Selective stimulation of translational expression by Alu RNA

Carol M. Rubin¹, Richard H. Kimura^{1,2} and Carl W. Schmid^{1,2,*}

¹Section of Molecular and Cellular Biology and ²Department of Chemistry, University of California–Davis, Davis, CA 95616, USA

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ABSTRACT

Human Alu and adenovirus VA1 RNAs each stimulate the translational expression of reporter genes in co-transient transfection assays without affecting either the rate of global protein synthesis or the abundance of the reporter mRNA. This selective, post-transcriptional stimulation of expression, which is observed in human and mouse cell lines and for three reporters, acts through a PKR-independent mechanism. The activity of Alu and VA1 RNAs in this assay is transient, causing a reduction in the lag time for the translational expression of the newly synthesized reporter mRNAs. The reduction in this lag time accounts for the relative selectivity of the effect upon the expression of the reporter and suggests novel roles for Alu and VA1 RNA in cell stress recovery and viral infection. Deletion analysis demonstrates that a specific region residing within the right monomer of the dimeric Alu consensus sequence is necessary for activity. Highly abundant left Alu monomer transcripts are inactive but the right Alu monomer is fully active, although its transcripts are scarce. Mouse B1 and B2 SINE RNAs stimulate reporter gene expression in mouse cells, suggesting that this activity is a general property of eucaryotic SINEs.

INTRODUCTION

Most eucaryotic genomes contain highly repetitive short interspersed sequences (SINEs) that are derived from genes for either SRP RNA or tRNAs (1,2). From their homology to these genes, SINEs contain an internal promoter for RNA polymerase (Pol) III. Cell stress and viral infection cause rapid and large increases in the abundance of Pol III-directed SINE transcripts (2). This response is conserved in evolution and occurs in animals subjected to non-lethal stresses (2–5). These observations prompted the proposal that SINE RNAs function during cell stress recovery and more specifically might affect translation (2). In agreement with this proposal, overexpression of the human SINE (Alu) RNA increases the expression

of a luciferase reporter gene in co-transient transfection assays (6).

Adenovirus VA1 RNA, which regulates protein synthesis in infected host cells, provides a possible model for understanding this activity of Alu RNA. In co-transfection assays, VA1 RNA also increases the expression of reporter genes, in some examples by more than 50-fold (7–15). VA1 RNA inhibits the activation of PKR, an eIF2 kinase, essentially making VA1 RNA an anti-inhibitor of global protein synthesis, and VA1 RNA overexpression does rescue protein synthesis in cells that are infected with VA1-deficient virus (11,15–17). Alu RNA also binds to PKR, raising the possibility that it might similarly regulate eIF2 kinase activity (6).

This well-established mechanism of blocking the inhibition of protein synthesis by inhibiting the activation of PKR (reviewed in 17) does not readily explain the activity of VA1 RNA that is observed in co-transient transfection assays. VA1 RNA can act through this mechanism only if global protein synthesis is inhibited by PKR, requiring an implausibly high default level of translational inhibition (e.g. 50-fold) in the examples cited above. Paradoxically, all available evidence indicates that VA1 RNA overexpression does not increase global protein synthesis in co-transient assays (7–15). Together these observations lead to the remarkable conclusion that VA1 RNA must selectively stimulate the expression of the reporter gene, raising another perplexing question of how the reporter and endogenous mRNAs are distinguished (10,12). There are conflicting reports concerning the obvious possibility that VA1 RNA increases the abundance of reporter gene mRNA (7–15).

In addition to being an eIF2 kinase, PKR has other functions (17) and the activity of VA1 RNA in co-transient transfection assays might still involve PKR. However, the additional observation that VA1 RNA increases reporter expression in PKR knockout cells disproves this possibility (14).

Since VA1 RNA provides a model for how Alu RNA functions during cell stress recovery, we have compared the activities of these two RNAs upon reporter gene expression in co-transient assays. Our results show that these RNAs stimulate the expression of the reporter by acting upon a novel regulatory target, which accelerates the expression of newly synthesized mRNAs. This activity explains the selectivity of the effect upon reporter gene expression. Furthermore, this activity resides within a limited region of the right monomer unit of the dimeric Alu consensus sequence (2), demonstrating its specificity and providing possible insights

*To whom correspondence should be addressed. Tel: +1 530 752 3003; Fax: +1 530 752 3085; Email: cwschmid@ucdavis.edu

into Alu evolution. The A and B box internal promoter elements for Pol III reside in the left monomer (2), suggesting that Alu consists of two functional domains: one directs transcription and the other has the cellular activity reported here.

