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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 a 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 monomeric 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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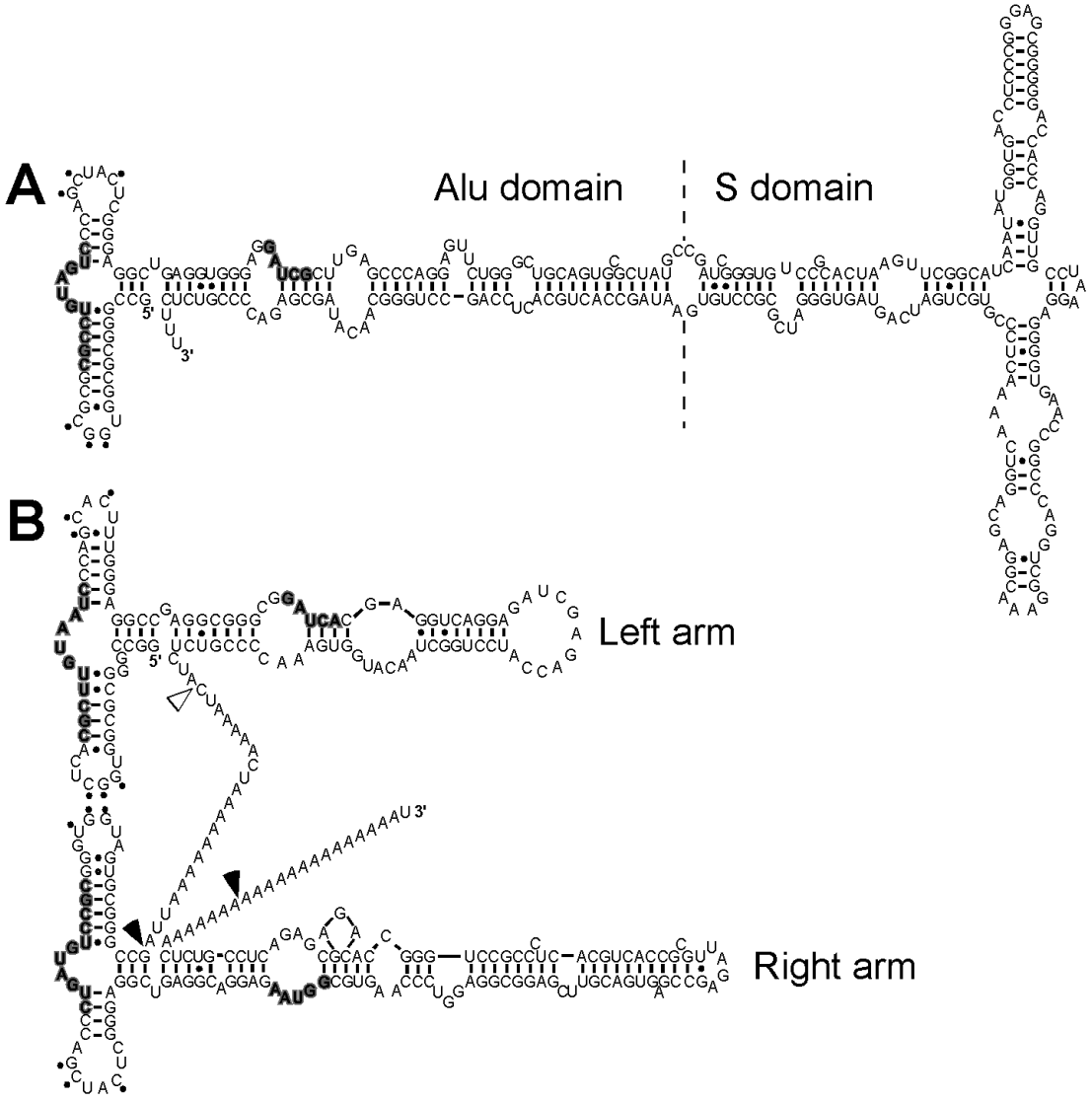

Figure 1. Secondary structure homology between *Alu* RNA and the SRP RNA *Alu* domain. (**A**) Secondary structure of the human SRP RNA. The SRP RNA is divided in two functional domains called S and *Alu*. The S domain of SRP binds nascent chains carrying a signal sequence while they emerge from the 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 sc*Alu* RNA (116 nt) and closed arrows the 5′ and 3′ ends of sRight RNA (155 nt). sc*Alu* and sRight RNAs represent monomeric left and monomeric right arms, respectively.

