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RNA polymerase III promoter and terminator elements affect *Alu* RNA expression

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ABSTRACT

Promoter elements derived from the 7SL RNA gene stimulate RNA polymerase III (Pol III) directed *Alu* transcription *in vitro*. These elements also stimulate expression of *Alu*s transfected into 293 cells, but transcripts from these same constructs are undetectable in HeLa cells. A terminator resembling the terminator for the 7SL RNA gene has no effect on *in vitro Alu* template activity, but increases expression *in vivo* in a position independent manner. *Alu* transcripts generated from templates with and without this terminator have identical half-lives, indicating that this terminator stimulates expression by increasing template activity. Together, these results show that *Alu* expression may be regulated at multiple levels and can respond to *cis*-acting elements. This new found ability to express *Alu* transcripts by transient transfection provides an opportunity to monitor their post-transcriptional fate. Primary *Alu* transcripts are not extensively adenylated or deadenylated following transcription, but are short-lived compared to 118 nt sc*Alu* RNA. In addition to *Alu* RNA, transfected templates encode sc*Alu* RNA, but very high levels of *Alu* RNA expression does not increase the abundance of sc*Alu* RNA. Sc*Alu* RNA is not merely a transient RNA degradation product, but is instead tightly regulated by factors other than the abundance of primary transcripts.

INTRODUCTION

Nearly one million *Alu* repeats interspersed throughout the human genome share a 282 nt consensus sequence consisting of two tandemly repeated monomer units homologous to 7SL RNA (1–4; Fig. 1A). Like the homologous 7SL RNA gene, *Alu* repeats contain an internal type 2 Pol III promoter and are active templates *in vitro* (reviewed in 5).

Despite the transcriptional potential of one million templates, corresponding *Alu* transcripts are barely detectable in cultured human cells (6,7). *Alu* transcripts are retrotranspositional intermediates and, like 7SL RNA, may have a function (1–4). Viral infection, cell stress, translational inhibition and other cell treatments dramatically induce *Alu* expression, demonstrating the latent activity of *Alu* repeats (8,9,10). Elsewhere, we suggest that an increase in *Alu* RNA is part of the cell stress response, so that *Alu* expression is normally repressed (10). Chromatin structure, DNA methylation and many other commonly encountered mechanisms for regulating eukaryotic gene transcription may collectively silence this vast number of potential transcription units (4,5,11,12).

An additional possibility, inherently weak promoters, may partially account for the low expression of *Alu*s *in vivo*. The 7SL RNA gene and pseudogenes provide a pertinent model system for *Alu* template activity (Fig. 1A). The 7SL RNA gene and retrotransposed pseudogenes are also transcribed *in vitro* from their internal A box and B box promoter elements (13,14). Despite their internal promoter elements, retrotransposed 7SL RNA sequences are relatively silent *in vivo* compared to the authentic 7SL RNA gene. 5′ flanking sequences stimulate the 7SL RNA gene's template activity *in vitro* and are required for efficient expression *in vivo* (14,15). Without the benefit of similar flanking sequences, *Alu*s should also be very weak templates *in vivo*.

Sequences flanking an *Alu* source gene stimulate its template activity *in vitro* , suggesting a molecular basis for its retrotranspositional success (16). However, this source gene like other *Alu* elements is not expressed at detectable levels upon transient transfection in to HeLa cells (16). While the importance of flanking elements is axiomatic, there is no evidence for their activity *in vivo*.

Steady state levels of transcripts are also regulated by RNA life time. Since endogenous *Alu*s are expressed at a very low level, and transfected *Alu*s are inactive in cell lines tested to date, the post-transcriptional fate of *Alu* transcripts is, with one exception (17), largely uninvestigated. The fate of *Alu* transcripts may involve both polyadenylation and processing into a shorter 118 nt transcript called sc*Alu* RNA:

(i) The 282 nt *Alu* consensus sequence is immediately followed by an A rich sequence reminiscent of an mRNA poly A tail. Pol III transcription reads through this region before terminating in the first run of four or more T residues present in the 3′ flanking sequence of the genomic insertion site, so that the resulting primary transcript fractionates as poly A plus RNA (6,7; Fig. 1A). In evolutionarily older *Alu*s, this A rich region includes all four bases, but in younger *Alu*s it consists exclusively of adenine residues (3,4,18). Upon retrotransposition, any templated A rich

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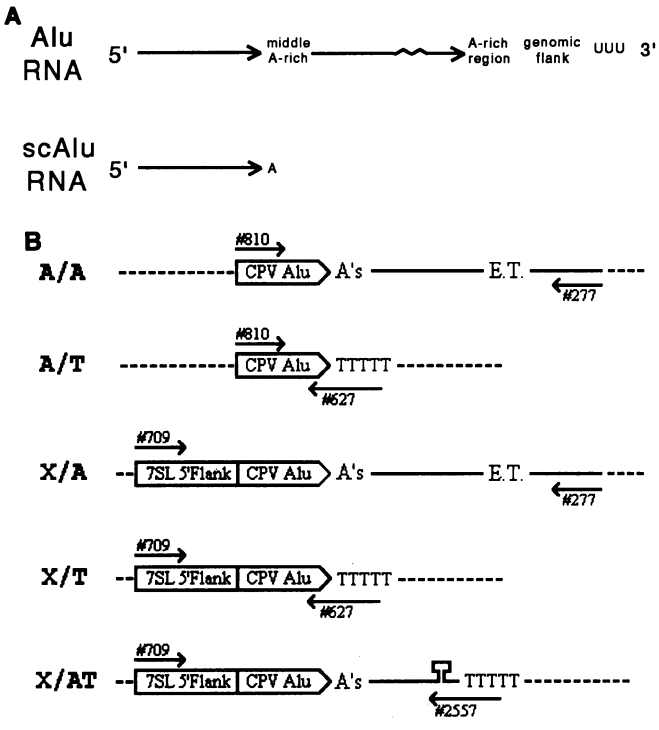