MATERIALS AND METHODS

Expression vectors

To compare modulator plasmid effects accurately all modulators were cloned into pUC19. Alu overexpression vectors that combine a 5' flanking sequence from the SRP RNA gene and a strong terminator have been previously described (6). Those constructs (XAT, XT and XAL), after recloning into pUC, are named pAlu +A, pAlu-A and pLAlu, identifying clones that express full-length Alu RNA with an A-rich 3' tail, without an A-rich 3' tail and the left monomer of Alu RNA, respectively. Sequences for these and other constructs described below are available (GenBank AF458106–AF458115). pLAlu uses the SRP flanking sequence, the strong terminator and incorporates six point mutations to create a functional B box for independent transcription of the right Alu monomer. Alu truncation and deletion constructs were made by PCR using pAlu-A as the parent template. The truncation series pAlut253, pAlut206 and pAlut175 introduces a Pol III transcription terminator (T_6) at Alu positions 253, 206 and 175. The constructs pDW (deletion, wild-type), pWD and pDD delete positions 206–229, 238–256 and their combination; pAU substitutes an AT dinucleotide for positions 230–238, which encode an RNA hairpin loop. B1 and B2 expression plasmids were cloned downstream of the SRP RNA gene flanking promoter elements. The VAI expression plasmid was created by excising the VAI gene from pAdvantage (Promega) with *NaeI* and cloning it into the *SmaI* site of pUC19. Luciferase (pGL3 Control) and β -galactosidase (β -gal) (pSV- β -galactosidase Control) expression plasmids were obtained from Promega. The E1a expression plasmid has been reported (18).

Plasmid DNAs were purified with a Qiagen MaxiKit, then treated with RNase, phenol extracted, ethanol precipitated and redissolved to a final concentration of 0.3–0.7 μ g/ μ l in water or 0.1 $\times$ TE buffer.

Cell culture and transfection

Cells were grown in high glucose DMEM supplemented with non-essential amino acids, 10% newborn calf serum and antibiotic/antimycotic cocktail (Invitrogen). The day before transfection cells were split into 24-well plates and the antibiotics were withdrawn. At 50–75% confluency, cells were transfected with Lipofectamine PLUS (Invitrogen) following the manufacturer's protocol with 100 ng reporter plasmid, 400 ng modulator plasmid (pUC or indicated RNA expression vector), 4 μ l PLUS and 2 μ l Lipofectamine per well. Serum in DMEM was added to 10% final concentration after 3–4 h transfection. Confluency, cell type, and duration of transfection all influence the timing of the maximum effect on expression and its magnitude. The maximum modulation of reporter expression was achieved with HeLa cells at 50–75% confluency harvested 8–10 h post-transfection.

Transfection efficiency was determined by substituting a GFP reporter in the above protocol and assaying expression by flow cytometry. The average (four samples) number of cells expressing more than the cut-off threshold response was $30.2 \pm 5.7\%$ (mean $\pm$ SD) for HeLa and $46.3 \pm 4.4\%$ for 293 cells. The number of cells expressing the reporter was uncorrelated with the identity of the modulator plasmids.

Cell lysis and reporter analysis

Each well was lysed with 100 μ l cell culture lysis reagent (Promega CCLR) and analyzed for reporter expression. Luciferase transfects were analyzed by luminometry using the Promega Luciferase Assay System and a Monolight 2000 luminometer on 5–20 μ l of lysate, 10 s assay duration. β -gal transfects were analyzed by the Promega β -Galactosidase Assay System, using 75–100 μ l of lysate.

