Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11533–11537, December 1993
Biochemistry

Translation of the human LINE-1 element, L1Hs

(repeated DNA/retrotransposon/bicistronic/gene expression)

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ABSTRACT **Full-length RNA transcribed from the human LINE-1 (L1) element L1 *Homo sapiens* (L1Hs) has a 900-nt, G+C-rich, 5′-untranslated region (UTR). The 5′ UTR is followed by two long open reading frames, ORF1 and ORF2, which are separated from each other by an inter-ORF region of 33 nt that includes two or three in-frame stop codons. We examine here the mechanism(s) by which the translation of L1Hs ORF1 and ORF2 is initiated. A stable hairpin structure (ΔG = −74.8 kcal/mol), inserted at nt 661 of the 5′ UTR, caused a 3- to 8-fold decrease in the *in vitro* and *in vivo* translation of either a *lacZ* reporter gene for ORF1 or the ORF1 polypeptide product, p40, but translation of a *lacZ* reporter gene in ORF2 was increased. The results are compatible with a model for ORF1 translation initiation in which the majority of ribosomes scan from a point 5′ of nt 661 but suggest that ORF2 is not translated by attached ribosomes that reinitiate after the termination of ORF1 translation. Our data are compatible with a model whereby the translation of L1Hs ORF2 is initiated internally.**

The human LINE-1 (L1) element (L1 *Homo sapiens*; L1Hs) is the only known transposable element that is endogenous to the human genome. The structural features of L1Hs (schematically presented in Fig. 1) have long suggested that it is a class II (non-long terminal repeat) retrotransposon that uses element-encoded proteins to reverse transcribe its own mRNA and integrates cDNA copies into new genomic locations (1, 2). At least one of the ≈3500 full-length (6-kb) elements (3), an allele at the *LRE1* locus on chromosome 22, appears to be actively transposing (4, 5). Additional active L1Hs elements are likely to exist because there are other examples of gene interruption caused by *de novo* insertions of different L1Hs sequences (6). The mobility of L1Hs elements may therefore have considerable impact on the genome and is, in at least some cases, of clinical importance. However, we still know little about the factors that influence L1Hs expression.

The first 600 bp of the 900-bp-long 5′-untranslated region (UTR) of full-length elements contains a complex array of transcription regulatory elements that are sufficient for efficient cell-specific transcription (7). Full-length L1Hs transcripts start upstream of the regulatory region at residue 1 and so far have been detected only in multipotent teratocarcinoma cells and the choriocarcinoma cell line, JEG3 (8–10).

The translation of L1Hs RNA has not been studied in detail. Full-length, cytoplasmic L1Hs mRNA has a number of features that are likely to affect the efficiency with which either or both of the two long open reading frames, ORF1 and ORF2, are translated (Fig. 1). First, there is the very long, G+C-rich 5′ UTR, which, according to computer analysis, has the potential to form stable secondary structures (G. Swergold and J.P.M., unpublished observations). Second, each of the full-length active elements and cDNA clones so far examined has at least one AUG in the 5′ UTR upstream of the initiation codon of ORF1. For example, the AUG codon at nt 16 is in a good context for translation initiation (11), is highly conserved, and begins a short reading frame of three codons. Some genomic elements have a second AUG at nt 607, in suboptimal context, followed by a short ORF of 20 codons. These features of the 5′ UTR are expected to impede the initiation of translation in ORF1 were it consequent to 40S ribosomal subunits scanning from a 5′-methylated cap according to the model of Kozak (11). We know, however, that ORF1 is translated since its product, p40, can be readily detected, using specific antiserum, in teratocarcinoma and choriocarcinoma cell lines (8, 12) and in certain tumors and tumor cell lines of epithelial origin (13, 14). Moreover, translation of ORF1 is known to initiate at the first AUG in the frame (8, 12).

While ORF1 and ORF2 are in the same frame, they are separated by an inter-ORF region, which contains at least two in-frame stop codons in all L1Hs sequences so far examined (see ref. 15, for example). Two alleles at the *LRE1* locus each contain a third in-frame stop codon in the inter-ORF region. ORF2 is open for 24 nt before the first AUG, which is in a suboptimal context (AUAAUGA) for translation initiation (11). To date no ORF2-specific polypeptides have been identified in human cells.

In this paper, we address questions related to the translation of both reading frames of L1Hs. We asked whether the translation of ORF1 could be described by the scanning ribosome model. We inserted a very stable hairpin structure into a region of the 5′ UTR known to be unnecessary for full activity of the internal transcriptional promoter (7) and examined the effect on translation of both a reporter gene for ORF1 and the p40 product itself. We concomitantly examined the effect of the same hairpin insertion on the translation of a reporter gene for ORF2.