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 of 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).

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/dentritic domains (21,22). Recently, both BC200 and BC1 RNAs have been shown to inhibit protein synthesis *in vitro* and *in vivo* (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 pP*Alu*RNA (9) with oligos 5′-GGAATTCCTAATACGACTCACTATAGGCCG-GGCGTGATGG-3′ and 5′-CCCAAGCTTGGGAATATTT-TTTTGAGACGGAG-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), pSP-BP4 (12) and pDB5202 (27) linearized with EcoRI, EcoRI and 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, sc*Alu* RNA, sRight RNA, BC200 RNA and (−)h14mRNA were synthesized with T7 RNA polymerase (28) from plasmids pP*Alu*RNA (9), pPsc*Alu*/α feto (9), pSPsRight , pPBC200 (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 OD_{260}. 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* transcribed *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 Centricon 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 electophoresis 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-^{35}S]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 phosphoImager 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.

Polysome 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 deacylation 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-CATGTTAGCCAGGATGGT-3′; right arm probe, 5′-GCAATCTCGGCTCACTGCAAG-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 phosphoImager 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

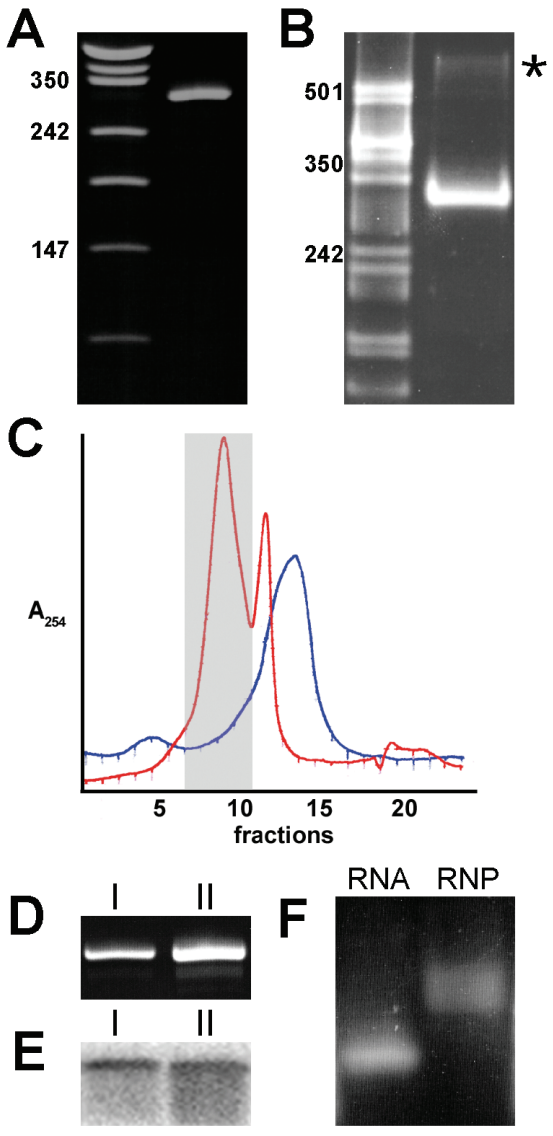

Figure 2. *Alu* RNP purification on *Superdex 200*. (**A**) Denaturing acrylamide gel. Synthetic *Alu* RNA migrates as a single band with the expected size of 305 nt. (**B**) Native acrylamide gel. *Alu* RNA migrates in a defined band indicating that it is homogeneously folded. Trace amounts of an RNA 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.