RNA analysis

To determine the level of RNA modulators by northern analysis, total cytoplasmic RNA (5 μ g) was run on a 5% denaturing polyacrylamide gel and electroblotted onto Hybond N⁺ (Amersham) filters. Hybridization and washing conditions were standard (19). The oligonucleotide 5'-AAAAGGAGCGCTCCCCGTTG-3' was used to detect VAI RNA. Most Alu transcripts and SRP RNA were detected with the left monomer oligonucleotide 5'-TCCTGACCTCGTGATCCGCC-3'. The oligonucleotide 5'-ACGCCATTCTCCTGCCTCAG-3' was used in assays for transcripts of the right monomer.

In vitro translation with wheatgerm extract (Promega) was used to measure luciferase mRNA (20). In addition to its greater sensitivity, this functional assay obviates artifacts that might ensue in hybridization-based assays of nucleic acid samples isolated from transfected cells. Total cellular RNA was isolated using Trizol (Invitrogen) and the poly(A)⁺ fraction was selected with Oligotex (Qiagen). Typically, 200 ng poly(A)⁺ RNA was used for *in vitro* translation and the resulting products were analyzed for luciferase activity as described above but using a 120 s assay time. To test for equivalent RNA loading, an aliquot of the poly(A)⁺ RNA was assayed for actin mRNA by northern analysis.

Protein electrophoresis and immunoblotting

Protein content in cell lysates was assayed by the Bradford technique. Equal amounts were run on 10% SDS-PAGE (typically 15 μ g/lane) and transferred to nitrocellulose by standard methods (21). The Amersham ECL Western Blot system was used for analysis. The E1a primary antibody (Santa Cruz Biotechnology) was used at 1:200 dilution; the actin antibody (Sigma) was used at 1:100 dilution. For pulse labeling experiments, at 8 h post-transfection 10–100 μ Ci/ml [³⁵S]methionine (Amersham cell labeling grade) was added to the cellular medium for 0.5–4 h. Cells were lysed as above and cytoplasmic extracts were run on SDS-PAGE.

RESULTS

Modulation assayed by co-transient transfection

Plasmids that express either Alu or VAI RNAs ('modulator RNAs') are co-transfected into mammalian cells along with

plasmids that express a reporter gene (e.g. luciferase). The resulting level of reporter expression is compared to that obtained in a parallel transfection assay in which the pUC vector is substituted for the modulator plasmid. Using this definition of activity, luciferase expression in HeLa cells is stimulated 10-fold by a VA1 RNA expression plasmid (pVA) and 8-fold by an Alu expression plasmid (pAlu+A) (Fig. 1A). As negative controls, plasmids which express either left Alu monomer transcripts (pLAlu) or mouse B2 RNA have no effect or slightly inhibit luciferase expression (Fig. 1A). Constructs which express only the right Alu monomer (pRAlu) or delete the 3' A-rich tail (pAlu-A) of Alu RNA approximate the activity of pAlu+A (Fig. 1A). These constructs are active both in HeLa and human 293 cells, showing that these effects are not restricted to a particular cell line (Fig. 1A).

Selective stimulation of reporter gene expression

Labeling with [35 S]methionine assays whether the modulators affect the rate of global protein synthesis (Fig. 1B). Although VA1 RNA co-expression stimulated luciferase expression 10-fold or more in this experiment (Fig. 1A), it had no effect upon [35 S]methionine incorporation (Fig. 1B). Since 30–40% of the cells are transfected (Materials and Methods), this assay should be sufficiently sensitive to detect an increase in protein synthesis that corresponds to the effect of VA1 RNA upon the luciferase reporter. Similar results were obtained for the various Alu RNA expression plasmids (Fig. 1B). In agreement with previous studies on VA1 RNA (7–15), Alu RNAs selectively stimulate reporter gene expression without increasing the rate of global protein synthesis.

Another formal possibility deserves consideration: The transfection procedure might cause a stress-induced inhibition of protein synthesis and VA1 RNA might relieve this inhibition, thereby stimulating the expression of the co-transfected reporter (14). However, the level of [35 S]methionine incorporation in cells that were transfected (and serum starved) is only slightly less than or even equal to that of untransfected cells (Fig. 1B). Thus the effects of VA1 and Alu RNAs upon reporter gene expression greatly exceed the level that could result from relieving the putative transfection-induced inhibition of protein synthesis.

