end processing and adenylation of SRP RNA occurs in the nucleus, before SRP RNA enters the cytoplasm.

**DISCUSSION**

The main observation made during this investigation is that an 87-nucleotide-long region, corresponding to the 5′ and 3′ regions out of the 300-nucleotide-long mammalian SRP RNA, is necessary and sufficient for accurate 3′ end processing and adenylation. Studies from other laboratories have shown that this domain of SRP RNA has a tRNA-like structure and binds to two (9/14-kDa) SRP proteins (30). This domain was also recently shown to be necessary and sufficient for transport of SRP RNA from nucleolus to the nucleolus and then to the cytoplasm (32). These data show that the domain of SRP RNA, in addition to arresting translation of proteins containing the signal peptide, which are destined for translocation across the endoplasmic reticulum, has multiple functions. These functions include binding of 9/14-kDa SRP proteins, 3′ end processing where extra sequences on the 3′ end are removed, adenylation where a single adenylic acid residue is added, and migration of SRP RNA from nucleolus, where SRP RNA is synthesized, to nucleolus and then to the cytoplasm.

SRP RNA is synthesized by RNA polymerase III and terminates with four uridylic acid residues on its 3′ end. However, SRP RNA characterized from several mammalian sources including human, rat, Drosophila, and frog show that several of these uridylic acid residues on the 3′ end are not present in most of the mature cytoplasmic SRP RNA molecules. In addition, a single post-transcriptionally added adenyl acid residue is found in over 50% of the human and rat SRP RNA population (10). Data presented in this paper describe an accurate in vitro system that removes the terminal uridylic acid residues from exogenously added SRP/Alu RNAs and adds one adenyl acid residue. It is interesting to note that Alu RNAs with longer 3′ ends containing 7 or 11 extra nucleotides were accurately processed and then adenylated. We also used Alu RNAs with ~200 extra nucleotides on the 3′ end, and this RNA was also accurately processed and adenylated, though less efficiently (data not shown). These data indicate that human cells have the ability to accurately process and adenylate longer read-through SRP RNA transcripts that failed to terminate at the first pol III termination site. It is relevant to note that, in the case of human 5 S RNA genes, read-through transcription in vivo through the first pol III termination site is well documented (40). While there is no evidence that longer read-through SRP RNA transcripts are actually made in vivo, it is known that Alu
RNAs are made as longer transcripts and processed to yield mature Alu RNAs. Only the Alu RNA sequences capable of binding SRP 9/14-kDa protein heterodimer were found to be processed accurately and accumulated in the cytoplasm (33). These data are consistent and support the results obtained during this investigation.

Fig. 6. A, diagrammatic representation of Alu variants with 3’ end nucleotide(s) changes. Residue(s) marked with red are changed nucleotide(s). The “+” symbol represents the Alu variants that can be adenylated, and “-” represents those RNAs that cannot be adenylated. B, adenylation of Alu RNA and Alu variants. Alu RNAs with 3’ end variations were constructed by PCR and transcribed by T7 RNA polymerase. RNAs were incubated with [α-32P]ATP in HeLa cell nuclear extract for 3 h, extracted, and fractionated on a 10% polyacrylamide gel. C, analysis of 3’ end sequence of adenylated Alu RNA and Alu variants. Adenylated Alu RNA and Alu RNAs with 3’ end variation were eluted from polyacrylamide gel and subjected to T1 RNase digestion. The digestion products were fractionated on a 20% polyacrylamide gel, dried and subjected to autoradiography.
Although 300-nucleotide-long SRP RNA, 161-nucleotide-long Alu RNA, and also the 87-nucleotide-long deletion mutant (Alu$^{D68–141}$) were all processed and adenylated, the efficiencies of adenylation were dramatically different. The SRP RNA was the least efficient and Alu$^{D68–141}$ was the most efficient. The adenylation efficiency of Alu RNA was consistently better than SRP RNA (Fig. 1, lanes 3 and 4; Fig. 3C, lanes 3 and 6). The reason for this might be that smaller RNAs may renature and acquire native conformation much more readily compared with longer RNAs.