MATERIALS AND METHODS

Plasmid Constructs. A diagrammatic representation of the plasmids used for the hairpin constructs is given in Fig. 1. L1Hs sequences are numbered according to those of the L1.2 sequence [GenBank accession number M80343 (4)]. Plasmids p1LZ and p3LZ (7, 12) and pL1.1 and pL1.2A (4, 8, 16) have been described. The 5′ UTR of some of these plasmids was modified to introduce a self-complementary segment that would form stem–loop structures in the mRNAs. A duplex 41-bp oligonucleotide, duplex a, with one cohesive end for *Bam*HI and another for *Hin*dIII was prepared by annealing the following single-stranded, 5′ phosphorylated oligonucleotides: 5′-GATCCTACACTTAGCACGGCGGGAACGT-GTGACTGATTCGA-3′ and 5′-AGCTTCGAATCAGTCA-CACGTTCCCGCCGTGCTAAGTGTAG-3′. This duplex

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Abbreviations: L1Hs, LINE-1 (L1) element from *Homo sapiens*; ORF, open reading frame; UTR, untranslated region; β-gal, β-galactosidase.

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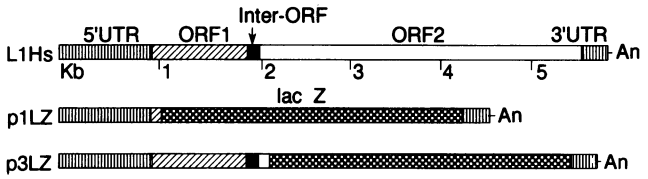

FIG. 1. Schematic representation of the L1Hs and plasmids p1LZ and p3LZ, indicating the 5′ and 3′ UTRs (vertical lines), ORF1 (diagonal lines), inter-ORF region (solid), ORF2 (open), *lacZ* (plaid), and poly(A) tail (An).

was self-ligated and cut with *Bam*HI to produce an 82-bp oligonucleotide consisting of a tandem inverted repeat of the 41-bp oligonucleotide, which can form a stable stem–loop structure [$\Delta G = -74.8$ kcal/mol; calculated by the method of M. Zuker (17) using the FOLD program of the Genetics Computer Group (Madison, WI) package]. This was inserted into the *Bgl* II site at residue 661 in the 5′ UTR of plasmids p1LZ and p3LZ to yield p1LZloop and p3LZloop1, respectively. p3LZloop2 was similarly constructed but using a duplex oligonucleotide, duplex b, which has the same base composition as duplex a and the *Bam*HI and *Hin*dIII cohesive ends but a different sequence (sequence from the multiple cloning site of the pRSET A vector; Invitrogen). The control plasmids p1LZunloop and p3LZunloop were similarly constructed using the duplex oligonucleotides a and b ligated together to yield an 82-bp oligomer with potential to form relatively unstable secondary structure ($\Delta G = -13.4$ kcal/mol).

Plasmid pL1.1Z was constructed by substituting the L1 sequences of p3LZ, from the *Bgl* II site in the 5′ UTR to the *Age* I site at residue 1889 of ORF 1, with the corresponding sequences of pL1.1; pL1.1Z is therefore almost identical to p3LZ except that it is unable to direct the synthesis of p40 from its ORF1 sequence.

Plasmid pΔORF1 is a derivative of pL1.2A from which the entire ORF1 and inter-ORF regions were precisely deleted such that the first methionine codon of ORF2 is juxtaposed to the 3′ extremity of the 5′ UTR. It was constructed by exploiting PCR to splice sequences by overlap extension (18). A fragment extending from 500 to 910 in the 5′ UTR joined to the sequence 1991–2281 in ORF2 was produced by using two flanking primers (L1.2A residue numbers: 5′-500–519 and 5′-2281–2262) and two complementary primers to provide a joint between sequences 895–910 of the 5′ UTR and sequences 1991–2206 of ORF2. This amplification product was digested with *Bgl* II and used to replace the smaller *Bgl* II fragment (nt 661–2173) of pL1.2A.

pT7-LZ was constructed by inserting the *Bam*HI–*Hin*dIII fragment of pCH110 (Pharmacia) into *Bam*HI/*Hin*dIII-digested pSK$^-$ (Stratagene) such that the entire *lacZ* gene (together with the 40 codons of *gpt* and the 28 codons of *trpS* present in pCH110) is just downstream of the T7 promoter.

The relevant sequences of all clones were confirmed using the dideoxynucleotide method (19).