Transient effect of modulation: eliminating a lag time in expression

Variability in the activities of VA1 and Alu RNAs was observed from experiment to experiment. To determine the optimum modulatory effect, reporter expression was examined as a function of time following transfection (Fig. 2). At 8.5 h post-transfection, luciferase activity was 45-fold greater in HeLa cells co-expressing VA1 RNA than in the pUC control (Fig. 2). This stimulation dropped to 5-fold at 31.5 h post-transfection (Fig. 2). Most studies of VA1 RNA stimulation of reporter gene expression have been performed 1 or 2 days post-transfection (6–15).

Similarly, co-expression of Alu RNA transiently stimulates the reporter expression (Fig. 2). However, the effect of Alu RNA in human cells is always less than that of VA1 RNA (achieving a maximum of 9-fold stimulation at 8.5 h). Consequently, Alu modulatory activity (1.8-fold stimulation) nearly disappears 1 or 2 days post-transfection (Fig. 2).

Figure 1. Effects of modulator plasmids upon luciferase and total protein expression. (A) HeLa or 293 cells were co-transfected with luciferase expression vector and modulator plasmids as shown. Eight hours after transfection cells were harvested and luciferase activity measured. Relative luciferase activity in the presence of pUC is standardized as 1; activities in other modulator-transfected cells in this and all subsequent figures and in Table 2 are shown relative to the pUC baseline. (B) HeLa cells which had been transfected as discussed above were treated with 100 μ Ci/well (24-well plate) [35 S]methionine for 1 h (total time post-transfection 8 h). Cell lysates were run on SDS-PAGE and an autoradiograph made of 35 S incorporation. A replicate set of SDS-PAGE gels stained with Coomassie Blue demonstrated equal protein loading (not shown). In experiments not shown, protein synthesis in both HeLa and 293 cells was assayed starting at various times post-transfection and for 0.5–4 h labeling with 10–100 μ Ci/ml 35 S. Under no conditions were significant differences in total protein synthesis observed.

Reporter expression in 293 cells exhibits a similar temporal progression after transfection, though the maximum fold stimulation is only about one-third as great as in HeLa cells (data not shown). Since both Alu and VA1 RNAs selectively stimulate the expression of the reporter and act with similar kinetics, it is likely that they act upon a common pathway.

The actual rate of luciferase production can also be estimated as the differential rate from this data set (Table 1). At the later times (i.e. 21–31.5 h), the rate of luciferase synthesis in the pUC control cells [$\sim$ 21 000 luminosity units

Figure 2. Time dependence of modulation. HeLa cells were co-transfected with luciferase expression vector and pUC, Alu+A or VAI modulator plasmid. At various times post-transfection luciferase activity was determined; the stimulation provided by the modulator RNAs relative to the pUC controls in that experiment (Table 1) is reported above.

(l.u.)/h) approaches the rate of luciferase production that occurs during this same interval in Alu-treated cells (~28 000 l.u./h). Similarly, this rate approximates the rate (~27 000 l.u./h) that is observed in the VAI-treated cells at the optimum time of 8.5 h, at which time luciferase expression is stimulated 45-fold (Table 1 and Fig. 2). Thus at later times reporter expression in the pUC control begins to 'catch up' to the cells that co-express the modulator RNAs (Table 1 and Fig. 2). More importantly, this experiment shows that the modulator RNA does not increase the maximum rate of luciferase synthesis *per se* but instead accelerates the kinetics of expression so that a high rate of synthesis is attained at an earlier time. In other words, it decreases a lag time in the kinetics of luciferase synthesis. Since newly synthesized endogenous mRNAs are produced at relatively low levels compared to those which are already actively being translated, the stimulatory effect is most apparent for the newly synthesized reporter mRNA. According to this interpretation, the selectivity that is apparent in this effect is not attributable to a special feature of exogenous reporter mRNA but rather to its recent appearance.

Reporter mRNA abundance is not modulated

Conceivably, the modulator RNA selectively increases the abundance of the transfected reporter gene mRNA. Using

in vitro translation to assay the relatively scarce luciferase mRNA (20), we observe no such effect (Table 2). For example, at 7 h post-transfection, VAI RNA increases luciferase activity by 37-fold compared to the pUC control but its mRNA abundance is equal (0.93) to that of control cells. Similar results are obtained for cells co-expressing Alu RNA and at a later time (Table 2).

