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Flanking Sequences of an Alu Source Stimulate Transcription In Vitro by Interacting with Sequence-Specific Transcription Factors

Igor Chesnokov,[1] Carl W. Schmid[1,2]

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

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Abstract. An Alu source gene, called the EPL Alu, was previously isolated by a phylogenetic strategy. Sequences flanking the EPL Alu family member stimulate its RNA polymerase III (Pol III) template activity in vitro. One *cis*-acting element maps within a 40-nucleotide region immediately upstream to the EPL Alu. This same region contains an Ap1 site which, when mutated, abolishes the transcriptional stimulation provided by this region. The flanking sequence, as assayed by gel mobility shift, forms sequence-specific complexes with several nuclear factors including Ap1. These results demonstrate that an ancestral Alu source sequence fortuitously acquired positive transcriptional control elements by insertion into the EPL locus, thereby providing biochemical evidence for a model which explains the selective amplification of Alu subfamilies.

Key words: Alu — Transcription factors — Flanking Sequence — Retrotransposition

Introduction

Human Alu repeats are thought to be generated by the retrotransposition of new members through RNA intermediates (Weiner et al. 1986; Schmid et al. 1990). Alu repeats belong to distinct sequence subfamilies each of which appeared at a different evolutionary time (Deininger et al. 1992; Schmid and Maraia 1992). A recognizable subfamily must ultimately result from a single ancestral source sequence, provoking the question of why certain sequences became sources.

Retrotransposition requires many discrete steps, each of which may impose additional demands on retrotranspositional success (Schmid and Maraia 1992). Transcription is a prerequisite to retrotransposition. Almost all Alu repeats are transcribed in vitro by virtue of their internal *Pol*III promoter; however, the corresponding transcripts in HeLa cells are surprisingly sparse given the potential transcriptional activity of nearly 1 million human Alu repeats. The extensive methylation of Alu repeats in certain cell lines and tissues might repress their template activity in vivo. Also, the in vitro template activity of Alu elements varies greatly, with older Alus being inactivated by accumulated mutations (Liu and Schmid 1993; Liu et al. 1992, and references therein). Additionally, the promoter activity of many *Pol*III transcription units is stimulated or even directed by flanking sequences (Bredow et al. 1990; Ullu and Weiner 1985; Murphy et al. 1987; Lobo and Hernandez 1989; Carbon et al. 1987; Pruzan et al. 1992; Baer et al. 1990; Topper and Clayton 1990; Howe and Shu 1989); 7SL RNA genes are homologous to human Alu repeats and therefore provide the most pertinent example of the importance of flanking sequences for the in vivo activity of *Pol*III-directed templates (Bredow et al. 1990; Ullu and Weiner 1985). Included among 7SL RNA genes are many retrotransposed pseudogenes which, like Alu repeats, terminate in A-rich 3′ ends, are flanked by short direct repeats, and are ac-

Correspondence to: C.W. Schmid

tively transcribed in vitro by *Pol*III (Ullu and Weiner 1984, 1985). However, compared to the authentic 7SL RNA gene which is activated by positive *cis*-acting elements provided by its 5′ flanking sequences, these pseudogenes are relatively inactive in vivo (Bredow et al. 1990; Ullu and Weiner 1985). The internal *Pol*III promoter for the 7SL RNA gene is necessary but not sufficient for a high level of expression in vivo. Positive *cis*-acting elements for an Alu source sequence might be acquired by the Alu fortuitously inserting within a favorable sequence context.