Cell Lines and Transfection. NTera2D1 cells (20), obtained from P. W. Andrews (MRC, Sheffield, England) were cultured and transfected as described (7, 10, 21).

β-Galactosidase (β-gal) Assays and Histochemical Stain. A sensitive chemiluminescent assay for β-gal (Galactolight; Tropix, Bedford, MA) was performed on extracts of transfected cells that were prepared according to the kit's manufacturer. All assays were performed on 1, 0.1, and 0.01 μl (as a decimal dilution series) of extract, and the activity curve for the two higher volumes had the expected slope. Chemiluminescent emission was measured for 10 sec using a Monolight 2001 luminometer (Analytical Luminescence Laboratory, San Diego). The relative numbers of cells expressing β-gal from ORF2 after transfection with the appropriate plasmids were counted using light microscopic examination of cells fixed *in situ* and stained with 5-bromo-4-chloro-1-indolyl-β-D-galactopyranoside (22).

RNA Extraction and Northern Blotting. Cytoplasmic RNA was prepared from transfected cells using the guanidinium isothiocyanate method (23). Northern blots were performed according to ref. 24 using 20 μg of total cellular RNA per sample. After transfer, RNA was fixed to the Nytran membrane (Schleicher & Schuell) by exposure to UV light (254 nm) for 5 min. The relative amounts of RNA from each sample were estimated by comparing the intensities of the ethidium bromide-stained rRNAs transferred to the membrane. The 539-bp *lacZ* hybridization probe was amplified by PCR and labeled as described (7). Membranes were treated for 2 hr at 50°C in 7% SDS/1 mM EDTA/0.263 M Na_2HPO_4/1% (wt/vol) bovine serum albumin. Labeled probe was then added (1×10^6 cpm/ml in the above solution), and hybridization was performed for 20 hr at 50°C. Membranes were sequentially washed at 50°C for 15 min once in 2× standard saline citrate (SSC)/0.1% SDS, once in 0.5× SSC/0.1% SDS, and twice in 0.1× SSC/0.1% SDS.

Other Methods. Protein electrophoresis, Western blotting, and *in vitro* transcription and translation were performed as described (8, 25).

RESULTS

Translation of L1Hs ORF1. The scanning ribosome model predicts that translation initiation will be profoundly inhibited by the presence of stable secondary structure in the 5′ UTR of an mRNA, and such inhibition has been observed (26). We asked whether the translation of the L1Hs ORF1 would be similarly inhibited. In these studies we used two previously described plasmids. p1LZ has a *lacZ* reporter gene inserted, in frame, after 15 codons of ORF1. p3LZ contains an intact L1Hs ORF1 encoding the product p40 and *lacZ* fused, in frame, after the first 15 codons of ORF2 (Fig. 1; refs. 7 and 8). Into these plasmids, we inserted a stable hairpin of 82 bp ($\Delta G = -74.8$ kcal/mol) after nt 661 of the 5′ UTR to yield plasmids p1LZloop and p3LZloop, respectively. For controls we constructed plasmids p1LZunloop and p3LZunloop by inserting a relatively unstructured oligomer of the same length ($\Delta G = -13.4$ kcal/mol) into the same site. Each of the plasmids was used to transfect NTera2D1 cells, which are known to be permissive for L1Hs transcription (7) and ORF1 translation (7, 8). Table 1 shows the results of chemiluminescent β-gal assays performed on extracts of cells transiently transfected with the p1LZ set of constructs. There was considerable variation between the results of the different sets of transfections, but the amount of β-gal produced in cells transfected with p1LZloop was always less (3- to 8-fold) than the amount produced in cells transfected with p1LZ or p1LZunloop.

Table 1. Relative expression of β-gal from L1Hs ORF1 in transfected NTera2D1 cells

Plasmid	β-gal activity		
	Exp. 1	Exp. 2	Exp. 3
p1LZ	100	100	100
p1LZloop	30	20	14
pLZunloop	60	140	100
Untransfected	5	<5	<5
Vector only	ND	ND	<5

The results were normalized to the activity of p1LZ-transfected cells, which were considered 100. Assays were performed on 1, 0.1 and 0.01 μl of whole-cell extract; the 0.1 μl results are presented here. ND, not determined.

To confirm that the results were due to an effect on translation rather than transcription, the amounts of plasmid-specific mRNA were examined on Northern blots hybridized with a *lacZ*-specific probe. The expected size of the full-length 5′ UTR–β-gal transcripts is 4.4 kb and, as shown in Fig. 2, lanes 1–3, hybridizing RNA of the expected size is present in cells transfected with any of the three plasmids. No decrease in the amount of plasmid-specific RNA in the cells transfected with p1LZloop was apparent (lane 2). We can conclude therefore that the decreased amount of β-gal produced by cells transfected with the stem–loop construct is a consequence of decreased translation and not due to an effect of the insertion on transcription or RNA degradation.