PKR-independent modulation

Cells derived from a PKR mouse knockout were used to test whether the activity of Alu RNA requires this kinase (Fig. 3). Previous studies using these same cell lines showed that luciferase stimulation by VAI RNA is PKR independent (14). Alu RNA (pAlu+A) stimulates the expression of the reporter gene in both PKR^{+/+} and PKR^{-/-} mouse cells at 6.5 h post-transfection (Fig. 3). As observed for human cells, the modulation is transient, so that at 22.5 h the effect nearly disappears (Fig. 3). Virtually identical results are obtained for pRAlu, which expresses the active right Alu monomer (Fig. 3). As reported for VAI-treated cells (14), the modulatory effect of Alu RNA is greater in PKR^{-/-} than in PKR^{+/+} cells (Fig. 3). Although the reason for this difference is unknown, the modulatory activity is PKR independent.

Other aspects of the modulation observed in human and mouse cells deserve comment. The plasmid expressing the left Alu monomer (pLAlu), which is inactive in human cells, is nearly inactive in mouse cells. Although VAI RNA is always more active than Alu RNA in human cells (compare 45- to 9-fold, Fig. 2), Alu RNA is significantly more active than VAI RNA in mouse cells (compare 8- to 3-fold at 6.5 h in PKR^{-/-} cells, Fig. 3). Plasmids expressing B1 and B2 RNA, the two families of mouse SINES, stimulate luciferase expression in mouse cells but B2 is inactive in human cells. We have not examined the degree of overexpression of these two constructs in mouse cells, which endogenously express appreciable levels of both B1 and B2 RNA (2,3).

Activity upon other reporters

To determine whether the activity of Alu RNA is peculiar to luciferase, we tested its effect upon the expression of β -gal and E1a reporter genes (Fig. 4). Genes for luciferase and β -gal were co-transfected into HeLa and 293 cells so that their relative expression might be directly compared (Fig. 4A). Because the spectrophotometric assay of β -gal has a higher

Table 1. Time dependence of luciferase activity

Time post-transfection	4 h	5 h	6 h	7 h	8.5 h	11.5 h	21 h	31.5 h
Modulator								
	Luciferase activity (l.u. $\times$ 1000)							
pUC	0.106	0.155	0.327	0.872	1.28	4.47	61.9	280
Alu+A	0.131	0.543	1.88	4.72	11.3	23.3	215	509
VAI	0.100	0.846	2.85	17.1	57.5	161	788	1640
	Differential l.u./h ($\times$ 1000)							
pUC	0.0270	0.049	0.172	0.545	0.271	1.07	06.05	20.8
Alu+A	0.0330	0.412	1.34	2.84	4.41	3.97	20.1	28.0
VAI	0.0250	0.746	2.00	14.2	26.9	34.3	66.0	81.3

HeLa cells were co-transfected with luciferase expression vector and pUC, pAlu+A or pVAI modulator plasmid. Cells were lysed at various times post-transfection and luciferase activity was determined. The corresponding stimulation relative to the pUC controls provided by the RNA modulators is depicted in Figure 2. The rate of synthesis of luciferase is estimated as the differential for each successive time interval.

Table 2. Reporter mRNA abundance in modulator co-transfected cells

	pUC, 7 h	Alu+A, 7 h	VAI, 7 h	pUC, 9 h	Alu+A, 9 h	VAI, 9 h
Luciferase expression	1	14	37	1	4.2	18
Luciferase mRNA ($\pm$ SD)	1.00 $\pm$ 0.11	0.95 $\pm$ 0.05	0.93 $\pm$ 0.05	1.00 $\pm$ 0.51	1.06 $\pm$ 0.08	1.20 $\pm$ 0.11
Actin mRNA	1	0.82	0.87	1	1.0	0.93

HeLa cells were co-transfected with luciferase expression vector and pUC, pAlu+A or pVAI. Cells were lysed at 7 or 9 h post-transfection and luciferase activity relative to pUC control cells was determined. Poly(A)⁺ total RNA was prepared from the cell lysates and luciferase mRNA was measured by *in vitro* translation (20). Average measurements of three trials are reported. To test for equal RNA loading, aliquots of these RNA samples were assayed for actin mRNA by northern blot hybridization.