Exploiting an extreme speciation bottleneck in the expansion of the PV Alu subfamily, we have identified this subfamily's putative ancestral source, called the EPL Alu locus (Leeflang et al. 1993). EPL Alu is the single recognizable PV subfamily Alu to predate the divergence of the human, chimpanzee, and gorilla lineages. Also, the EPL Alu is present in gibbon, but the gibbon ortholog more closely resembles the sequence of the next older Alu subfamily. These findings indicate that the EPL Alu was originally templated by a source gene for an older subfamily and acquired the sequence substitutions characteristic of the PV subfamily by drift. Shaikh and Deininger show that EPL Alu inserted after the divergence of the ape and monkey lineages (Shaikh and Deininger 1995). According to this interpretation, EPL Alu was the first member and therefore the presumptive founder of the PV subfamily. Sequence comparisons show that the human EPL Alu is not the contemporary source for the newest members of its subfamily (Leeflang et al. 1993; Shaikh and Deininger 1995). Also, this particular Alu is now rather old, predating the divergence of gibbon and hominoids, and the human EPL Alu has a point mutation in its *Pol*III promoter which possibly depresses its template activity (Liu and Schmid 1993; Leeflang et al. 1993). Within these qualifications, the template activity of the EPL Alu was investigated to learn if it has the necessary transcription signals presumed to be required for an Alu source. Because the *Pol*III promoter elements of the gorilla EPL Alu exactly match the consensus sequence for the youngest Alu repeats, this representative of the EPL locus is selected for the present study.

Materials and Methods

Nuclear Extracts and Chromatographic Purifications. Nuclear extracts for *Pol*III transcription were prepared from HeLa cells (Dignam et al. 1983). Nuclei were resuspended in an equal volume of buffer containing 20 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 20% glycerol, and 1 mM PMSF. Concentrated NaCl was added to adjust the final concentration to 0.4 M. After homogenizing and incubation on ice for 30 min, nuclei were pelleted at 15,000*g* and the supernatant was dialyzed against 20 mM HEPES (pH 7.8), containing 0.1 M KCl, 1 mM DTT, 0.1 mM EDTA, and 20% glycerol. Following dialysis, insoluble material was removed by centrifugation at 15,000*g*. Protein concentration was generally about 5–6 mg protein per ml of extract.

Nuclear extracts for electrophoretic mobility shift assays (EMSA) were prepared from HeLa cells by extraction of isolated nuclei with buffer containing 0.4 M NaCl without dialysis. A DEAE-cellulose column (DE52, Whatman) was equilibrated with 20 mM HEPES (pH 7.8) containing 50 mM NaCl, I mM DTT, 0.1 mM EDTA, 5% glycerol. The nuclear extract was diluted with buffer containing no NaCl to yield 50 mM NaCl and loaded onto the column, which was then washed with the equilibration buffer. Steps of 0.3 M NaCl (DE-0.3), 0.5 M NaCl (DE-0.5), and 1.0 M NaCl (DE-1.0) in the equilibration buffer were then used to elute the bound material.

Oligonucleotides. Oligonucleotides used for constructing deletions and other subclones by PCR are listed according to their map position in Fig. 1:

−315 GTGAGGCTTTCATAGGAATT
−118 TGAAATGCATATAAGGCTTG
−85 CTGCCGCACCCTGCATCAAAC
−39 CAGAGG**TGAGTCA**GACTTAT
−39mut CAGAGG**CAGACTG**GACTTAT
1 AAAGCTTGGCCGGGCGCGGTGGCT
2 CCTGTAAAATCATTCTTGC
351 CATCTGCATAGTAGGGTCT

The double stranded −39 and −39mut oligonucleotides were used in DNA-binding experiments.

Construction of Templates and Flanking Probes. Deletion constructions were obtained by PCR using oligonucleotide 351 as the primer from the 3′ end of the EPL Alu in conjunction with oligonucleotides 1 through −315, which were used as primers from the indicated sites within the 5′ flanking sequence. The resulting PCR products were subcloned into the vector pCR II using a TA cloning kit (Invitrogen). The −315mut construction with the mutated Ap1 site was specifically mutated by sequential PCR steps (Ausubel et al. 1994). The authenticity of the insertions was checked by sequencing; all deletion templates used here have the same orientation. Closed circular plasmid templates were purified by the alkaline lysis method and CsCl ethidium bromide centrifugation (Maniatis et al. 1989).