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

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; (−)h14 mRNA and BC200 RNA. (−)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 (−)h14 mRNA represents the same mass as 300 nM *Alu* RNA. Neither (−)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

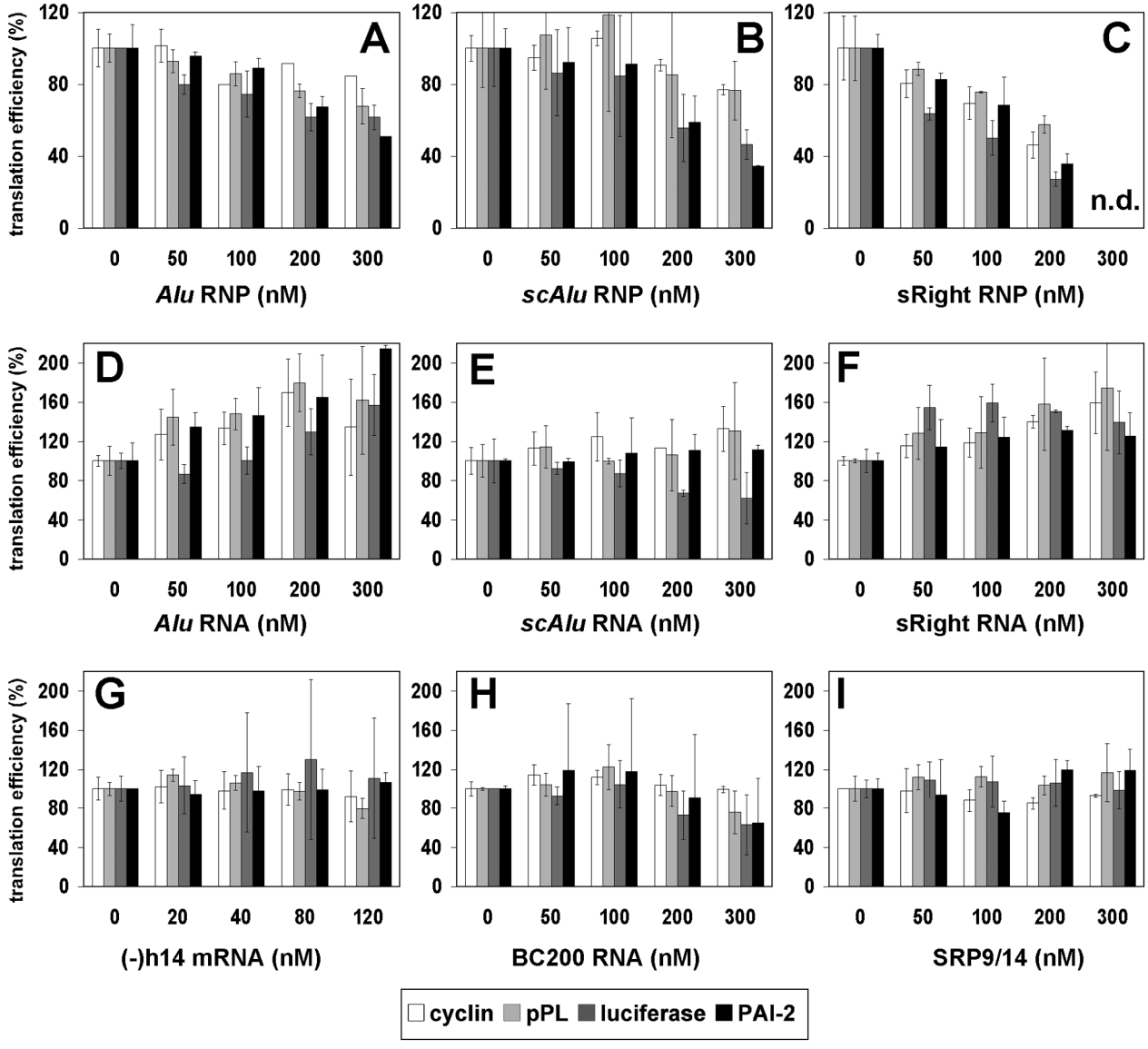

Figure 3. Quantification of the effects of purified *Alu* RNPs and *Alu* RNAs on protein synthesis. Wheat germ translation reactions programmed with cyclin, preprolactin (pPL), luciferase and PAI-2 mRNAs were supplemented with increasing amounts of (**A**) *Alu* RNP, (**B**) sc*Alu* RNP, (**C**) sRight RNP, (**D**) *Alu* RNA, (**E**) sc*Alu* RNA, (**F**) sRight RNA, (**G**) (−)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 sc*Alu* 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 sc*Alu* and sRight RNAs *in vitro* and purified their respective RNPs 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 sc*Alu* RNAs as indicated by its diffuse migration in native gels (Supplementary Figure S3B). sc*Alu* and sRight RNPs 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