Figure 3. Effect of PKR on modulator activity. PKR^{+/+} and PKR^{-/-} cells (14) (a gift from Dr R. B. Williams) were co-transfected with luciferase expression vector and the modulator plasmids shown. At two times after transfection cells were harvested and luciferase activity measured. Data are averages of three replicate transfections.

baseline, these two assays have inherently different sensitivities. With this qualification, Alu+A and VAI stimulate β -gal and luciferase expression similarly in the two cell lines (Fig. 4A). As previously observed for luciferase, the left Alu monomer transcript (pLAlu) has no effect upon β -gal expression but the right monomer transcript (pRAlu) and an Alu transcript without the A-rich 3' tail (pAlu-A) are fully active (Fig. 4A).

Unlike the exogenous luciferase and β -gal reporters, the adenovirus E1a gene is normally expressed in the presence of high concentrations of both Alu and VAI RNA (22). We therefore used this gene as an additional reporter to test the modulatory activities of Alu and VAI RNAs upon an 'endogenous', albeit viral, gene product. As shown by western analysis, both Alu and VAI RNAs stimulate E1a expression at early times (7 and 10 h) (Fig. 4B). At a later time (24 h), the level of E1a expression in the pUC-transfected control approaches the levels in Alu and VAI RNA overexpressing cells, providing another example of the time-dependent nature of the effect (Fig. 4B)

In summary, luciferase and β -gal respond similarly to the different modulators and the degree of modulation is transient

for both luciferase and E1a protein. The observed activities of Alu and VAI RNA are not limited to a particular reporter gene.

Activity resides in the right Alu monomer

The abundance of the Alu modulator RNAs in this experiment was compared by northern analysis (Fig. 5A and B). The internal B box element in pRAlu has been repaired by site-directed mutations and all of the Alu constructs have the identical A box and the 5' flanking sequence of the SRP RNA gene (Materials and Methods). However, unpublished studies (this laboratory) indicate that Alu left monomer transcripts are stable and accumulate to relatively high levels whereas unstable right monomer transcripts do not. In agreement with these expectations, the left monomer Alu transcript from pLAlu accumulates to twice the level of the dimeric transcripts from either pAlu+A or pAlu-A whereas the right monomer transcript from pRAlu accumulates to less than one-tenth of that of the dimeric transcripts (Fig. 5A and B). The modulatory activity of these plasmids (Figs 1, 3 and 4) does not simply correlate with the steady-state abundance of their transcripts (Fig. 5A and B). Furthermore, this striking difference indicates that the activity of Alu RNA is not an artifact resulting from the overexpression of non-specific transcripts but requires a particular structure.

In addition to other sequence differences, the natural right monomer contains an insertion of an additional ~30 nt with respect to the left monomer (23,24). A series of 3' truncations and internal deletions of the Alu sequence were employed to test the effect of this insertion upon activity (Fig. 6).

As previously observed, the left Alu monomer is inactive and the right monomer is fully active (Fig. 6). Progressive 3' truncations of the right monomer eliminate this activity (Fig. 6). As one example, truncation at position 175 deletes most of the right monomer and the transcripts resulting from this construct are as inactive as pLAlu left monomer transcripts (Fig. 6). Less severe truncations, such as that at position 253, have reduced activities. Northern analysis shows that the corresponding Alu transcripts from these constructs, as well as from internal deletion constructs discussed below, accumulate to approximately the same level as the full-length Alu transcript (data not shown). Presumably, structural differences in these Alu transcripts are responsible for their different activities.

The 30 nt insertion that is specific to the right monomer enlarges a stem-loop structure which is present at homologous positions in both monomers (23; Fig. 6). Internal deletions (~20 nt) of this stem that are positioned on either the 5' side

Figure 4. Modulation of three reporter genes. (A) HeLa or 293 cells were co-transfected with both luciferase and β -gal expression vectors and the indicated modulator plasmid. Luciferase and β -gal activities were assayed 8 h after transfection. Four replicates performed for pUC, Alu+A and VAI transfections demonstrate reproducibility. (B) HeLa cells were co-transfected with an adenovirus E1a12S expression plasmid and modulator plasmids. Cells were harvested at the indicated times post-transfection and assayed by western analysis for E1a protein and for actin to compare protein loading. To demonstrate activity and specificity of the antibody, a lysate of adenovirus-infected (AV inf) cells was included. These cells, harvested late (2 days) after AV infection at high multiplicity, express mostly 13S E1a, though a small amount of the 12S form is visible. Unxf, untransfected control.

