Alu RNP and Alu RNA regulate translation initiation in vitro

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ABSTRACT

Alu elements are the most abundant repetitive elements in the human genome; they emerged from the signal recognition particle RNA gene and are composed of two related but distinct monomers (left and right arms). Alu RNAs transcribed from these elements are present at low levels at normal cell growth but various stress conditions increase their abundance. Alu RNAs are known to bind the cognate proteins SRP9/14. We purified synthetic Alu RNP, composed of Alu RNA in complex with SRP9/14, and investigated the effects of Alu RNPs and naked Alu RNA on protein translation. We found that the dimeric Alu RNP and the monomeric left and right Alu RNPs have a general dose-dependent inhibitory effect on protein translation. In the absence of SRP9/14, Alu RNA has a stimulatory effect on all reporter mRNAs. The unstable structure of sRight RNA suggests that the differential activities of Alu RNP and Alu RNA may be explained by conformational changes in the RNA. We demonstrate that Alu RNPs and Alu RNAs do not stably associate with ribosomes during translation and, based on the analysis of polysome profiles and synchronized translation, we show that Alu RNP and Alu RNA regulate translation at the level of initiation.

INTRODUCTION

With more than one million copies, Alu elements are the most abundant repetitive elements in the human genome; they represent ~10% of the genome mass and belong to the SINE (short interspersed elements) family of repetitive elements. Alu elements emerged ~55 million years ago from a fusion of the 5' and 3' ends of a 7SL RNA gene, which encodes the RNA moiety of the signal recognition particle (SRP). The first Fossil Alu Monomers (FAMs) arose from this fusion (1); they were ~160 bp long and are poorly represented in the human genome (1). According to the current model, modern Alu elements emerged from a head to tail fusion of two distinct FAMs (2) that gave rise to the dimeric Alu structure composed of two similar but distinct monomers (left and right arms) joined by an A-rich linker. Modern Alu elements are ~300 bp in length and are classified into subfamilies according to their relative ages [for a review see (3)]. Dimeric Alu elements are unique to primates but similar elements, called B1, exist in rodent genomes. B1 elements are monomorphic FAM-like monomers (4), which are present in ~150 000 copies in the mouse genome. Modern Alu elements amplified throughout the primate genomes to reach the present number of 10^6 copies. They are mobile but non-autonomous, and amplified via RNA intermediates by a mechanism of retrotransposition that remains rather unclear. As they do not encode any protein, their amplification has been most likely dependent on the transposition machinery of other retrotransposing elements; it has been shown recently that they could use LINE-1 elements for this purpose (5).

Alu RNAs, transcribed from Alu elements, are present in the cytosol of primate cells. Alu elements inherited the internal A and B boxes of the RNA polymerase III (Pol III) promoter from the 7SL RNA gene. These internal promoter elements significantly diverge from the consensus (6) and the efficient transcription of Alu elements is then dependent on sequences flanking their site of insertion (7). At normal cell growth, Alu RNAs accumulate at very low levels (10^3–10^4 molecules per cell), but their abundance increases up to 20-fold under various stress conditions, such as adenovirus infection or heat shock (8). The typical Alu RNA is a dimer of related but non-equivalent arms that are joined by an A-rich linker and followed by a short poly(A) tail (Figure 1). Each arm is related to the Alu portion of SRP RNA in terms of sequence and secondary structure and can bind the cognate SRP protein SRP9/14 in vitro (9) and in vivo (10). However, the left arm shows a higher affinity for these proteins than the right one (9).

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SRP is a ribonucleoprotein complex that fulfills an adaptor function between translation and translocation of proteins into the endoplasmic reticulum (ER). It interacts with translating ribosomes and samples the nascent polypeptide chains for the presence of a signal sequence, a hallmark of ER-targeted proteins. SRP then tightly binds to the ribosome–nascent chain complex and transiently blocks nascent chain elongation until the complex reaches the ER membrane where its interaction with the SRP receptor (SR) releases the ribosome to the translocon; protein synthesis is then resumed at normal speed across the ER membrane [for a review see (11)]. SRP is a particle composed of a 300 nt long RNA (7SL RNA) and 6 protein subunits. The signal sequence recognition and targeting activities of SRP where assigned to SRP54 bound to a conserved RNA helix of the S portion of SRP RNA. The arrest or delay in nascent chain elongation is mediated by the complete SRP but it specifically requires the presence of the Alu domain (12,13), which contains the Alu portion of 7SL RNA and the proteins SRP9/14. Consistent with its function, the Alu domain is positioned at the interface between the two ribosomal subunits in the elongation factor-binding site (14,15) in ribosome–nascent chain complexes arrested in elongation by SRP.

The striking structural similarity between the Alu RNA bound to SRP9/14 and the Alu domain of SRP (Figure 1) indicated a role for Alu RNAs in the regulation of protein synthesis. This hypothesis was further supported by previous data showing that an overexpressed Alu RNA stimulates the translation of co-transfected reporter genes in mammalian cells (16,17).