For use as a DNA probe in binding experiments, the entire 315-nt flanking sequence was subcloned as a PCR product using oligonucleotides −315 and 2 as primers. This fragment was subcloned into pCR 1000 (Invitrogen). Exploiting a unique *NsiI* site at position −110, proximal (−110 to 2) flanking fragment was released by digestion with *NsiI-Hin*dIII.

*Transcription Reactions. Pol*III transcription reactions were carried out for 1 h at 37 C in 50 µl containing 50–100 µg of protein, 10 mM HEPES (pH 7.8), 10% glycerol, 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.6 mM ATP, GTP, and UTP, 0.03 mM CTP, 5 µC α-P^{32} CTP, and 100–500 ng of template DNA. In all experiments reported here, 200 ng of template is employed but similar results were obtained at both higher, 500 ng, and lower 100 ng, amounts of template. Reactions were stopped by the addition of 50 µl 0.5 M NaCl and 1% SDS, extracted with phenol-chloroform, chloroform and precipitated with ethanol. The products were resuspended in formamide and analyzed by electrophoresis in 6% polyacrylamide denaturing gels which were then autoradiographied. A Fuji BAS 1000 Bio-Imaging Analyzer was used to measure radioisotope incorporation into RNA transcripts.

DNA-Binding Experiments. Binding reactions were performed in 20 mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.1 mM EDTA in the presence of indicated amounts of lambda DNA which served as a nonspecific competitor. Nuclear extracts or the DE-0.3 fraction were used in DNA-binding experiments. Recombinant AP1 (Promega) was also employed to test the specificity of the binding conditions. Labeled DNA probes, 2–5 ng, were incubated with 2–5 µg of protein at room temperature for 30 min and then loaded on 5% polyacrylamide gel (run in TAE for 2 h at 10 V/cm). Competition

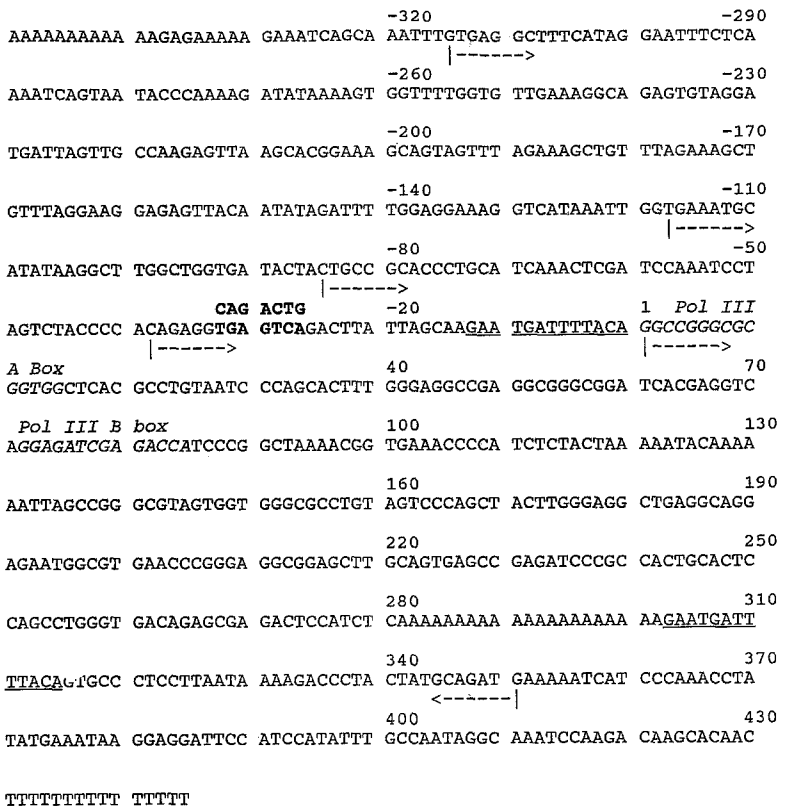

Fig. 1. Base sequence of the EPL locus (L). The direct repeats surrounding the Alu repeat, which begins at position 1, are *underlined. Arrows* indicate the end points of the PCR-generated deletions examined below. The Ap1 site at position −33 is indicated by *bold type* and the sequence of the transition mutant of the Ap1 site is written above its sequence.

experiments were performed by the addition of indicated amounts of unlabeled specific oligonucleotide.