Figure 1. (**A**) Comparison of *Alu* related transcripts. The 282 nt *Alu* consensus sequence consists of two distinguishable monomer units, depicted here as arrows, that are separated by an A rich middle sequence (1,2). The wavy line in the right monomer indicates a small insertion that is absent in the left monomer. The 282 nt consensus sequence is immediately followed by a longer A rich region and genomic flanking sequence, such that the resulting transcript terminates in the first adventitious run of four T's, producing a U-terminated transcript as depicted. Sc*Alu* RNA resembles the left *Alu* monomer; its 3′ end is not yet exactly defined and is depicted here as an A residue (17). (**B**) Identification of *Alu* templates used in this study. Vector sequences are indicated by dashed lines. The 7SL RNA gene's 5′ flanking promoter elements and the CPV *Alu* element are indicated by boxed arrows. Oligonucleotides, which define each construct, are shown by arrows with the appropriate orientations. The endogenous A rich region and the endogenous terminator present in clones A/A, A/T and X/A are labeled 'A's' and 'ET' respectively. The newly introduced terminator is labeled 'TTTTT' in clones A/T, X/T and X/AT. The synthetic palindrome in clone X/AT is schematically depicted and its sequence is reported in Materials and Methods.

sequences would have to be corrected to the perfect A runs which are present in the resulting young *Alu* insertions (3,4,18). Polyadenylation, while unprecedented for Pol III transcripts, plausibly accounts for the poly A-like sequences in recently transposed *Alu*s.

(ii) A short A rich region located in the middle of the 282 nt *Alu* consensus sequence separates the two *Alu* monomer subunits (Fig. 1A). Sc*Alu* RNA resembling the left *Alu* monomer unit may result from processing of the primary transcript by cleavage within the middle A rich region (17,19). Lacking the primary transcript's 3′ A rich region, sc*Alu* RNA fractionates as poly A$^-$ RNA. Sc*Alu* RNA is stabilized by specifically binding SRP proteins (20). An *Alu* template microinjected into *Xenopus* oocytes produces both *Alu* RNA and sc*Alu* RNA (17). Also, incubation of *Alu* transcripts with nuclear extract results in the appearance of sc*Alu* in a time dependent manner (17). Sc*Alu* RNA is derived post-transcriptionally from dimeric *Alu* templates. However, as an alternative to a defined processing pathway, sc*Alu* RNA might simply be a major RNA degradation product resulting from non-specific cleavage within the relatively unstructured middle A rich region of *Alu* transcripts.

Unlike primary *Alu* transcripts, the 7SL RNA gene employs a more conventional Pol III terminator consisting of four T residues immediately preceded by a potential hairpin consisting of a 5 bp palindrome (13–15). Four T residues are sufficient to define a Pol III terminator, but the palindrome may also assist in termination or have a post-transcriptional role by interacting with specific factors. Because of its homology with 7SL RNA, an identical palindrome is present at the 3′ end of the 282 nt *Alu* consensus immediately preceding the A rich region, rather than the T residues that define the 7SL RNA gene terminator. Conceivably, the 3′ A rich region destabilizes *Alu* transcripts relative to 7SL RNA and sc*Alu* RNA, or the 3′ terminator of the 7SL RNA gene stabilizes its transcript relative to either *Alu* RNA or sc*Alu* RNA. The terminator structure alters the efficiency with which B1 transcripts are processed into scB1 RNA (21). Also, by increasing the rate of template clearance, an efficient terminator increases B1 transcriptional activity *in vitro* (22).

Using chimeric constructs, we test the effects of 5′ and 3′ flanking sequences on *Alu* RNA expression by transcription *in vitro* and transient transfection assays. Chimera are constructed using the 7SL RNA gene's 5′ promoter elements and a defined 3′ termination signal to examine whether *cis* elements can stimulate *Alu* expression *in vivo*, to test the effects of a defined transcriptional terminator on *Alu* expression and to monitor the post-transcriptional fate of the resulting transcripts.

MATERIALS AND METHODS

Construction of chimeric *Alu* clones

Using oligonucleotides defined below, five constructs (Fig. 1B) were generated by PCR strategies from CPV *Alu* (23) and the human 7SL RNA gene using 7SL 30.1 plasmid (14). The resulting PCR products were cloned into TA vector (Invitrogene Inc.) and verified by base sequence.

Clone A/A was generated using oligonucleotides #810 and #277 in conjunction with the CPV *Alu* clone. The resulting 433 bp PCR product consists of a *Hin*dIII site (see oligonucleotide #810) immediately abutting the 5′ end of the *Alu* repeat, the 282 nt CPV *Alu* and 145 nt of endogenous 3′ flanking sequence (Fig. 1B). Clone A/T is defined by oligonucleotide #810, described above, and oligonucleotide #627 which introduces five T residues, followed by a *Bam*HI site immediately on the 3′ end of the CPV *Alu* element.