(AluDW) or the 3' side (AluWD) of the loop reduce the activity of the resulting Alu transcripts (Fig. 6). These deletions may cause misfolding of the resulting transcripts. In contrast, a double deletion (AluDD) of the same bases from both sides of the stem is almost fully active (Fig. 6). This double deletion, which is symmetrically positioned about the loop, eliminates mostly paired bases from the stem. Evidently, the larger size of this stem within the right monomer is not required for activity. However, the loop is important; substituting an AU dinucleotide for the 9 nt loop (pAUloop) reduces

activity (Fig. 6). Again, this substitution might alter RNA folding.

Mouse B1 and Alu each belong to the SRP superfamily of SINEs (1,2); we therefore compared their activities on reporter expression (Fig. 6). Interestingly, the activity of B1 approximates that of pAlu+A in human cells (Fig. 6), whereas the tRNA-derived SINE, B2, is inactive (Fig. 1). As a further control, a construct containing the SRP RNA gene is inactive (Fig. 6). This gene is homologous to both Alu and B1 (Fig. 6) and is the source of the *cis*-acting transcription elements used

Figure 5. Northern analysis of modulator RNA abundance. (A) HeLa cells were transfected with luciferase expression vector and the indicated modulator plasmids or were untransfected (unxf). Modulation of luciferase expression at 8 h after transfection was verified and total RNA from these cell lysates was hybridized with oligonucleotides specific for the right Alu monomer, VAI and U6 RNAs. Hybridization to U6 RNA as well as weak cross-hybridization of this Alu probe to SRP RNA provide internal controls for RNA loading equivalence. The Alu-A transcript co-migrates with and obscures this additional SRP RNA reference band. The modulator RNAs are identified on the right of the figure. (B) The blot described in (A) was stripped and hybridized with an oligonucleotide specific to the left Alu monomer.

in the SINE constructs compared here (Materials and Methods).

Figure 6. Activity of Alu deletion constructs. The effect upon luciferase expression of the constructs relative to the pUC control is the average of three replicates. The alignment of Alu constructs to the dimeric Alu consensus sequence is schematically depicted. Breaks in the lines and numbering indicate positions of deletions relative to the full-length Alu sequence. The positions of these deletions are also shown with respect to the secondary structure of Alu RNA (23). The approximate alignments of SRP RNA and B1 to the Alu sequence are also shown (24).

DISCUSSION

The issue of whether Alu and other eucaryotic SINEs have a function is an important consideration in understanding their accumulation within gene-rich regions of the genome (25). The SINE RNA stress response raises the possibility that these transcripts serve a function. One clue to that possible function is the observation that Alu RNA stimulates expression of a reporter gene in a transient transfection assay (6). The fact that VAI RNA has a similar activity provides assurance that this observation is meaningful; VAI RNA regulates protein synthesis in virally infected cells (Introduction).

However, the activity of Alu and VAI RNA upon reporter gene expression cannot be reconciled with the one established mechanism by which VAI RNA regulates translation in infected host cells (Introduction). By inhibiting PKR activation, VAI RNA prevents the inhibition of global protein

synthesis. This mechanism would require either that the cell exists in a default state of extreme translational inhibition or that severe inhibition inadvertently results from the transfection procedure. As evidence against this putative inhibition, VA1 RNA has never been observed to increase global protein synthesis in cells which are not infected by virus (7–15). Most convincingly, VA1 RNA stimulates reporter expression in PKR knockout cells (14; Fig. 3). Similarly, we find that Alu RNA stimulates reporter gene expression in a PKR-independent manner without affecting global protein synthesis.