Figure 1. Secondary structure homology between Alu RNA and the SRP RNA Alu domain. (A) Secondary structure of the human SRP RNA. The SRP RNA is divided in two functional domains called S and Alu. The S domain of SRP binds nascent chains carrying a signal sequence while they emerge from the ribosome; the Alu domain mediates a transient delay in elongation. Boldface indicates the binding sites of SRP9/14 according to Refs (61) and (46). Three base pairs are formed between two loops and are indicated by dots. (B) Secondary structure of the synthetic Alu RNA used in this study. It was drawn based on a previously determined secondary structure (62) and adapted to the sequence of the Alu element of intron 4 of the α-Fetoprotein gene (Alu Y) (33). Boldface and dots indicate the binding sites of SRP9/14 and the tertiary base pairing between the two loops, respectively, by analogy to SRP RNA. Open arrow indicates the 3' end of sAlu RNA (116 nt) and closed arrows the 5' and 3' ends of sRight RNA (155 nt). sAlu and sRight RNAs represent monomeric left and monomeric right arms, respectively.
Another non-coding small RNA, which is related to the Alu portion of SRP RNA, is the neuron-specific BC200 RNA. It is expressed from a single gene (18), is monomeric and has an Alu-like fold (19). Its putative functional analogue in mice is BC1 RNA, which is derived from a tRNA gene (20). Both RNAs are specifically expressed in nerve cells and are localized to the somatic/dendritic domains (21,22). Recently, both BC200 and BC1 RNAs have been shown to inhibit protein translation in vitro (23,24).

To investigate a role of transcribed Alu elements in regulation of protein synthesis, we produced Alu RNPs composed of Alu RNA bound to SRP9/14 and tested the effects of Alu RNP and naked Alu RNA on protein translation in vitro. Our results support a role of transcribed Alu elements in translation regulation. Alu RNP inhibits whereas Alu RNA stimulates protein translation, and both act at the level of initiation.

**MATERIALS AND METHODS**

**pSPsRight construction**

The plasmid pSPsRight containing the Alu Right arm sequence under T7 promoter has been constructed by PCR amplification of Alu Right arm from the plasmid pAluRNA (9) with oligos 5'-GGATCCCTAATACGACTCACTATAGGCCG-GGCGTGATGG-3' and 5'-CCCAAGCCTTGGGAATATGTTTTTGGACCGGAG-3'. The PCR fragment was inserted in the vector pSP64 (Promega) at the EcoRI/HindIII site.

**In vitro transcription and mRNA isolation**

Cyclin, preprolactin and PAI-2 mRNAs were synthesized with SP6 RNA polymerase (25) from plasmids pCyclin (26), pSPsRight (12) and pDB5202 (27) linearized with EcoRI, HindIII, respectively. After transcription, mRNAs were purified on G50 columns, ethanol precipitated and resuspended in water. Luciferase mRNA was provided by Promega (L456A). Alu RNA, scAlu RNA, sRight RNA, BC200 RNA and – jh14mRNA were synthesized with T7 polymerase (28) from plasmids pPAuRNA (9), pPscAlu/teta.ilo (9), pSPsRight , pPBCC200 (9) and pGhSRP14/Sp6 (29) linearized with SspI, SpeI, SspI, DraI and EcoRI, respectively. After transcription, RNAs were run on a preparative 8 M urea, 10% acrylamide gel, visualized by UV shadowing, eluted in 0.5% SDS, 0.3 M sodium acetate, ethanol precipitated and resuspended in water. RNA concentrations were determined by OD260. The full sequences of these RNAs are shown in Supplementary Figure S4. Cytoplasmic RNA from HeLa cells was prepared as described previously (30).

**Alu RNPs purification**

SRP9/14 heterodimer was purified as described previously (31). In vitro heterodimer Alu RNAs were reannealed 10 min at 65°C and slowly cooled down prior to be mixed with an excess of recombinant SRP9/14, incubated 10 min on ice and 10 min at 37°C and then loaded on a Superdex 200 column in a buffer containing 20 mM HEPES, 500 mM potassium acetate, 5 mM magnesium acetate, 0.1% Nikkol and 10 mM DTT. The fractions containing Alu RNA complexed with proteins were pooled and dialysed in a buffer of the same composition supplemented with 10% glycerol. These fractions were then concentrated 2 h in PEG 20000 and 2 h by centrifugation in a Centrificon 10 (Amicon). The purified RNPs were stored at –80°C. The concentration of RNP was determined by in-gel quantification of the RNA of the fractions after digestion with proteinase K.