Clone X/A was constructed by first ligating a 180 bp *Hin*dIII–*Bst*UI fragment from 7SL 30.1 plasmid with the 600 bp *Bst*UI–*Pst*I fragment derived from CPV *Alu*. This region from the 7SL RNA gene includes all of its known flanking promoter elements (14,15). Ligation of the *Bst*UI sites defining these two fragments recreates the exact spacing between the 7SL RNA gene's flanking promoter elements and the internal A box of the resulting chimeric. The ligation product was PCR amplified using oligonucleotide #709, which exactly matches positions −174 to −158 in the 7SL RNA gene and oligonucleotide #277. The resulting chimera, clone X/A, has the 5′ flank of the 7SL RNA gene fused to the *Alu* and its attendant 3′ flanking sequence as described for clone A/A (Fig. 1B).

Clone X/T is derived by PCR amplifying clone X/A using oligonucleotides #709 and #627 described above. The resulting chimera consists of the 7SL RNA gene's 5′ promoter elements and the *Alu* element from CPV *Alu* terminated by five T residues (Fig. 1B). In clones A/T and X/T, five T residues (indicated by small letters) are preceded by the *Alu* consensus sequence (indicated by capital letters) forming the sequence: GAGACTCCGTCTCttttt (see for example the complement oligonucleotide #627). The 5 bp palindrome positioned immediately before the T residues is highly conserved in *Alu* evolution and a similar structure is present in its homologue, the 7SL RNA gene (1,2,13–15). For brevity, we refer to the resulting structure as a 'T-terminator'. Using oligonucleotide #709, described above, and oligonucleotide #2557, the endogenous *Alu* terminator is converted to a T-terminator in clone X/AT (Fig. 1B). Specifically, endogenous 3′ sequence in clones A/A and X/A (30As,GAAAGGTTCTGAGATGTTAATTCATTTT) is converted to the sequence 30As,GAAAGGTTCTGAGACGTTGTCTCTTTTT in clone X/AT by the five base substitutions indicated by bold type. The T-terminator has the same palindrome as that present in *Alu* repeats and the 7SL RNA gene, but substitutes three bases between the palindromic inverted repeat (GTT versus TCC). Also, the T-terminator has five Ts, whereas four Ts are present in the 7SL RNA gene terminator (13).

Oligodeoxyribonucleotides

Table 1 shows the custom-synthesized oligonucleotides used for synthesis of chimeric clones, primer extension and Northern blot analysis.

Cell culture and transfection

HeLa cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 5% calf serum and 5% fetal bovine serum; 293 cells (human transformed primary embryonal kidney cells, ATCC) were grown in α-minimal medium supplemented with 2% fetal calf serum and 8% new-born calf serum. All cells were maintained in 5% CO_2 at 37°C. One day prior to transfection, cells were seeded at a density of 5×10^4 on 100 mm plates. DNA (20 μg of plasmid containing a cloned *Alu* repeat, 5 μg of plasmid containing a luciferase reporter gene and 15 μg of salmon sperm DNA carrier) was transfected by calcium phosphate precipitation (24). After 40 h, cells were harvested for RNA or RNP extraction, retaining an aliquot for luciferase assay (25). For RNA lifetime determinations, cells were treated with actinomycin D (5 μg/ml) for the indicated periods of time.

RNA preparations and transcription assays

In vitro transcription using crude HeLa extracts was performed as previously described (5). Cytoplasmic and nuclear RNAs were prepared and separated into poly A^+ and poly A^- fractions (7). For primer extension assays, RNA in 12.5 μl of annealing buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.3 M KCl) and 5–10 pmol (~10^5 c.p.m.) of radiolabelled primer was incubated at 80°C for 3 min and then allowed to anneal for ≥ 1 h at 56°C for *ALU* 21, 46°C for PVcomp. or 64°C for P-S *Alu*. Following addition of 50 μl of extension buffer [10 mM Tris–HCl (pH 8.6), 5 mM $MgCl_2$, 5 mM DTT, and 1 mM dNTP], the reaction mixture was incubated with 10 U AMV reverse transcriptase (Life Science Inc.) for ≥ 1 h at 42°C. Analysis of the primer extension product has been previously described (7). Quantitation of the primer extension was done with the Fujix BAS 1000 PhosphorImager.

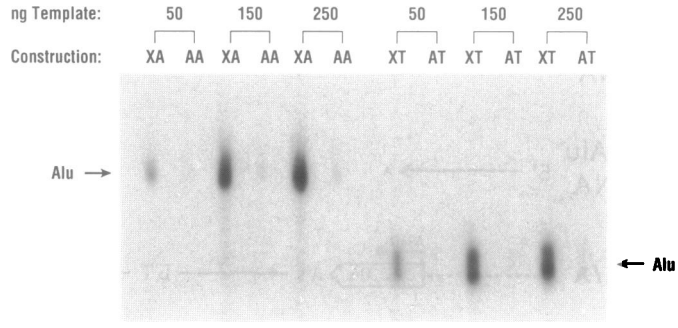


Figure 2. *In vitro* template activity was assayed by gel electrophoresis for the indicated quantities of each construct; total DNA is adjusted to a constant amount (2 μg) by the addition of pUC18 carrier. The sizes of the transcripts determined in parallel experiments is consistent with Pol III terminated transcription. The clone A/A and A/T products are underexposed (45 min) to demonstrate the dramatic difference in template activities between clones X/A and A/A, as well as clone X/T and A/T. By PhosphorImager analysis, the template activities of clones X/A and X/T are equal and 30-fold higher than the template activities of clones A/A and A/T, which are also equal.