Results

Structure of the EPL Alu Flanking Sequences

Like almost all members of the PV Alu subfamily, the EPL Alu contains consensus A and B boxes for the *Pol*III bipartite promoter (Paolella et al. 1983; Fig. 1). The four T's at position 312 are expected to provide a leaky, endogenous terminator for *Pol*III transcription (Platt 1986; Fig. 1). A second run of four T's positioned 120 nt further downstream is supplied by the vector. Additionally, sequences flanking the EPL Alu include several possible transcriptional control elements for *Pol*II. A perfect consensus binding site for AP1 is located at position −33 (Fig. 1). Imperfect matches to other transcriptional control sites, such as an OCT-1 site at −61, an NF1 site at −230, and a C/EBP site at −252, can also be recognized in this flanking region (Mitchell and Tjian 1989).

Transcriptional activating sequences can be far from the promoter on which they act (Mitchell and Tjian 1989). However, the presence of other Alu repeats almost immediately preceding (position −340) and following (position 430) the EPL Alu constrains the present investigation to sequences immediately flanking this locus (Fig. 1).

Flanking Sequences Stimulate In Vitro Pol*III Transcription of EPL Alu*

PCR was used to construct a progressive series of deletion templates to decide which, if any, 5′ flanking sequences stimulate transcription of EPL Alu (Fig. 1). These constructs are named according to the positions of their 5′ start sites. Each construct yields two principal transcription products having approximate lengths of 320 and 420 nucleotides, in agreement with the predicted lengths of 310 and 430 nucleotides for termination within the flanking sequence and downstream plasmid sequence (Fig. 2; Materials and Methods). Transcription of these products is also resistant to low concentrations of α-amanitin, providing further evidence that transcription is directed by *Pol*III.

The 420-nucleotide transcript from the −315 construct is 10.3-fold more abundant than the corresponding product from the +1 construct, which is totally devoid of 5′ flanking sequences (Fig. 2). The level of stimulation is estimated to be slightly less (7.2-fold) for the less abundant 320-nucleotide transcripts for which the background correction is more significant. For this reason, we restrict our analysis to the longer transcription product with the understanding that qualitatively similar results are obtained for the less abundant shorter transcript (Fig. 2). The other templates (−118, −85, and −39 constructs) are approximately equally active and each is transcribed about 2.0-fold more than the +1 construct (Fig. 2). Evidently there are distal *cis*-acting elements located be-

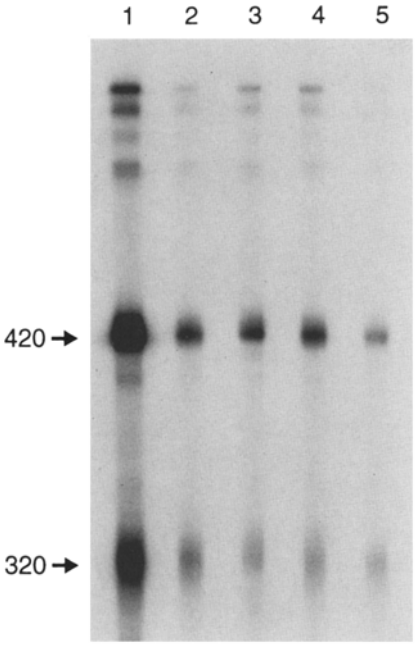

Fig. 2. Sequences upstream from EPL Alu stimulate its in vitro transcription. The templates used for transcription are −315 (*lane 1*), −118 (*lane 2*), −85 (*lane 3*), −39 (*lane 4*), and +1 (*lane 5*). The incorporation of radiolabeled substrate in the major transcriptional product corrected for background was determined in phosphorescence units (pu) by Bioimage analysis to be 29,300 pu (*lane 1*), 6,500 pu (*lane 2*), 5,700 pu (*lane 3*), 5,600 pu (*lane 4*), and 2,800 pu (*lane 5*).