Fig. 8 shows a proposed pathway for the biogenesis of SRP RNA. The SRP RNA is made in the nucleoplasm with four uridylic acid residues on the 3′ end. This RNA binds to SRP9/14-kDa protein heterodimer, followed by removal of three uridylic acid residues and adenylation. Since SRP RNA in the nucleolus is already adenylated, it is clear that this processing and adenylation is occurring in the nucleus. Whether it happens in the nucleolus or in the nucleolus is not known. Purification of nucleoplasmic SRP RNA without nucleolar and/or cytoplasmic SRP RNA contamination is necessary to answer this question. It is very likely that the processing and adenylation pathway shown in Fig. 8 is common to SRP RNA and to Alu RNAs.

The fact that SRP RNA in the nucleolus is already adenylated shows that 3′ end processing and adenylation is an early and nuclear event in the biogenesis of SRP particle. However, Alu RNAs injected into frog oocyte cytoplasm were accurately processed indicating that machinery for 3′ end processing of SRP RNA can occur both in the nucleus and the cytoplasm. It is known that the 3′ ends of some RNAs, like human U6 snRNA, are constantly trimmed and rebuilt (41). The adenyl acid residue added on the 3′ end of SRP RNA also appears to be turning over constantly. For example, Alu$^{160A}$ RNA, which has an adenyl acid on the 3′ end, was labeled in the in vitro adenylation system by removal of unlabeled adenyl acid residue and replacement with a labeled adenyl acid (Fig. 5B, lane 4). Therefore, it is possible that SRP RNA adenylated in the nucleus is also adenylated in the cytoplasm during turnover. This process is reminiscent of CCA turnover on the 3′ end of tRNAs where terminal nucleotides are constantly turning over. Although the necessity of CCA turnover in tRNAs can be rationalized on the basis of the need for complete CCA sequence for aminocacylation, the need for A turnover in SRP is not known.

We investigated whether SRP/Alu RNA itself is the substrate or whether binding of 9/14-kDa protein is required for Alu/SRP to be recognized as a substrate for 3′ end processing and adenylation. It is known that in the case of U1, U2, and U4 snRNAs, precursor RNAs first associate with snRNP proteins to form snRNPs and then get processed (1, 3, 5). It is clear from the immunoprecipitation data shown in Fig. 4 that adenylated SRP and Alu RNAs are bound to the 9/14-kDa proteins. The Alu RNA substrate added to the adenylation reaction is in large
excess compared with the 9/14-kDa protein available in the HeLa cell extract (1 μg of substrate RNA for 60 μg of total protein in the nuclear extract). Therefore, if RNA alone is a suitable substrate for processing and adenylation, only a small fraction of the adenylated RNA would be immunoprecipitable. The fact that virtually all the adenylated Alu RNA was precipitable under conditions of excess antibody strongly suggests that 9/14-kDa protein binding is required for processing and adenylation. This conclusion is consistent with the observation that Alu RNA mutant AluΔ60–147, which cannot bind 9/14-kDa protein heterodimer, was neither processed nor adenylation (Fig. 3D). Only Alu RNAs capable of binding SRP 9/14-kDa protein heterodimer accumulate in the cytoplasm of HeLa cells (33). Together, these data show that SRP RNA first binds to the 9/14-kDa heterodimer, three uridylic acid residues are removed, and then a single adenylic acid residue is added in the nucleus.

Adenylation in the 3′ end is not unique to SRP RNA. Adenylation occurs on the 3′ end of many RNAs including human U2 snRNA, 7SK RNA, and ribosomal 5 S RNA (10). The 3′ end processing of U1, U2, and U4 snRNAs has been extensively characterized both in vitro and in vivo using the frog oocyte system and transfection into cells (1–9). In the case of U2 snRNA, processing and adenylation appear to be a late event where precursor RNA is first transported to the cytoplasm for 3′ end processing (5). Whether the adenylation of U2 snRNA occurs in the cytoplasm or the nucleus after import is not known. In our in vitro adenylation system, the U2 snRNA and 7SK RNA were not substrates for adenylation (data not shown). It is possible that some essential binding proteins were missing in the HeLa nuclear extract used for the adenylation reactions. It is not known whether all these small RNAs are adenylated by a common adenylation machinery or different enzymes are responsible for the adenylation of other RNAs. Purification and characterization of the SRP RNA adenylylating enzyme will help answer this question.

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