For Northern blots, RNA was separated on 1.5% agarose containing 7% formaldehyde and 1 × MOPS (7). After soaking in 2 × SSC to remove formaldehyde, the gel was transferred to Hybond-N+ membrane (Amersham) overnight and the membrane was dried at 80°C. Blotting from 5% polyacrylamide gels (40:1 acrylamide:bis) was performed as previously described (7,10). The membrane was incubated with prehybrization buffer (50 mM phosphate buffer, pH 6.8, 5 × SSC, 5 × Denhardt's solution , 0.5% SDS and 100 μg/ml yeast tRNA) at 44°C for 6 h, and then was hybridized with the labeled oligonucleotide (10 ng/ml) (10) in hybridization buffer (25 mM phosphate buffer, pH 6.8, 5 × SSC, 5 × Denhardt's solution, 0.5% SDS) for 20 h at 44°C for *Alu* #71, PVcomp. and 7SL oligonucleotides and at 52°C for oligonucleotide *Alu* 21mer. The membrane was washed twice with 5 × SSC, 0.1% SDS at room temperature and once at the hybridization temperature for 20 min.

RESULTS

7SL RNA gene promoter elements stimulate *Alu* template activity *in vitro*

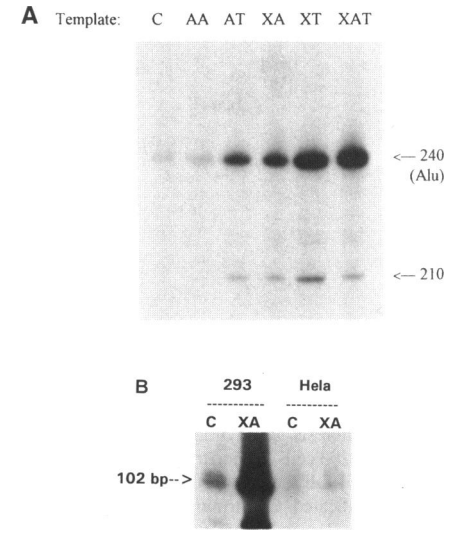
The PV subfamily *Alu* repeat (CPV *Alu*) from chimpanzee has four base substitutions, compared to the PV subfamily consensus sequence, and we emphasize that the species of origin of this clone should not affect any properties examined in this study. The five *Alu* clones examined here are named as follows (Fig. 1B): clone A/A is a subclone of CPV *Alu* including its endogenous 3′ A rich region and downstream terminator. In chimeric clone X/A, the 5′ promoter region flanking the 7SL RNA gene replaces the endogenous 5′ flanking sequences of clone A/A. In chimeric A/T, a 3′ terminator called a 'T-terminator' (Materials and Methods) is positioned at the 3′ end of the 282 nt *Alu* consensus. Chimeric X/T consists of CPV *Alu* flanked by both the 5′ 7SL promoter region of clone X/A and the T-terminator of clone A/T. Chimeric X/AT resembles clone X/T except that a T-terminator has been recreated at exactly the same site as that of the endogenous terminator; i.e., downstream from the A rich region.

Table 1.

Name	Sequence 5′→ 3′	Position	Size of product
PVcomp. (23)	ACCGTTTTAGCCGGGATG	104–84	104
Alu 21mer (7)	GCGATCTCGGCTCACTGCAAG	238–218	238 (240)
P-S *Alu* (8)	TTAGTAGAGA$^{C}/_{G}$GGGGTTTCACCATG	120–96	120
Alu #71 (10)	GGTTTCACCGTGTTAGCCA	89–107	107
7SL (13)	ATGCCGAACTTAGTGCGG	129–112	129
#277	CATGTCTCTGGCCAAGCTG		
#627	AAAGGATCCAAAAAGAGACGGAGTCTCGC		
#709	TCCCAACCCTGTAAGCA		
#810	AAAGCTTGGCCGGGCGCGGTGGCT		
#2557	AAAAAGAGACAACGTCTCAGAACC		

Clone A/A is transcribed by Pol III *in vitro* (Fig. 2). The assignment of this product to Pol III directed transcription is consistent with its length (Fig. 2), and primer extension product lengths described in experiments reported below. This particular PV *Alu* is relatively active compared to other *Alu* templates (unpublished). Fusion of this *Alu* with the 7SL RNA gene's 5′ promoter region to form the chimera, clone X/A, stimulates its template activity *in vitro* by 30-fold (Lanes A/A and X/A, Fig. 2). Similarly, the template activity of clone X/T is 30-fold greater than that of clone A/T (Lanes A/T and X/T, Fig. 2). This promoter region has a comparable effect on the activity of the 7SL RNA gene (15).

Transcripts from clones A/T and X/T resolve into a doublet (Fig. 2). The lower band corresponds to the product expected for termination at the T-terminator. The other product is 10 nt longer and corresponds to termination at the T residues encoded by the 5′ end of oligonucleotide #627. The first run of T's in clones A/T and X/T serve as inefficient terminators. Transcripts shown in Figure 2 account for the major products from these templates as only minor amounts of RNA are seen at higher molecular weights. As presented below, the terminator increases expression *in vivo* in contrast to these *in vitro* results. The experimental conditions used here have been optimized for *Alu* transcription *in vitro*, and we have not investigated whether changes in these conditions can reveal the terminator's effect *in vitro* (5). With these qualifications, the chimeric T-terminated *Alu*, clone A/T, has the same template activity as its parent, clone A/A (Fig. 2). Similarly, the template activities of clones X/A and X/T are identical (Fig. 2). Thus, the position and structure of the terminator and the presence or absence of the A rich region does not significantly affect *in vitro Alu* template activity.