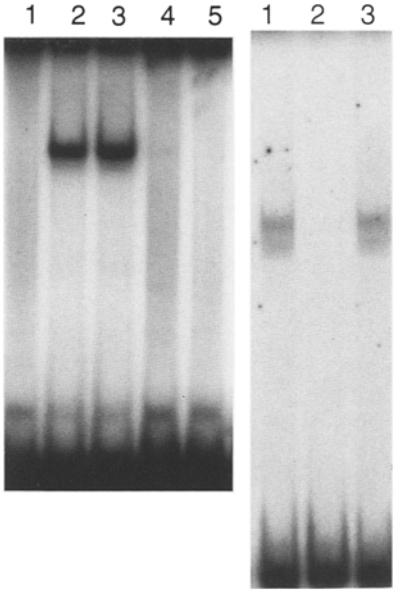

Fig. 3. Electrophoretic mobility shift experiments. **A** Double-stranded −39 (*lanes 1–3*) and −39mut (*lanes 4, 5*) oligonucleotides were used as probes; 3 μg of protein from the DE-0.3 fraction were added in all lanes except for *lane 1;* 0.5 μg (*lanes 2, 4*) or 1 μg (*lanes 3, 5*) of lambda DNA was added. **B** Competition experiment; 50 ng of unlabeled double-stranded −39 (*lane 2*) and −39mut (*lane 3*) oligonucleotides were added to the labeled *Nsi*I-*Hin*dIII fragment along with 3 μg of protein from the DE-0.3 fraction; 0.5 μg of lambda DNA was added in all lanes.

tween positions −315 and −118 and proximal *cis*-acting elements located between positions −39 and −1. The physical separation of these *cis*-acting elements suggests that this region probably interacts with several different transcription factors. Also the degree of transcriptional stimulation provided by the flanking sequences is somewhat variable with the −315 construct being transcribed as much as 30-fold more than the +1 construct (data not shown). This variability may be attributable to the complexity of assembling the different factors that bind to the 5′ flanking sequence as well as the difficulty of making precise background corrections. The level of stimulation is independent of a threefold change in either template or extract concentration (data not shown).

The Ap1 Site Is Essential for In Vitro Transcription of the Alu Source

A perfect Ap1 site is located at position −33 of the flanking sequence. To examine whether this site binds with a protein we performed a number of electrophoretic mobility shift assays (EMSA) using both oligonucleotides containing this site and a fragment of the flanking sequence overlapping this region. A double-stranded −39 oligonucleotide forms a specific complex with a protein as detected by EMSA (Fig. 3A) in the presence of increasing amounts of nonspecific lambda DNA. The double-stranded −39mut oligonucleotide does not form any noticeable complex under the same conditions. In the reverse experiment, we used a *Nsi*I-*Hin*dIII fragment overlapping the region from −110 to 2 of the 5′-flanking sequence. As detected by EMSA this fragment forms a specific DNA-protein complex (Fig. 3B), which can be disrupted upon addition of "cold" double-stranded −39 oligonucleotide, but not −39mut oligonucleotide. To test the whether Ap1 site is important in transcriptional reactions we used constructions in which the Ap1 site is abolished, −39mut and −315mut. The −39 construct is transcribed twofold more abundantly than the +1 construct (Fig. 2). While a twofold effect is difficult to quantitate accurately, the level of stimulation provided by the −39 flanking sequence is qualitatively reproducible. As examples, the −39 constructs are transcribed 1.9-fold and 1.7-fold more abundantly than the +1 constructs in replica experiments using independent preparations of extracts (Fig. 4A). Specific mutation of the Ap1 site, TGAGTCA, to CAGACTG, essentially abolishes the transcriptional stimulation provided by the −39 flanking sequence (Fig. 4A). The transcriptional activity of the −39 construct is essentially indistinguishable from that of the −118 and −85 constructs so this Ap1 site is evidently responsible for most of the transcriptional stimulation provided by the proximal *cis*-acting elements. The same mutation within the full flank significantly decreases the