Native gel electrophoresis was performed on 8% acrylamide—10 mM magnesium acetate gel containing 50 mM Tris–acetic acid (pH 7.5); gels were stained with GelStar (Cambrex). Denaturing gel electrophoresis was performed on 10% acrylamide—8 M urea gel in TBE and stained in ethidium bromide. Agarose gel shifts have been performed on 2% agarose gels containing 50 mM Tris–acetate and 5 mM magnesium acetate, ran for 4 h at 60 V at 4°C; gels were stained with GelStar. Western blots have been performed using anti-SRP14 antibodies, affinity purified as described previously (29).

**In vitro translation**

In vitro translation reactions containing [L-35S]methionine (Amersham) were performed using wheat germ extract (Promega) at 80 mM potassium acetate and 2.5 mM magnesium acetate salt conditions. Synthetic mRNAs were used at 5 nM final concentrations. Cytoplasmic RNA was used at a final concentration of 5 ng/µl. After 25 min incubation at 26°C, a 10 µl aliquot of each reaction was TCA-precipitated and subjected to SDS–PAGE. The amount of translated protein was determined by phosphomager analysis (GS-363; Bio-Rad). Histograms represent the translation efficiency relative to the buffer control (100%). Each value represents an average of at least two independent experiments.

**Polysomes profile**

Polysomes profiles were determined mainly as described previously (32) with the following modifications: translation reactions containing radiolabelled methionine were incubated for 20 min at 26°C before the addition of cycloheximide at 0.5 mM final concentration. They were then loaded on to a 11 ml of 10–30% continuous sucrose gradient [50 mM HEPES (pH 7.5), 100 mM potassium acetate and 5 mM magnesium acetate] and centrifuged for 2 h at 39 000 r.p.m. in a TST41.14 rotor (Kontron instruments). After centrifugation, 500 µl fractions were collected from the gradient using an Auto Densi-Flow II (Buchler instruments). An aliquot of each fraction was spotted on a filter and dried before precipitation in 10% TCA and decalcification in boiling 5% TCA. Samples were then counted using a Betamatic V (Kontron instruments). Position of 80S was determined by immunoblotting with anti-L9 and anti-S15 antibodies (data not shown).

**Northern blotting**

RNA containing fractions were digested with proteinase K (Roche Diagnostics), 30 min at 55°C before ethanol precipitation in the presence of 5 µg glycogen. RNA samples were separated in 2% agarose–formaldehyde gel and transferred on to a nylon membrane in 1.5 M NaCl and 150 mM sodium citrate. The following radiolabelled oligonucleotides were used as probes: left arm probe, 5'-TCAC- CATGTGAGCCAGAGTGT-3'; right arm probe, 5'-GCAATCTCGGCTCAGGAAG-3'; and 28S rRNA.
probe, 5′-GGGCTAGTTGATTCGGCAG-3′. Hybridizations were carried out in 250 mM Tris–HCl (pH 7.5), 750 mM NaCl, 5 mM EDTA, 20% formamide, 0.2% SDS and 100 µg/ml sheared salmon sperm DNA. Membranes were washed in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 0.2% SDS, three times before exposure on the film.

Edeine-synchronized translation
Wheat germ translation reactions containing cyclin mRNA and radiolabelled methionine were allowed to initiate for 2 min at 26°C before the addition of edeine (kind gift from Dr D. Belin, CMU, Geneva) at 5 µM final concentration. After incubation for two more minutes at 26°C, Alu RNAs and Alu RNPs were added individually to the reaction at final concentrations of 300 and 100 nM, respectively. An aliquot of the reaction was then removed every minute, TCA-precipitated and subjected to 15% SDS–PAGE. The amount of translated protein was quantified by phoshoImager analysis (the average of time points 12 and 14 was taken as 100% of cyclin synthesis).

RESULTS
Purification of synthetic Alu RNPs
In order to purify synthetic Alu RNPs, composed of Alu RNA in complex with human SRP9/14, we synthesized in vitro an Alu RNA representing the Alu element contained in intron 4 of the α-Fetoprotein gene (9,33). This Alu element belongs to the Alu Y subfamily. The synthetic RNA migrated as a single band with the expected size in denaturing gel electrophoresis (Figure 2A). Under native conditions, most of the RNA also migrated in a discrete band indicating that the RNA is composed of a population of homogeneously folded molecules. In addition, a faint slower-migrating band appeared (Figure 2B), which most probably represents a RNA dimer. We know from our previous work with small Alu RNAs (34) that they have a propensity to form dimers because base pairing of the 3′ sequences may occur intermolecularly instead of intramolecularly. The same RNA fractionated on a molecular sizing column eluted in a major single peak in fractions 10–15 (Figure 2C, blue curve) confirming that the RNA did not form aggregates. We presumed that the minor peak in fractions 4 and 5 represented the dimeric form of the RNA that we already observed in native gel electrophoresis. After complex formation with recombinant SRP9/14 proteins, Alu RNA was found in faster migrating peaks, consistent with its binding to SRP9/14 (Figure 2C, red curve). Since both arms of the Alu RNA can bind SRP9/14, the major and minor peaks were expected to contain Alu RNA bound to two or one proteins, respectively. Fractions 7–10 were pooled and chosen as the Alu RNP fraction. To ensure that the purified RNP contained both RNA and proteins, we analysed aliquots by denaturing gel electrophoresis and by immunoblotting with anti-SRP14 antibodies, respectively (Figure 2D and E). Native agarose gel electrophoresis further corroborated that all detectable RNA was bound to SRP9/14 (Figure 2F).