Effects of *cis* elements on *in vivo Alu* expression

The expected 240 nt primer extension product demonstrates the high level of *Alu* expression in 293 cells transfected with clone X/T (Fig. 3A; Materials and Methods). In addition to the principal 240 nt product, a minor 210 nt product is observed in transfected, but not control cells (Fig. 3A).

The following observations indicate that this 210 nt product is an artefact and that it does not result from *Alu* transcripts which have been shortened by 30 nt. Oligonucleotide primers positioned elsewhere within the *Alu* consensus sequence do not reveal the presence of transcripts truncated by 30 nt, but yield predicted full length products (Fig. 3B, data not shown). Also, Northern blot results reported below (Fig. 4) reveal only homogenous, full length transcripts, confirming this conclusion. Potential misprimming of this particular *Alu* sequence at a site that is positioned 30 nt 5′ to its targeted priming site, plausibly accounts for the 210 nt product. Albeit an artefact, this 210 nt product provides an internal control to distinguish endogenous and transfected template *Alu* transcripts (lane C, Fig. 3A).

As judged by the intensity of the 240 nt product, *Alu* expression in clone A/A transfected cells is marginally greater (1.4-fold) than in control cells (lanes C and AA, Fig. 3A, Table 2). Similarly, the 210 nt primer extension product is barely detectable in clone A/A transfected cells (data not shown, and Fig. 3A). Chimeric clones A/T and X/A are each highly expressed compared to the parent clone A/A (lanes A/A, A/T and X/A, Fig. 3A). 5′ flanking sequences from the 7SL gene and the T-terminator stimulate *Alu* expression to a similar extent (Table 2). To monitor the

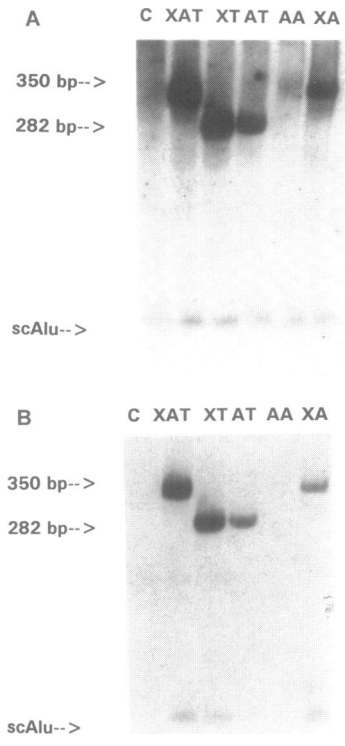


Figure 4. (**A**) Northern blot analysis of cytoplasmic RNA (100 µg) separated by acrylamide gel electrophoresis and hybridized with oligonucleotide, *Alu* #71. RNAs are from control 293 cells 'C' and transfected cells as indicated. The positions of sc*Alu* RNA and full length *Alu* transcripts corresponding to the transfected templates are indicated. Confirming these assignments, hybridization of the same blot to *Alu* 21mer oligonucleotide, which complements the right *Alu* monomer, gives the same pattern of high molecular weight *Alu* bands, but does not hybridize to sc*Alu* RNA (data not shown). Table 2 reports the intensities of these bands as *Alu* and sc*Alu* RNAs. (**B**) Re-hybridization of the blot described in (A) with oligonucleotide PVcomp. which exactly complements PV subfamily *Alu* template employed in the transfection. Table 2 reports the intensities of these bands as PV *Alu* and scPV *Alu* RNAs.

reproducibility of transfection, a luciferase reporter gene was co-transiently transfected in this and subsequent experiments (Table 2, data not shown). The maximum variation in luciferase activity is ~30%.

Clone X/T, combining both the 7SL RNA gene's 5′ promoter region and its 3′ terminator, is expressed at a higher level than either clones A/T and X/A (Fig. 3A, Table 2). These two elements independently increase expression.

Pol III terminates in the first run of four or more Ts so that the primary transcription product from clone X/A should include the A rich 3′ sequence, whereas the primary products from clones A/T and X/T should not. (This expectation is verified by Northern blot results discussed below.) To test whether the T-terminator, as such, increases expression, or the presence of the A rich 3′ sequence decreases expression, clone X/AT was constructed and assayed by transient transfection. By substituting five bases (see Materials and Methods for an exact sequence comparison), this construct creates a T-terminator at exactly the same position as the endogenous terminator present in clones A/A and X/A (Fig. 1B). The level of expression of clones X/AT and X/T are similar (Fig. 3A, Table 2). Thus, the efficient T-terminator increases expression of clones A/T, X/T and X/AT, as opposed to the competing possibility that the presence of the A rich sequence or other 3′ flanking sequences decrease expression of clones A/A and X/A. We have not investigated whether the positive effect of the T-terminator depends on the number of T residues (five in X/AT versus four in X/A), the palindrome, or some combination of both features. However, five preselected base substitutions positioned far from known promoter elements significantly increase *Alu* expression (Table 2, Discussion).