To estimate the effect of the insertion on the formation of the ORF1 p40 product, extracts made from cells transfected with the p3LZ set of constructs were examined by Western blotting using the p40-specific antibody AH40.1 (8, 14). The p40 synthesized from the transfecting plasmids is known to have a slower mobility than endogenous p40 in SDS/polyacrylamide gels (8, 12), so the two products can readily be distinguished. In these experiments, therefore, the amount of endogenous p40 serves as a convenient control for the amount of extract loaded in each lane. As shown in Fig. 3, cells transfected with the p3LZloop (lane 3) construct produced appreciably less p40 than cells transfected with p3LZ (lane 2) or p3LZunloop (lane 4).

Thus, translation of ORF1 in NTera2D1 cells, be it a reporter gene or p40 sequence, was inhibited by the presence of a stable hairpin structure at nt 661 of the 5′ UTR. These results suggest that most of the translation of the L1Hs ORF1 initiates after the loading of 40S ribosomal subunits at some point 5′ of the site of the hairpin insertion at nt 661, followed by scanning.

Translation of ORF2. We next wanted to determine the effect of the stable hairpin on ORF2 translation in NTera2D1 cells transfected with these plasmids. We have already shown that considerably less p40 was synthesized after transfection

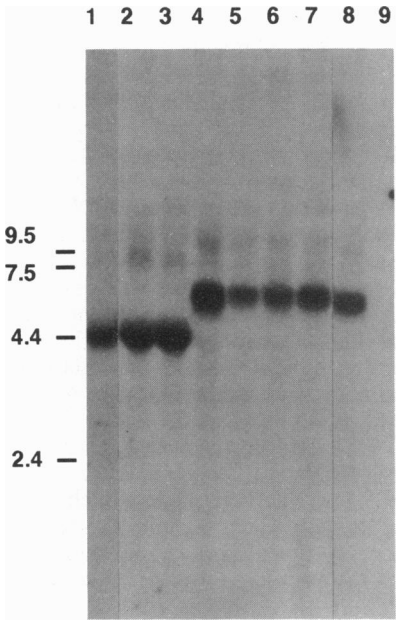

FIG. 2. Detection of RNA synthesized in transfected NTera2D1 cells. Approximately 20 μg of total cellular RNA was loaded in each lane. After electrophoresis and blotting, the membrane was probed with a 576-bp fragment of the *lacZ* gene. RNA was from cells transfected with p1LZ (lane 1); p1LZloop (lane 2), p1LZunloop (lane 3), p3LZ (lane 4), p3LZloop1 (lane 5), p3LZloop2 (lane 6), p3LZunloop (lane 7), pL1.1Z (lane 8), and nothing (untransfected; lane 9). Location and sizes (in kb) of markers are indicated on the left.

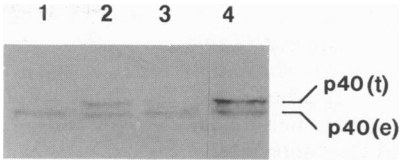

FIG. 3. The effect of a stable hairpin in the 5′ UTR of L1Hs on the translation of the ORF1 polypeptide, p40, *in vivo*. Aliquots (20 μg of total protein in each lane) of whole-cell extracts made from transfected NTera2aD1 cells were separated on 8.5% Laemmli gels, which were then blotted and immunostained with the p40-specific antibody AH40.1. Lane 1, untransfected control. In lanes 2–4, cells transfected with p3LZ (lane 2), p3LZloop2 (lane 3), and p3LZunloop (lane 4). The location of endogenous p40 polypeptide [p40(e)] and p40 encoded by RNA of the transfecting plasmid [p40(t)] are indicated on the right.