Effects of Alu RNPs and Alu RNAs on protein translation
The effects of Alu RNP and Alu RNA on protein translation were assessed using a wheat germ translation system programmed with four different mRNAs encoding the proteins cyclin, preprolactin, plasminogen activator inhibitor (PAI-2) and luciferase. Although two of the synthetic mRNAs code for secretory proteins, we did not expect Alu RNPs to have a specific effect on the synthesis of these proteins. The signal sequence, a common hallmark of ER-targeted proteins, is recognized by SRP54, a component of the S domain of SRP (35) and not by components of the Alu domain of SRP. The translation reactions were programmed individually

Figure 2. Alu RNP purification on Superdex 200. (A) Denaturing acrylamide gel. Synthetic Alu RNA migrates as a single band with the expected size of 305 nt. (B) Native acrylamide gel. Alu RNA migrates in a defined band indicating that it is homogeneously folded. Trace amounts of an RNA dimer are also observed (star). (C) OD_{254} elution profile of a Superdex 200 column. Free Alu RNA, blue; Alu RNA bound to recombinant SRP9/14, red. Fractions 7–10 (grey box) containing Alu RNA in complex with two SRP9/14 proteins were pooled for subsequent experiments. The second RNP peak most likely represents Alu RNA bound to one protein and free RNA. (D) Aliquots of 1 and 2 µl (I and II, respectively) of the purified RNP fraction were subjected to denaturing acrylamide gel electrophoresis after proteinase K digestion. (E) Aliquots of 1 and 2 µl (I and II, respectively) of the purified RNP fraction were subjected to immunoblotting with anti-SRP14 antibodies. (F) Native agarose gel electrophoresis of the purified RNP fraction and free RNA.
with equal molar amounts of the mRNAs and were supplemented with increasing concentrations of either Alu RNP or Alu RNA. Protein synthesis was monitored by following the accumulation of the \(^{35}\)S-labelled protein displayed by SDS–PAGE (a series of representative experiments is shown in Supplementary Figure S1). The effects on protein synthesis were quantified and the results of all experiments are summarized in Figure 3. Alu RNP had a general dose-dependent inhibitory effect on the translation of all four reporter mRNAs in a concentration range varying from 50 to 300 nM (Figure 3A). Its inhibitory effect went up to 50% in the case of PAI-2 mRNA supplemented with 300 nM Alu RNP. Purified free SRP9/14 had no significant effect on protein translation (Figure 3I), demonstrating that the inhibitory effect observed with Alu RNP is not accounted for by SRP9/14 alone.

Alu RNA had a general and dose-dependent stimulatory effect on the translation of all four mRNAs in the same concentration range as Alu RNP (Figure 3D). For example, Alu RNA, added at 300 nM in a PAI-2 translation reaction, increased the translation efficiency >2-fold. To confirm that the stimulatory effects were specific for Alu RNA, we did the same experiments with two control RNAs; \((\kappa)\)h14 mRNA and BC200 RNA. \((\kappa)\)h14 mRNA represents the antisense strand of the human SRP14 mRNA. It was added to translation reactions in a concentration range varying from 20 to 120 nM. As it is much longer than Alu RNA (\(~700\) nt), 120 nM of \((\kappa)\)h14 mRNA represents the same mass as 300 nM Alu RNA. Neither \((\kappa)\)h14 mRNA nor BC200 RNA had a significant effect on protein translation (Figure 3G and H). BC200 RNA even appeared to inhibit translation at the highest RNA concentrations, consistent with previous studies (23). Notably, we

**Figure 3.** Quantification of the effects of purified Alu RNPs and Alu RNAs on protein synthesis. Wheat germ translation reactions programmed with cyclin, preprolactin (pPL), luciferase and PAI-2 mRNAs were supplemented with increasing amounts of (A) Alu RNP, (B) scAlu RNP, (C) sRight RNP, (D) Alu RNA, (E) scAlu RNA, (F) sRight RNA, (G) \((\kappa)\)h14 mRNA, (H) BC200 RNA and (I) SRP9/14. The translation products were analysed by SDS–PAGE (Supplementary Figure S1), quantified and normalized to the buffer control, which was set to 100%. The results represent the average of at least two independent experiments.
observed a higher error rate in the effects of Alu RNA on translation as compared with the effects observed with Alu RNP (compare Figure 3A and D). This difference might be explained by a reduced stability of Alu RNA in the absence of SRP9/14 (36) and see below). Despite the significant standard error, there is a clear drift to increased translation rates upon the addition of Alu RNA (compare Figure 3D and Figure 3G and H).