We did not observe any increase in *Alu* RNA above its basal level in HeLa cells transfected with these clones (data not shown). For reasons presented in the Discussion, we regard this negative result as being remarkable. The oligonucleotide used in the experiments of Figure 3A primes most endogenous *Alu* transcripts (3,4). To reduce this background, the activity of clone X/A in HeLa cells was reinvestigated using an oligonucleotide, PVcomp., that targets two diagnostic base substitutions of the PV *Alu* subfamily. Members of this subfamily account for only 0.1% of all *Alu* repeats so that corresponding transcripts are rare (6,7; also see Figs 4B and 5B as discussed below). The abundance of PV *Alu* RNA is 80-fold higher in clone X/A transfected 293 cells than in both control and transfected HeLa cells (Fig. 3B). Clone X/A's *in vitro* template activity is 30-fold higher than that of its parent *Alu*, clone A/A, underscoring the low level at which ordinary *Alu* elements are expressed in transfected HeLa cells.

Table 2.

Cells	Primer extension		Northern blot analysis				
	Alu	Luciferase	*Alu*	sc*Alu*	PV*Alu*	scPV*Alu*	7SL RNA
X/AT	1	1	1	1	1	1	1
X/T	0.78	1.22	0.8	0.89	0.97	0.87	1.32
X/A	0.36	0.99	0.22	0.52	0.21	0.64	0.91
A/T	0.27	0.91	0.20	0.68	0.26	0.17*	1.23
A/A	0.07	N.A.	0.02	0.52	0.01	0.02*	1.04
293	0.05	1.13	0.01	0.68	0.01	0.11*	0.92

Intensities of primer extension products observed for *Alu* RNAs in transfected cells (Fig. 3A) were measured by PhosphorImager analysis and are reported relative to the intensity of the X/AT product. Luciferase activity relative to its activity in clone X/AT is also reported to compare transfection efficiencies. Intensities of bands on Northern blots corresponding to *Alu* RNA, sc*Alu* RNA (Fig. 4A); PV*Alu* RNA, scPV*Alu* RNA (Fig. 4B) and 7SL RNA (data not shown) were determined by PhosphorImager analysis and are reported relative to the intensity of the corresponding RNA in clone X/AT transfected cells. Endogenous *Alu* RNAs do not give a definite band length on a Northern blot; the hybridization intensity for 293 cells was measured at the same RNA length as that of the clone X/A transcripts for purpose of comparison. The small amounts of scPV*Alu* RNA marked by an asterisk are within experimental error of the background correction; i.e. zero (Fig. 4B).

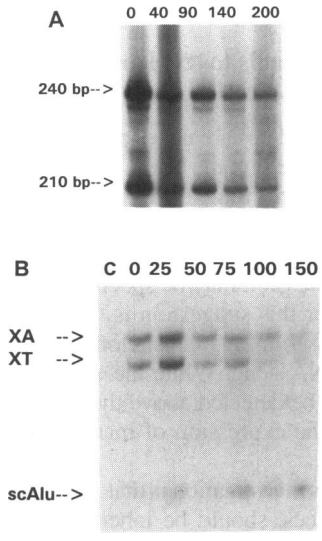


Figure 5. (**A**) Primer extension assay of cytoplasmic RNA (20 µg) using *Alu* 21mer oligonucleotide of *Alu* transcripts in X/A transfected 293 cells treated with actinomycin for the indicated period of time. (**B**) Northern blot analysis of cytoplasmic RNA (75 µg) from 293 cells co-transiently transfected with clone X/T and three equivalents of clone X/A and treated with actinomycin for the indicated periods of time. The probe, PVcomp., hybridizes to both full length transcripts and sc*Alu* RNA present in transfected cells, but gives a negligible signal when hybridized to RNA from control cells 'C'. The ratio of intensities of the X/T and X/A bands and the intensities of the sc*Alu* RNA bands are reported as experiment 2 in Table 3.

Post-transcriptional fate of *Alu* RNA

Northern blot analysis using agarose gel (data not shown) and polyacrylamide gel (Fig. 4A) electrophoresis confirms and extends the primer extension assay results. The hybridization probe used in this experiment, oligonucleotide *Alu* #71, does not discriminate among different *Alu* subfamilies. In agreement with the primer extension results, transcripts from clone A/A are essentially undetectable by Northern blot analysis (Fig. 4A). For each of the four remaining transfected clones, a single principal band is observed at the expected transcript length. For example, the transcript resulting from the X/T template is shorter than that from the X/A template (Fig. 4A). The relative intensities of transcripts observed by blot analysis are in excellent agreement with the primer extension results (Table 2). Specifically, the 5′ 7SL RNA gene promoter region and the T-terminator each independently stimulate *Alu* expression. Also, the T-terminator works equally well when positioned immediately 3′ to the *Alu* element (clone X/T) or when it replaces the endogenous terminator downstream from the *Alu* element (clone X/AT).

The homogeneity of these bands on a high resolution acrylamide gel and their correspondence to the relative sizes predicted for their templates suggest that there is no gross alteration of the resulting transcripts' 3′ ends. These RNAs were separated into poly A^+ and poly A^- fractions for both Northern blot and primer extension assays (data not shown). The transcripts resulting from clones X/T and A/T fractionate as poly A^- transcripts, whereas those from clones X/AT, X/A and A/A fractionate as poly A^+ RNA. While these results do not preclude subtle changes in the 3′ ends of these transcripts or major changes in the 3′ ends of a minor fraction of these transcripts, most *Alu* transcripts are not grossly altered with respect to their adenylation.