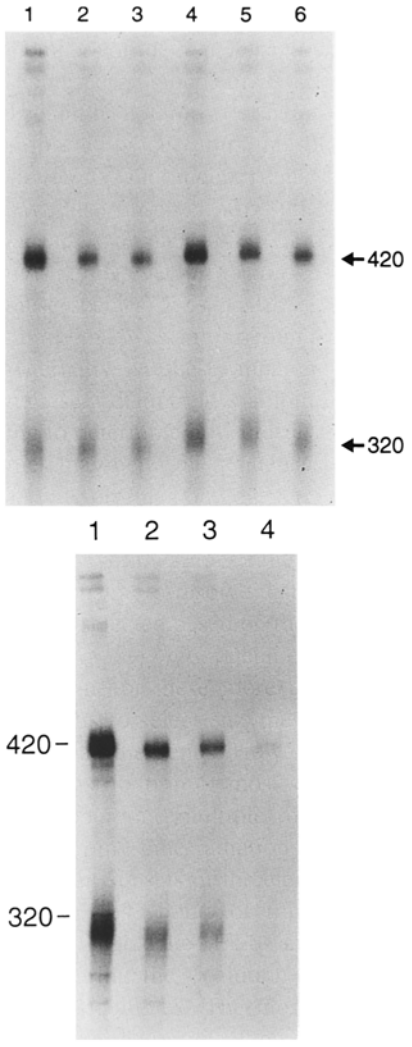

Fig. 4. The AP1 site stimulates transcription. **A** The template activities of the –39 (*lanes 1* and *4*), –39mut (*lanes 2* and *5*), and +1 (*lanes 3* and *6*) constructs are compared. The incorporation of radiolabeled substrate in the major product corrected for background is 5,300 and 5,600 pu (*lanes 1* and *4*), 3,150 and 3,700 pu (*lanes 2* and *5*), and 2,800 and 3,250 pu (*lanes 3* and *6*). Different preparations of nuclear extract were used for transcription in these two sets of experiments. **B** The template activity of the –315 (*lane 1*), –315mut (*lane 2*), –40 (*lane 3*), and +1 (*lane 4*) constructs are compared.

efficiency of Alu transcription (Fig. 4B). In a number of experiments, the –315mut construct was transcribed approximately five times less efficiently than –315 construct. From these observations we are confident that Ap1 activity present in the extracts used here is responsible for the formation of the DNA-protein complexes observed in EMSA. Also the Ap1 site is important for transcriptional stimulation provided by the flanking sequence.

We attempted to assay the effect of the EPL flanking sequence in vivo by transient transfection into HeLa cells. We find expression of transfected Alu constructs is effectively blocked in HeLa cells and are unable to test the biological significance of the flanking sequences using this system (data not shown). Since the in vivo regulated expression of Alu repeats is poorly understood, we are presently unable to design incisive experiments to test the role of these flanking sequences in vivo.

Discussion

*Pol*III template activity is determined by many traditional *Pol*II factors such as TBP, Octl, Sp1, and, of special significance in this discussion, ATF (Bredow et al. 1990; Murphy et al. 1987; Lobo and Hernandez 1989; Carbon et al. 1987; Pruzan et al. 1992; Baer et al. 1990; Topper and Clayton 1990; Howe and Shu 1989; Rigby 1993). Here we find that an Ap1 site stimulates *Pol*III transcription in vitro of an Alu repeat which has a conventional, internal split *Pol*III promoter (Paoletta et al. 1983; Geiduschek and Tocchini-Valentini 1988). Ap1 joins the increasingly long list of transcription factors shared by *Pol*II and *Pol*III. While Ap1 and ATF are distinct activities, they are both leucine zipper proteins which have very similar DNA recognition sites (Mitchell and Tjian 1989). Thus there is already ample precedent to suspect that Ap1 might stimulate *Pol*III transcription in vitro. Furthermore, as discussed below, ATF has a special role in activating the transcription of an Alu homolog, the 7SL RNA gene (Bredow et al. 1990; Ullu and Weiner 1985).