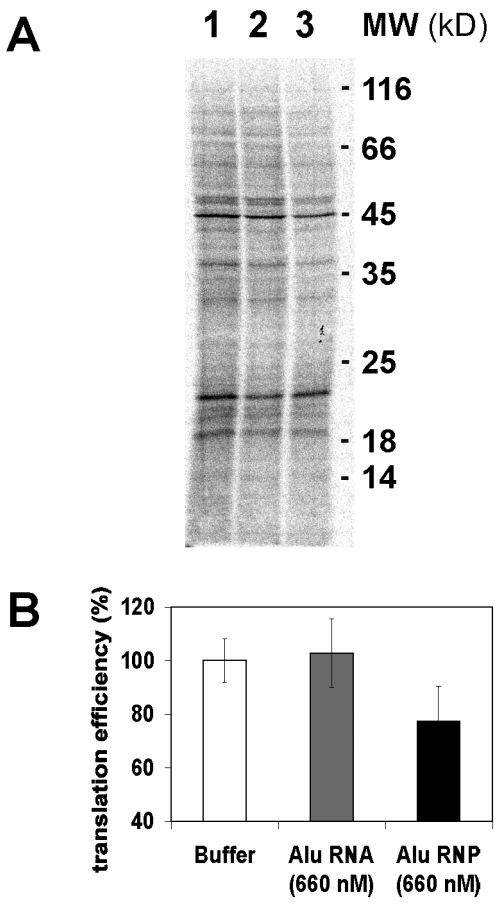

Figure 4. Effects of *Alu* RNP and *Alu* RNA on the translation of cytoplasmic RNA from HeLa cells. Wheat germ translation reactions were programmed with 5 ng/μl^{-1} of cytoplasmic RNA and the translation products were analysed by SDS–PAGE (**A**). Lane 1, buffer control; lane 2, 660 nM *Alu* RNA; and lane 3, 660 nM *Alu* RNP. (**B**) Quantification of the results shown in (A). Total protein synthesis was determined by measuring the intensities of identical elongated squares covering the translation products in the approximate size range of 10–100 kDa in all three lanes. The results represent the average of two independent experiments and were normalized to the buffer control, which was set to 100%.

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 presence of a significant amount of dimeric RNA in the sample used in the experiments (Supplementary Figure S3B). sc*Alu* 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 OD_{260}. In order to consider only functional ribosomes, we determined polysome profiles by monitoring [^{35}S]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 [^{35}S]methionine bound to tRNA was removed from the collected fractions to ensure that only [^{35}S]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.