To examine whether the observed effects could be generalized, we did two more series of experiments. First, the effects of Alu RNP and Alu RNA were repeated with two of the four reporter mRNAs using the rabbit reticulocyte translation system. These results confirmed the inhibitory and the stimulatory effects of Alu RNP and Alu RNA, respectively, on the translation of cyclin and luciferase mRNAs (Supplementary Figure S2A and B). Like in wheat germ extract, SRP9/14 had no effect on translation (Supplementary Figure S2D) and the control RNA, (−)h14 mRNA, had a slightly stimulatory effect at low concentrations but not at higher concentrations (Supplementary Figure S2C). These results demonstrate that stimulatory and inhibitory activities of Alu RNA and Alu RNP are not limited to wheat germ extract but also exist in rabbit reticulocyte lysate. Second, we used cytoplasmic RNA isolated from HeLa cells to program wheat germ translations. Translation of the cytoplasmic RNA resulted in a large variety of translation products ranging in sizes from 14 to 120 kDa (Figure 4, lane 1). Upon the addition of Alu RNP, we observed a moderate but significant overall inhibition of translation (Figure 4A, lane 3, and B). In contrast, we failed to observe a general stimulatory effect of Alu RNA on translation (Figure 4A, lane 2, and B). The lack of an overall response to Alu RNA may indicate that the effect is specific for certain mRNAs. However, the negative result may also be explained by differences in the translation efficiencies of the synthetic and authentic mRNAs used in the experiments (Discussion).

To further characterize the inhibitory and stimulatory effects of Alu RNP and Alu RNA on the reporter mRNAs, we investigated whether their respective activities reside specifically in one of the two arms of the Alu RNA. Therefore, we produced Alu RNAs representing either the left or the right arm of the dimeric Alu RNA, called scAlu and sRight RNAs, respectively (Figure 1B). These isolated arms, which result from processing of dimeric Alu RNA (37), are known to exist in the cytoplasm of cells and both of them have been shown to bind SRP9/14 in vitro (9).

We produced scAlu and sRight RNAs in vitro and purified their respective RNP s the same way as we did for Alu RNP (Supplementary Figure S3). Notably, both RNAs had a higher propensity to form RNA dimers and the sRight RNA appeared to be less stably folded than Alu and scAlu RNAs as indicated by its diffuse migration in native gels (Supplementary Figure S3B). scAlu and sRight RNP s and RNAs were then assayed in translation reactions as before. Both RNPs had an inhibitory effect on protein translation (Figure 3B and C) and the effects appeared to be equal or more prominent than the effect observed for the dimeric Alu RNP. Of the two RNAs, only sRight had a noticeable but not significant stimulatory effect on translation (Figure 3F). The previously reported stimulatory effect of sRight RNA in vivo (17) could therefore not convincingly be reproduced in vitro by this study. However, the absence of a significant activity of the synthetic sRight RNA could also be explained by its reduced capacity to fold properly as indicated by its diffuse migration in a native gel and by the absence of a significant amount of dimeric RNA in the sample used in the experiments (Supplementary Figure S3B). scAlu RNA had no significant stimulatory effect on the translation of all reporter mRNAs (Figure 3E) and luciferase translation was even slightly inhibited at higher RNA concentrations. The latter result was consistent with our previous observations with BC200 RNA (Figure 3H), which also represents a left arm monomer (19).

Alu RNP and Alu RNA influence translation initiation

To examine more closely the mechanism by which Alu RNP and Alu RNA influence protein translation, we compared polysome profiles of translation reactions containing or not Alu RNP and Alu RNA. Even under optimized translation conditions and with saturating mRNA concentrations only ~10% of the ribosomes are active in wheat germ translations (15). The non-functional ribosomes interfere with the analysis...
of polysome profiles by OD260. In order to consider only functional ribosomes, we determined polysome profiles by monitoring [35S]methionine incorporation into the nascent chains (32). Translation reactions were prepared as before and split into two aliquots one of which served as the control reaction whereas the other was complemented with the analyte, Alu RNP or Alu RNA. The [35S]methionine bound to tRNA was removed from the collected fractions to ensure that only [35S]methionine incorporated into nascent chains was taken into account (Materials and Methods).

As seen in the control reactions, the majority of the nascent chains were found in fractions close to the 80S peak demonstrating that protein synthesis in wheat germ extract takes mainly place in small polysomes (Figure 5). This finding is consistent with the previous observation that typically only two to three polypeptides are synthesized from each mRNA in cell-free translation systems (38). Addition of Alu RNP to translation reactions programmed with cyclin and PAI-2 mRNAs lead to a marked decrease in the levels of monosomes and polysomes (Figure 5A and B). The observed decrease was strongest for monosomes, consistent with the idea that fewer mRNAs were able to recruit ribosomes. Alternatively, the same profiles could be explained by assuming an increase in the elongation rate in the presence of an unchanged initiation rate. However, this interpretation would not be in agreement with the reduced protein synthesis as shown in Figure 3A.