The ability of Alu and VA1 RNA to stimulate the expression of a reporter gene selectively introduces another puzzle in interpreting results from this assay. Historically, observations of the effect of VAI RNA on reporter mRNA abundance have been varied and conflicting. Although all investigators agree that VAI RNA increases co-expression of a reporter gene, some observe an accompanying increase in reporter mRNA (7,12,14) and others do not (8–11,13). These observations were all made later than 22 h post-transfection; we observe that the maximum transient effect on reporter expression occurs at least 12 h earlier without any increase in mRNA abundance.

The transient nature of this post-transcriptional response provides insight into the basis for its selective stimulation of reporter expression. We observe that the modulator does not increase the maximum rate at which the reporter product is synthesized but instead reduces the lag time at which this rate is achieved. Reducing this lag time would presumably increase the expression of any newly synthesized mRNA. The effect is apparent for the reporter since all of its mRNAs are recently synthesized. These observations support a previous proposal that VA1 RNA acts selectively upon newly appearing mRNAs to increase their expression (9). The mechanism of this response is unknown but there is precedence for the possibility. The translation initiation factor eIF4G has been shown to discriminate between new and old mRNAs and to cause similar effects upon luciferase expression in HeLa cells (26).

Although the physiological significance of this activity remains to be determined, an activity which reduces the lag time for the expression of new mRNAs (and perhaps more generally for any mRNAs which are not translationally engaged), could be advantageous during cell stress. Considering heat shock as one example of a cell stress, the rapid expression of newly transcribed heat shock mRNAs is essential for stress recovery and, after recovery is complete, the translation of the pre-heat shock mRNA cohort is reinitiated. SINE RNA abundance increases following heat shock (3–5).

Similarly, an ability to accelerate the translational initiation of newly synthesized mRNAs provides an obvious survival advantage for a virus, enabling it to rapidly and selectively stimulate expression of its own new messages in competition with pre-existing host cell mRNAs (9). VA1 RNA is required for the selective expression of viral mRNAs in adenovirus-infected cells (9). As a pertinent example, E1a protein is an early gene product, which adenovirus requires to gain regulatory control of the host cell (27). Significantly, E1a is endogenously co-expressed in infected cells with high levels of both Alu and VA1 RNA. This causes us to believe that the

stimulated expression of E1a, which is observed here, accurately models physiological events that normally occur in virally infected cells. Adenovirus directs the increase in Alu RNA that occurs in infected cells (22) which, according to this model, would also favor the expression of viral gene products.

The dimeric Alu consensus sequence consists of two distinct functional domains: The left monomer contains the essential A and B box promoter elements for Pol III transcription and the right monomer is both necessary and sufficient for its modulatory activity. The right monomer contains an additional 30 nt with respect to the left monomer, enlarging a stem-loop structure that is present in both monomer units (Fig. 6; 23,24). Mapping studies indicate that this region is necessary for the activity of Alu RNA, suggesting that the right monomer contains a specific structure which presumably interacts with cellular factors.

B1 RNA, representing the SRP RNA-related SINE superfamily in rodents, shares this activity, suggesting its evolutionary conservation. The mouse B1 consensus sequence can be represented as containing additional nucleotides with respect to the human left Alu monomer, enlarging the same stem-loop structure discussed above (23,28). In this sense, the structure of B1 RNA more nearly resembles that of the right Alu monomer, conceivably accounting for its activity in the transfection assay.

The SINE RNA stress response is conserved in evolution, prompting speculation that the corresponding function of the transcripts, if any, would also be conserved (Introduction). However, in the co-transient expression assay the activity of rodent B2 RNA in human cells is so low that we regard it as being an excellent negative control. Since B2 represents the tRNA-related superfamily of SINEs, its inactivity raises the possibility that the modulatory activities of Alu and B1 RNAs are a more recent adaptation of SRP-related SINEs, as contrasted to an ancestral property of all eucaryotic SINEs. Alternatively, the activity of tRNA-related SINE transcripts might have been lost following the divergence of humans from other mammals. Supporting this latter possibility, B2 over-expression appears to stimulate reporter gene expression in mouse cells. While this issue requires further investigation, SRP-related SINE transcripts, like VA1, have a specific cellular activity enabling them to selectively stimulate the expression of at least some newly synthesized mRNAs.

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