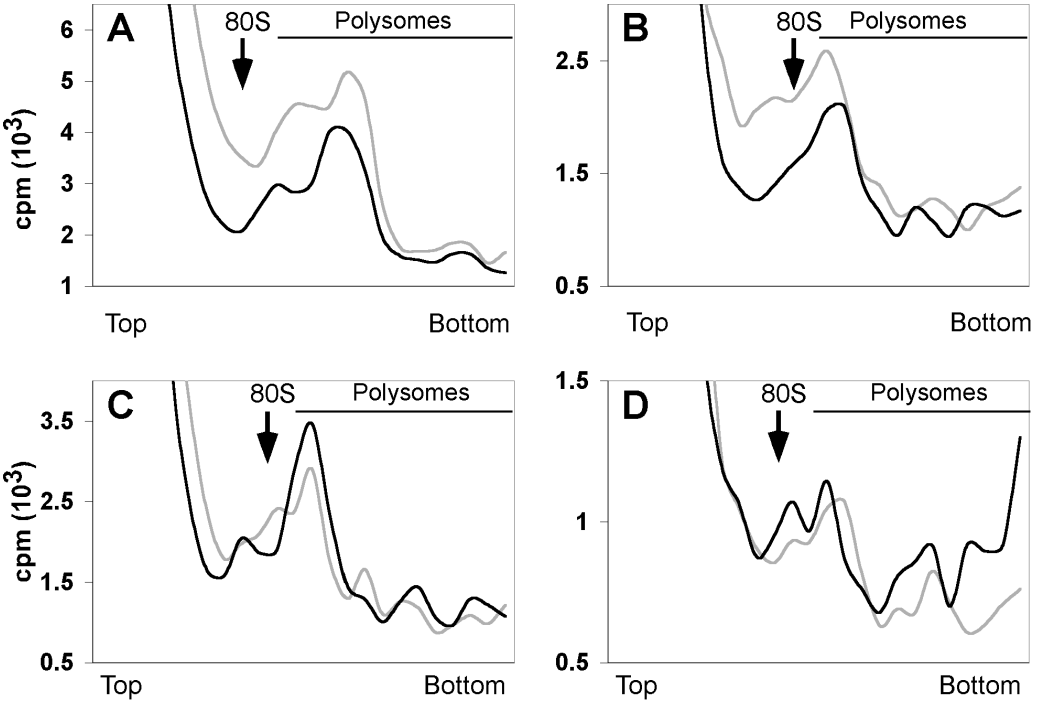

Figure 5. *Alu* RNP and *Alu* RNA act at the level of translation initiation. (**A**) Polysomes profile of wheat germ translation reactions programmed with cyclin mRNA in presence (black) or absence (grey) of 100 nM *Alu* RNP. (**B**) Idem (A) with PAI-2 mRNA. (**C**) Polysomes profile of wheat germ 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 [^{35}S]methionine into the nascent chains (cpm).

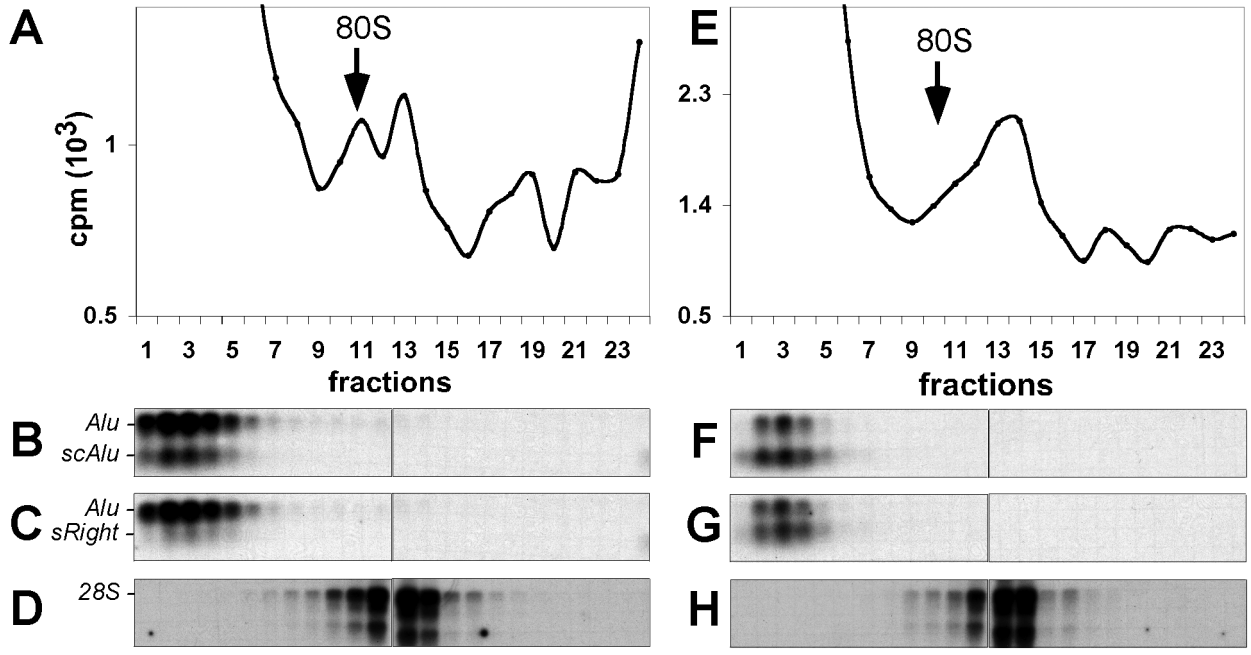

Figure 6. *Alu* RNP and *Alu* RNA migrate in different fractions than ribosomes. (**A**) Polysome profile of a wheat germ translation reaction programmed with PAI-2 mRNA and supplemented with 300 nM *Alu* RNA. Northern blot analysis of the gradient fractions with probes against sc*Alu* (**B**) and sRight RNAs (**C**) as well as against 28S rRNA (**D**). (**E**) Polysome profile of a wheat germ translation reaction programmed with PAI-2 mRNA and supplemented with 100 nM *Alu* RNP. Northern blot analysis of the gradient fractions with probes against sc*Alu* (**F**) and sRight RNAs (**G**) and against 28S rRNA (**H**).