118 nt sc*Alu* RNA is also detected by Northern blot analysis (Fig. 4A). The hybridization intensity of sc*Alu* RNA is increased less than 2-fold in transfected cells although the steady state concentration of *Alu* RNA in cells transfected by clones X/AT and X/T exceeds the concentration of endogenous *Alu*s in control cells by as much as 100-fold (Fig. 4A, Table 2). Even this small difference in the intensity of the sc*Alu* RNA band should be disregarded for two reasons: first, the sc*Alu* RNA hybridization signal is only about twice that of background, so that differences of less than two approximate the uncertainty in its background correction (Fig. 4A). As an internal control, the relative abundance of 7SL RNA has also been determined for these same samples and exhibits a maximum variation of 40% (Table 2). Secondly, the hybridization probe exactly matches the sequence of the transfected clones, thereby biasing the hybridization signal toward their transcripts (see below). With these qualifications, the level of sc*Alu* RNA is virtually independent of the level of primary *Alu* transcripts.

Hybridization of oligonucleotide PVcomp. to RNA from transfected cells reveals a very different pattern compared to the results obtained using the non-specific *Alu* probe (Fig. 4B). Because this oligonucleotide targets diagnostic PV subfamily base substitutions, we report its hybridization as being to PV *Alu* and scPV*Alu* RNA (23). The specificity of this hybridization probe is confirmed by the absence of any significant hybridization to control 293 cell RNA. ScPV*Alu* RNA is abundant in cells transfected by chimeric clones X/AT, X/T and X/A, is essentially undetected in control cells and cells transfected by clone A/A, and is expressed at intermediate levels in cells transfected by chimeric clone A/T (Fig. 4B, Table 2). Transfected templates encode sc*Alu* RNA. The relative abundance of *Alu* and sc*Alu* RNAs in cells transfected with clones X/AT and X/T is similar to the relative abundance of PV*Alu* and scPV*Alu* RNAs in these same cells (Fig. 4A and B). Most sc*Alu* RNA in these cells results from the transfected templates. Presumably, primary transcripts derived from the transfected templates in these cells swamp out other sc*Alu* RNA precursors (Table 2). As discussed above, the minor increase in the hybridization intensity of sc*Alu* RNA observed in transfected lines could be caused by this substantial change in the subfamily composition of sc*Alu* RNA (Table 2).

The relative abundance of scPV*Alu* RNA is 4-fold less in clone A/T transfected cells than in clone X/A transfected cells, although the amount of PV*Alu* RNA is similar in clone A/T and clone X/A transfected cells (Table 2). This observation has been independently replicated, leading us to conclude that the T-terminated transcript may be less efficiently converted to sc*Alu* RNA (Discussion).

X/A and X/T *Alu* transcripts have identical half lives

Actinomycin has been used to block new transcription in cells transfected with clone X/A to observe the decay of *Alu* RNA (Fig. 5A). Within 40 min of actinomycin addition, the abundance of this transcript decreases to 40% of its initial value and within 90 min is further reduced to 20% of its initial value. Allowing for the time required for actinomycin to take effect, the lifetime of this transcript is as short as 30 min.

Differences between the lifetimes of T-terminated and endogenously terminated *Alu* transcripts might cause the difference in

their expression reported above. Since clone X/T is expressed 3-fold higher than clone X/A (Table 2), this model requires the half-life of X/T transcripts to be three times longer than that of X/A transcripts. The following experiment was designed to provide an accurate measure of the relative lifetimes of these two transcripts. Clones X/T and X/A were co-transfected into 293 cells and RNA from these cells was assayed by Northern blot analysis at various times after the administration of actinomycin (Fig. 5B). This strategy permits a direct measure of relative abundance of the two transcripts that is unaffected by many experimental details which might preclude detecting a small difference in their absolute lifetimes. The relative amounts of clones X/T and X/A were adjusted such that the expression of X/A transcripts would initially exceed that of X/T transcripts (Table 3). In the event that X/A transcripts are shorter lived, the abundance of X/T transcripts would equal and then exceed that of X/A transcripts after several half lives.

Instead, the relative abundance of these two transcripts is virtually constant within an experimental variation of approximately ±10% for a period of 2.5 h following administration of actinomycin (Fig. 5B; Table 3). The two RNAs have essentially identical half lives. The difference in expression of clones X/T and X/A must be attributed to differences in their transcriptional activity.

Alu transcripts are short-lived relative to sc*Alu* RNA

The abundance of sc*Alu* was also monitored during these RNA lifetime experiments (Fig. 5B; Table 3). Sc*Alu* RNA is constant within an experimental range of ±20% for 2.5 h following actinomycin addition (Table 3). As a control for RNA loading, the amount of 7SL RNA in these same experiments has been determined. Variations in the amounts of sc*Alu* RNA and 7SL RNA are very similar (Table 3). Sc*Alu* RNA has a half life that greatly exceeds 2.5 h and is long lived compared to *Alu* RNA.

DISCUSSION

Alu expression responds to *cis* elements and is regulated at multiple levels

Transfected *Alu* templates are not expressed at readily detectable levels in HeLa cells but the same templates are abundantly expressed in adenovirus transformed 293 cells. 293 cells, unlike HeLa cells, are poised to express available *Alu* templates. Among other possible explanations for this difference between the two cell types, *Alu* expression may be specifically repressed in HeLa cells. The basis for this suggestion is that, in addition to being abundantly expressed in 293 cells, clone X/T closely resembles the active 7SL RNA gene. While the expression of transfected 7SL genes cannot be detected above the background of endogenous 7SL RNA, the expression of marked templates is readily detected (15,26).