Upon addition of Alu RNA, there was a general and noticeable increase in monosome and polysome levels consistent with an enhanced translation initiation (Figure 5C and D). The observed changes were also in agreement with the results shown in Figure 3. In PAI-2 translation, which was greatly stimulated by Alu RNA (Figure 3D), protein synthesis was shifted to bigger complexes indicating that, in average, mRNAs were loaded with more ribosomes (Figure 5D). Cyclin translation was only moderately stimulated by Alu RNA (Figure 3D) and, consequently, the changes in the profiles were less pronounced (Figure 5C). In summary, these results support the interpretation that both Alu RNP and Alu RNA affect protein synthesis at the level of translation initiation, but in opposite ways.

Alu RNP and Alu RNA are not stably associated with ribosomes during translation

Based on the results that the Alu domain of SRP is positioned at the interface of the two ribosomal subunits in ribosome-nascent chain complexes arrested in elongation by SRP (14,15), it was feasible that Alu RNPs and, possibly, also Alu RNAs may exert their functions through interactions with the ribosome. To localize Alu RNP and Alu RNA, we performed Northern blot analysis with the same gradient fractions that were used to establish the polysome profile. Alu RNA was present in the five top fractions of the gradient obtained with the translation reaction programmed with PAI-2 mRNA and supplemented with 300 nM Alu RNA (Figure 6A–D). In such gradients, the pre-initiation complexes migrate immediately before 80S (32) and the accumulation of Alu RNA in the top fraction is therefore not consistent with its binding to pre-initiation complexes. A similar result was obtained for Alu RNP (Figure 6E and H). Alu RNP was present in fractions 2–4 while 28S rRNA appeared only in fraction 10.

![Figure 5](https://academic.oup.com/nar/article-abstract/34/8/2374/1072110)

Figure 5. Alu RNP and Alu RNA act at the level of translation initiation. (A) Polysomes profile of wheat germ translation reactions programmed with cyclin mRNA in presence (black) or absence (grey) of 100 nM Alu RNP. (B) Idem (A) with PAI-2 mRNA. (C) Polysomes profile of wheat germ translation programmed with cyclin mRNA in presence (black) or absence (grey) of 300 nM Alu RNA. (D) Idem (C) with PAI-2 mRNA. Profiles were monitored by the incorporation of [35S]methionine into the nascent chains (cpm).
These results showed that there is no stable interaction between ribosomes and Alu RNP and Alu RNA during translation and that their effects on translation initiation might therefore be regulated through interactions with cytosolic factors.

Notably, Alu RNAs and Alu RNPs are partially processed in scAlu and sRight monomers upon incubation in wheat germ lysate (Figure 6B, C, F and G). A similar processing event has already been observed when Alu RNA was mixed with HeLa cell nuclear extracts (37). The sRight RNA appeared to be degraded in the absence of the proteins (Figure 6C) consistent with our finding that it is less stably folded (Supplementary Figure S3B). This observation further supports our interpretation that the lower activity of the sRight RNA as compared with the complete Alu RNA and the higher error rates observed in the reactions supplemented with Alu RNAs in general might be explained by the extent to which the RNA is degraded during translation. Not surprisingly, SRP9/14 stabilized the sRight RNA (Figure 6G), most probably by inducing a conformational change of the RNA that prevents its degradation.

Translation elongation remains unchanged in the presence of Alu RNP and RNA

Based on our previous results, it was unlikely that Alu RNA and Alu RNP would have an effect on the elongation rate of translation. However, because of the striking similarity between the Alu RNP and the Alu domain of SRP, we wanted to address this question experimentally. To this end, we synchronized translation reactions using edeine. It specifically blocks translation initiation in eukaryotes but not elongation (39) (Figure 7E). Translation reactions programmed with cyclin mRNA were allowed to initiate for 2 min before the addition of edeine. After supplementing with Alu RNA or Alu RNP, translation was resumed and aliquots removed at different time points (Figure 7A–C). Quantifications of the results revealed that there were no significant differences in the elongation rates of the different translation reactions (Figure 7D). These results also substantiated our interpretation of the polysome profiles.

DISCUSSION

Repetitive elements account for nearly half of the human genome (40); they were considered as ‘junk’ DNA for a long time but, nowadays, several lines of evidence strongly suggest that they play an important role in the regulation of gene expression at various levels. Alu elements, for example, have been shown to be an important source of alternative splicing when present in intronic regions of genes [for a review see (41)]. In this study, we investigated another potential function of Alu elements, namely one in regulation of protein synthesis. Using the wheat germ in vitro translation system, we showed that Alu RNAs specifically influence translation initiation in two distinct manners: Alu RNA bound to SRP9/14 generally inhibits protein synthesis whereas free Alu RNA enhances protein translation of reporter mRNAs.