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 sc*Alu* 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 sc*Alu* 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

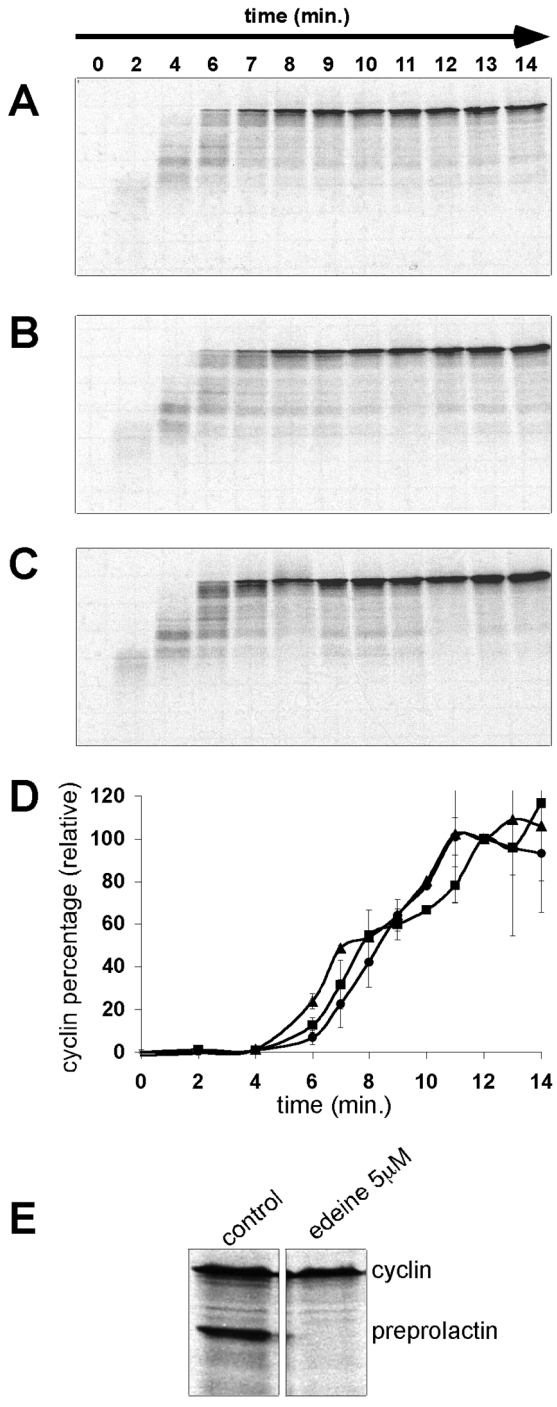

Figure 7. *Alu* RNP and *Alu* RNA do not influence translation elongation. Translation reactions containing [^{35}S]methionine and programmed with cyclin mRNA were allowed to initiate 2 min before the addition of edeine at a final concentration of 5 μM. After two more minutes at 26°C, *Alu* RNA and *Alu* RNP were added at final concentrations of 300 and 100 nM, respectively. Aliquots of the reaction were removed at the time points indicated and analysed by SDS–PAGE. Autoradiograms of reactions containing (**A**) buffer control, (**B**) *Alu* RNA and (**C**) *Alu* RNP. (**D**) The signals were quantified and normalized to the average of the time points 12 and 14, which were arbitrarily set to 100%. Circles, buffer control; triangles, *Alu* RNP; squares, *Alu* RNA. (**E**) Negative control reactions for translation initiation. Translation reactions programmed with cyclin mRNA were allowed to initiate 2 min before the addition of edeine at a concentration of 5 μM. After two more minutes of incubation, a second mRNA encoding preprolactin was added to the reaction, which was then incubated 20 min before being subjected to SDS–PAGE.

can mediate the observed effects. Monomeric *Alu* RNAs are present in the cytosol of primate cells and at least sc*Alu* has been shown to be associated with SRP9/14 *in vivo* (10,29). sc*Alu* 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 sc*Alu* 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 *Alu*151 RNP, representing the *Alu* domain of SRP, was not able to bind ribosomes; and (ii) the inhibitory effect of *Alu*151 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 mRNAs 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 sc*Alu* 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 eIF4A (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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