A premise of this investigation is that *Alu*s, like retrotransposed 7SL RNA sequences, should be inherently weak transcription units *in vivo*. Like its homologue, *Alu* expression *in vitro* and *in vivo* can be driven by the powerful promoter element flanking the 7SL RNA gene. 3′ Prime flanking sequences, specifically the T-terminator, can also significantly increase *Alu* expression *in vivo*. The effect of this terminator is position independent with respect to the 3′ A rich region and other endogenous sequences flanking the *Alu* element. Thus the presence or absence of flanking sequences in the resulting transcripts does not significantly affect their abundance.

RNA lifetime is identical for *Alu* transcripts employing either the endogenous terminator or the T-terminator; evidently, this terminator increases expression by increasing transcription. The template activity *in vitro* of a B1 repeat is increased by increasing the efficiency with which nascent RNA clears the template (21). The terminator for the 7SL RNA gene (which resembles the T-terminators in the present study, see Materials and Methods) has been observed to be somewhat more efficient in this regard than an endogenous B1 repeat terminator (22). This template clearance mechanism provides a plausible explanation for how the T-terminator increases *Alu* expression *in vivo* without increasing RNA lifetime.

Table 3.

Time (min)	Experiment 1 Ratio of X/T to X/A	Amount of sc*Alu* RNA	7SL RNA	Experiment 2 Ratio of X/T to X/A	Amount of sc*Alu* RNA	7SL RNA
0	0.65	1	1	0.88	1	1
25	0.61	1.21	1.27	1.06	1.36	1.22
50	0.71	1.74	1.37	0.84	1.02	1.53
75	0.64	1.10	0.80	0.93	2.02	1.41
100	0.56	1.44	1.15	0.75	1.40	1.52
150	0.55	0.93	0.94	0.79	1.65	1.56
Average	0.62 ± 0.05	1.1 ± 0.2	1.1 ± 0.2	0.88 ± 0.08	1.4 ± 0.3	1.4 ± 0.2

In two independent experiments, cells were co-transfected with clone X/T and X/A (Fig. 5B, data not shown) and treated with actinomycin for the indicated period of time. The intensities of *Alu* RNAs, sc*Alu* RNA and 7SL RNA on the resulting Northern blots (Fig. 5B, data not shown) were determined by PhosphorImager analysis. The ratio of intensities is reported for clone X/T and clone X/A transcripts. (In the two experiments, the relative amounts of the two transfected clones was intentionally altered accounting for the two different ratios observed.) The abundance of sc*Alu* RNA and of 7SL RNA is each reported relative to its abundance at zero time. The averages of values and the arithmetic variation are also reported.

The relative advantage of one *Alu* compared to another might accrue at each step in the multi-step retrotranspositional pathway (4). Considering just transcription, the present results support this model, showing that a proper combination of 5′ and 3′ sequence elements markedly increases (~100-fold) *Alu* expression. The accidental juxtaposition of an *Alu* repeat with an element as powerful as the 7SL RNA gene's promoter is probably an unlikely evolutionary event. However, clone X/AT is expressed 5-fold higher than clone X/A although these two constructs differ by only five base substitutions, which are far removed from known promoter elements. Subtle changes in sequence can, as proposed (4), significantly alter *Alu* expression.

Sc*Alu* RNA is regulated independently of *Alu* RNA

The majority of *Alu* transcripts are not subject to any gross polyadenylation or deadenylation following transcription. The mechanism by which the A rich 3′ end is corrected to a perfect poly A tract in recently transposed *Alu*s is unknown. While *Alu* transcripts have a half life of ~30 min, sc*Alu* RNA which binds SRP proteins, is relatively long lived (17,20). Northern blot analysis unambiguously shows that the same transfected templates encode both *Alu* and sc*Alu* RNAs. This observation confirms results in which an *Alu* template microinjected into *Xenopus* oocytes also produced sc*Alu* RNA (17).

Previous results indicate that sc*Alu* RNA arises post-transcriptionally from primary *Alu* transcripts (17). Cell stress, translational inhibition and 5-azacytidine treatment each markedly increase *Alu* RNA expression without affecting the level of sc*Alu* RNA (7,10). Results presented here, using transfected cells, control for these more drastic treatments which cause other changes in cell physiology. The level of sc*Alu* RNA expression is virtually unaffected by a 100-fold increase in the abundance of the primary transcripts (Table 2). This finding, in conjunction with the relative life times of these two transcripts, shows that sc*Alu* RNA is not merely a transient intermediate in an *Alu* RNA degradation pathway, but that its level of expression is tightly regulated by factors other than the abundance of *Alu* transcripts.

Alu RNA turns over by either of at least two pathways, one of which produces sc*Alu* RNA. Differential 3′ processing of *Alu* RNA could determine the relative retrotranspositional success of different *Alu*s and 'the 3′-sequence environment into which they insert could determine the efficiency of processing' (4). Subtle sequence changes near the terminator altered the efficiency with which B1 RNA microinjected into *Xenopus* oocytes is processed into scB1 RNA (21). Similarly, X/T *Alu* transcripts are processed less efficiently than X/A transcripts. *Cis* elements can affect *Alu* transpositional expression and the fate of the resulting transcripts.

Alu RNA is evidently the precursor to sc*Alu* RNA as well as being the intermediate in *Alu* retrotransposition. The question of whether *Alu* RNA *per se* also has a cell function is more problematic. Presumably, sc*Alu* RNA processing occurs in the nucleus, raising the question of why *Alu* RNA appears in the cytoplasm (7). The rapid, dramatic, transient induction of *Alu* expression by cell stress and translational inhibition suggests that this transcript has other specific roles, in addition, to being an intermediate in RNA processing and retrotransposition (10).

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