The inhibitory effect of Alu RNPs on translation is very robust and observed with all mRNAs that we tested including the cytoplasmic RNA of HeLa cells. Both RNPs, the sRight and the scAlu RNPs, share the inhibitory activity indicating that it resides in the common part of the two RNPs, which includes the 5′ portion of the Alu RNA bound to SRP9/14. Since SRP9/14 alone had no effect on protein translation, only the composite structure formed by the RNA and the proteins...
can mediate the observed effects. Monomeric Alu RNAs are present in the cytosol of primate cells and at least scAlu has been shown to be associated with SRP9/14 in vivo (10,29). scAlu and sRight RNAs are most likely generated simultaneously by processing of a full-size Alu RNA (37). Because of its reduced stability (36) and its weaker affinity for SRP9/14 (9), the presence of sRight RNA and RNP in the cytosol is controversial and has been less studied. The inhibitory effect of Alu RNP is not dependent on the poly(A) stretch of its RNA moiety because it is absent in sRight and scAlu RNPs (Figure 1 and Supplementary Figure S4). Hence, although Alu RNA might bind poly(A)-binding protein (PABP), as it is present in wheat germ extract (42), it is implausible that PABP-binding alone accounts directly or indirectly for the inhibitory activity of Alu RNPs.

Further investigations about the mechanism by which Alu RNP influences protein translation lead us to the conclusion that it acts at the level of initiation. The analysis of the polysome profiles revealed changes in polysome levels whereas the elongation rate remained unchanged. Moreover, the absence of a stable interaction between ribosomes and Alu RNPs further excludes a role in elongation. Hence, one important conclusion that can be drawn from these studies is that despite the structural similarities between Alu RNPs and the Alu domain of SRP, the mechanisms of their respective inhibitory effects on translation is not the same. The capacity of the SRP Alu domain to slow down nascent chain elongation may then depend on the SRP S domain of which SRP54 has been shown to make close contacts to ribosomal proteins (43). Unpublished experiments from our group confirm this interpretation: (i) the Alu151 RNP, representing the Alu domain of SRP, was not able to bind ribosomes; and (ii) the inhibitory effect of Alu151 RNP on translation was not dependent on the C-terminal sequences of SRP14 that are required to confer elongation arrest activity to SRP (L. Terzi, J. Hasler and K. Strub, unpublished data). As opposed to direct interactions with the ribosome, the specific activity of Alu RNP is then most likely mediated by direct or indirect effects on soluble factors such as translation initiation factors, but the exact underlying mechanisms remain to be elucidated.

A strong stimulatory effect on translation of reporter RNAs was observed in mammalian cells upon episomal expression of Alu RNAs (17). In both in vitro translation systems, we reproducibly observed a stimulatory effect of the full-length Alu RNA on translation of reporter mRNAs. The extent to which stimulation occurred was dependent on the reporter mRNA used and the obtained values were associated with a considerable error rate. In addition and unlike in vivo, where a stimulatory role of sRight RNA on translation has been recognized (17), the effect of sRight RNA on translation in vitro was very small and not significant with respect to the error rate. Based on our results, we believe that the observed error rate and the weak effect of sRight RNA might be due to the intrinsic instability of the right arm of Alu RNA in the absence of SRP9/14 and to the reduced capacity of the synthetic sRight RNA to fold properly. As a consequence, Alu and sRight RNAs might be degraded to variable degrees during translation and the specific activity of the synthetic sRight RNA might be reduced. As mentioned above, an increased instability of sRight and Alu RNAs as
compared with scAlu RNA has also been observed previously in vivo (36).

Unlike with Alu RNP, we failed to see an overall effect of Alu RNA on the translation efficiency of cytoplasmic RNA indicating that the effect might be specific to certain mRNAs. This conclusion was also drawn from in vivo studies where a kinetic effect on translation was observed upon co-expression of the reporter mRNA and Alu RNA in HeLa cells (17). However, alternative models that could explain the negative result of our in vitro studies cannot be excluded. The cytoplasmic mRNA comprises authentic mRNAs with 5'- and 3'-untranslated regions (5'- and 3'-UTRs) and poly(A) tails whereas our synthetic mRNAs were devoid of either one or both of the UTRs and were not polyadenylated. The stimulatory effect of Alu RNA might in vitro only be detected at presumably reduced translation efficiencies of the synthetic mRNAs. In addition, cytoplasmic mRNA also contains small non-coding RNAs which may neutralize the stimulatory effects of Alu RNAs. Similarly, the specific effect on translation of the reporter mRNA observed in vivo is not fully understood; it might be due to differences in the regulation of episomally and chromosomally produced mRNAs as suggested previously (44). Hence, these possibilities need to be further explored but at this point our results together with the in vivo studies rather suggest that the effect of Alu RNA might be limited to certain mRNAs.