Despite the *Pol*III template activity of Alu repeats in vitro, their in vivo transcription products are not abundant (Matera et al. 1990; Sinnet et al. 1992; Maraia et al. 1993). By analogy to the example of 7SL RNA pseudogenes (Introduction), we imagine that among other contributions, the relative transcriptional silence of Alu members is caused by the absence of flanking *cis*-acting elements necessary to stimulate their transcription. Because 7SL RNA and Alu repeats are closely related in sequence and have intragenic promoters that are active in vitro, we regard the 7SL RNA gene as one of the best possible models for the expression of Alu repeats. The A box elements are identical in the Alu and 7SL RNA gene promoters, although the B box elements differ slightly. The authentic 7SL RNA gene includes an ATF site at position –45 which happens to be similar in structure and position to the Ap1 site located at –33 with respect to the EPL. Alu (Bredow et al. 1990). Deletion of this region from the 7SL RNA gene reduces its in vitro template activity by about 50% (Bredow et al. 1990; Ullu and Weiner 1985), which is similar to the twofold effect the AP1 site has on the in vitro transcription of the EPL Alu. The juxtaposition of an ATF/Ap1 site with a type 2-*Pol*III promoter element is not a peculiarity of the 7SL RNA gene and the EPL Alu. The Epstein-Barr EBER gene has functional, internal type 2-*Pol*III promoter A

and B boxes and also has an ATF site at position −46 which stimulates its transcription both in vitro and in vivo (Howe and Shu 1989). Additional, unidentified *cis*-acting elements further upstream are also necessary for in vivo expression of the 7SL RNA gene (Ullu and Weiner 1985). Interestingly, transcription of the EPL Alu is further stimulated by unidentified distal (−315 to −118) *cis*-acting elements so that the structure of this flanking sequence resembles the structure of the 7SL RNA and EBER genes.

We were unable to demonstrate the expression of the EPL Alu by transfection into HeLa cells. Our experience and that of other laboratories is that Alu expression is tightly and specifically repressed in HeLa cells, probably by multiple levels of control (Schmid and Maraia 1992). As just one mechanism by which Alu expression is repressed, the start site of an Alu element positions nucleosome assembly in vitro (Englander et al. 1993). This nucleosome-positioning signal could contribute to the transcriptional silencing of Alu repeats in vivo. A critical issue remaining to be investigated is the activity of EPL Alu and other Alus in germ-line cells in which retroposition occurs. HeLa cells may provide a poor model for germ-line expression, so the relative inactivity of EPL Alu in HeLa cells may not be significant.

The existence of sequence subfamilies shows that some Alus have been more successful source genes than others (Deininger et al. 1992; Schmid and Maraia 1992). The relative transpositional activity of an Alu repeat possibly depends on a number of factors including its transcriptional activity (Schmid and Maraia 1992). Regarding the origin of the PV subfamily source gene, the EPL Alu repeat acquired the PV subfamily diagnostic base substitutions by simple sequence drift (Leeflang et al. 1993; Shaikh and Deininger 1995; Introduction). Presumably, other Alu repeats were simultaneously drifting from their original base sequence but they did not successfully generate recognizable subfamilies. *Cis*-acting elements stimulate the in vitro transcription of EPL Alu and the action of these same elements in vivo could provide this locus with a selective transcriptional and subsequent transpositional advantage compared to other Alu members. Phylogenetic comparisons (monkey, gibbon, orangutan, gorilla, chimpanzee, and human) show that the EPL Alu acquired its *cis*-acting elements, including especially the Ap1, site from the sequences which flank its insertion site. The insertion of an Alu into the EPL locus fortuitously provided this Alu source with *cis*-acting elements and an overall structure resembling the functional 7SL RNA and EBER genes. While the effect of these *cis*-acting elements on the expression of this Alu in vivo have not yet been verified, the 7SL RNA gene's requirement of an ATF site for its in vivo expression suggests that these sequences would play a similar role in the expression of an Alu repeat.

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