As mentioned previously, studies with BC200 RNA and its putative murine functional analogue BC1 RNA demonstrated that these RNAs inhibit protein translation in vitro and in vivo. BC1 RNA interferes with the formation of 48S pre-initiation complex possibly via a direct interaction with elf4A (24). In other studies, it was shown that the inhibitory effects of BC200 and BC1 RNAs were dependent on the poly(A) stretches of the RNA and could be alleviated by the addition of PABP (23), suggesting that the suppression is mediated through binding of PABP by BC1 and BC200 RNAs. In the same studies, the authors found that Alu RNA also inhibited translation, albeit to a much lower extent than BC200 RNA. The apparent contradiction with our results is most likely explained by the different experimental conditions we used. The stimulatory effect is observed at RNA concentrations that are ~5- to 20-fold lower than the ones used in Ref. (23). In addition, the stimulatory effects are better observed at relatively short incubation times (<30 min) whereas long incubation times (90 min) were used in Ref. (23). Since the sRight RNA is labile, the incubation times might be very critical. In addition, the stimulatory effect of Alu RNA could not be explained by the depletion of SRP9/14 from wheat germ extract, since, unlike for PABP, SRP9/14 has never been shown to have a role in translation. Moreover, there is no evidence for free SRP9/14 in wheat germ extract that can bind the mammalian SRP RNA, since partially reconstituted particles lacking SRP9/14 are not complemented for elongation arrest activity (13).

The fact that Alu RNAs and Alu RNPs displayed opposite activities was at first quite puzzling. However, it is known from the structure of the SRP Alu domain that the binding of SRP9/14 induces strong conformational changes in the RNA. In the absence of protein, the RNA is in a loosely folded state (45) whereas in its presence it assumes a very compact structure. The three-way junction of stems in the 5' domain forms two helical stacks that are held in a defined orientation by the bound protein and through base pairing between the two loops. In addition, the central stem is flipped by 180° to bind SRP9/14 (46). Base pairing between the two loops is essential for proper folding of SRP RNA (31). In the left and the right arms of Alu RNA, some structurally important elements such as length of the stems, sizes of the loops and base-pairing interactions between the loops are not conserved; it is therefore conceivable that the entire Alu RNA or its right arm and the sRight RNA may adopt a different conformation in the absence of the protein. Such conformational rearrangements of the RNA could explain the differential activities of Alu RNA and Alu RNP.

In this study, we tested the effect of Alu RNP composed of Alu RNA in complex with only SRP9/14. In primate cells, Alu RNAs might be present in larger complexes, as suggested by their high sedimentation coefficient in sucrose gradients (29,47). BC200 RNA is known to exist in vivo in a complex as large as SRP (48) but only SRP9/14 (49), PABP (50) and FMRP (51) have been shown to be part of the complex. Although the association of FMRP protein with BC1 and BC200 RNAs in vivo is still controversial (52,53), binding of BC1 RNA has been mapped recently in vitro to a specific domain in the FMRP protein (54).

As compared with SRP RNA, which has a long life span (55), Alu RNAs are rather unstable, explaining their relative low accumulation in normal cells (56). Functions of Alu RNAs and Alu RNPs might therefore be spatially or temporarily controlled and therefore be limited to certain physiological condition, such as stress (8), cancerous transformation (57) and to the tissue-specific control of gene expression. The low level of expression might be the result of a selective pressure to prevent their accumulation and thereby their function in normal cells. Alu elements have been shown for a long time to behave like cell stress genes. Various stress conditions cause a transient expression of Alu RNAs, which rapidly decreases upon recovery from stress. Upon stress, regular cap-dependent translation of most proteins is greatly reduced (58) whereas the expression of a small group of proteins such as heat shock proteins is greatly enhanced (59). The exact mechanisms that account for the selective translation of certain mRNAs are still incompletely understood but they may include internal ribosome entry sites and ribosome shunting (60). The increased expression of Alu RNA under stress is consistent with a stimulatory role in the translation of certain mRNAs during stress as proposed previously (17). BC200 RNA is also of relatively low abundance and its presumed effect on protein translation in vivo is most plausibly explained by its accumulation at certain sites in neuronal cells (22). Likewise, the inhibitory effect of Alu RNPs may be spatially restricted to certain sites in normal cells or in cells with increased levels of Alu RNA.

In this study, we investigated the role of Alu elements transcribed by RNA polymerase III but the role of Alu elements in regulation of protein synthesis could be more widespread than anticipated previously, since they are also present in the 5'- and 3'-UTRs of mRNAs, which are synthesized by RNA polymerase II. As 5'- and 3'-UTRs are hot spots of translational regulation, Alu sequences within these regions could modulate translation initiation in a similar manner as dimeric and monomeric Alu RNAs and RNPs presented here.
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Supplementary Data are available at NAR Online.

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