with the plasmid p3LZloop than after transfection with p3LZ (Fig. 3). Extracts made from cells transfected with these plasmids were first assayed for β-gal activity using the chemiluminescent method previously described. However, we were unable to obtain reliably quantitative data even when using 100 times more extract than was used in the assays of cells transfected with the p1LZ plasmid. This observation is consistent with our unpublished experiments indicating that ORF2 translation products are not detectable in NTera2D1 cells by methods that readily detect p40. Therefore, we estimated β-gal activity by fixing and staining transfected cells *in situ* with a histochemical stain for the enzyme and counting the number of stained cells. No β-gal-containing cells were observed in untransfected cells or after transfection with empty vector. Small numbers of cells expressing β-gal were detected after transfection with p3LZ, indicating that ORF2 is translated in NTera2D1 cells but at least 100 times less efficiently than ORF1 or perhaps in 100 times fewer cells. The results of five separate transfection experiments with p3LZ, p3LZloop1, p3LZloop2, and p3LZunloop are shown in Table 2. There was considerable variation between experiments, but the number of β-gal$^+$ cells was at least 2-fold greater in cells transfected with the loop constructs than with p3LZ or p3LZunloop (except for p3LZloop2 in experiment 5). The set of results for each plasmid was compared to those obtained with p3LZ and analyzed by the Wilcoxon rank method (27) to determine whether the sets were significantly different. At a $P\{W\}$ value of <0.05, the sets of data are unlikely to be equivalent. As shown in Table 2, the data for the loop constructs, but not p3LZunloop, are significantly different from those for p3LZ.

We then wanted to determine the effect of the total absence of ORF1 translation on ORF2 translation *in vivo*. We constructed the plasmid pL1.1Z by substituting the ORF1 sequence of p3LZ with that from pL1.1. p40 cannot be translated from pL1.1 or pL1.1Z because of a single-base-pair deletion, which results in a frame shift and termination 40 codons from the first AUG (8). Transfections with pL1.1Z

Table 2. ORF2 expression in transfected NTera2D1 cells

Plasmid	Number of β-gal$^+$ cells per 100 mm^2* Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Wilcoxon rank† W	$P\{W\}$
p3LZ	23	57	107	93	257		
p3LZloop1	179	417	220	283	546	38	0.016
p3LZloop2	388	204	228	199	293	37	0.028
p3LZunloop	79	77	55	33	47	22	0.155
pL1.1Z	15	22	30	19	5	39	0.008

*No β-gal$^+$ cells were observed in the untransfected controls or in cells transfected with empty vector.
†Calculations and abbreviations are according to ref. 27.

resulted in significantly fewer numbers of β-gal$^+$ cells than transfections with p3LZ (Table 2).

Thus, our results show that although the translation of ORF2 is enhanced when the translation of p40 is decreased in NTera2D1 cells, the absence of ORF1 translation leads to a decrease in the expression of ORF2.

We investigated the possibility that the data in Table 2 resulted from differences in the level of transcripts of the various plasmids or from alternative RNA processing. Northern blots of total cellular RNA were hybridized to a *lacZ*-specific probe. Extracts from cells transfected with each of the constructs contained approximately equal amounts of a 5.4-kb hybridizing RNA (Fig. 2, lanes 4–8), the expected size of the full-length transcript. There was no evidence of spliced RNA products.

***In Vitro* Translation.** Although *in vitro* translation experiments can give results inconsistent with data obtained from intracellular translation (11), we did carry out experiments on the translation of *in vitro*-synthesized RNA in reticulocyte and wheat germ extracts (data not shown). The insertion of the stable hairpin caused the translation of either the full-length β-gal polypeptide from p1LZloop or p40 from p3LZloop to be decreased ≈5-fold compared to the RNA from plasmids with no hairpin. No such decrease was observed with the control construct p1LZunloop. Thus, in the reticulocyte lysate system, as in NTera2D1 cells, the hairpin inhibits ORF1 translation.

We note here that appreciably more β-gal was translated from pT7-LZ RNA (which has no 5′ UTR sequences upstream of *lacZ*) than from p1LZ RNA. This indicates that the L1Hs 5′ UTR itself inhibits the translation of a downstream open reading frame; this could be due to the presence of secondary structure or the short ORFs (or both).

Analysis of ORF2 translation *in vitro* again confirmed the results with NTera2D1 cells. Thus, although there was considerably less p40 translated from p3LZloop than from p3LZ, the amount of full length β-gal produced from each was similar. If ORF2 translation were dependent on, and consequent to, ORF1 translation (for example, following reinitiation by attached ribosomes), we would have expected less β-gal translation from p3LZloop than from p3LZ. Further, we note that there was no evidence of an ORF1–β-gal fusion polypeptide.

In vitro, deletion of ORF1 had no apparent effect on ORF2 translation as observed in a comparison of the translation products of RNA from pL1.2A and pΔORF1, a derivative of pL1.2A from which the ORF1 sequences have been precisely deleted. This is again consistent with independent initiation of ORF2 translation.

DISCUSSION

Previous results (8, 12), as well as those in this report, show that ORF1 is translatable both *in vitro* and *in vivo* despite the impediments to translation presented by the L1Hs 5′ UTR. Nevertheless, the L1Hs 5′ UTR has a deleterious effect on translation *in vitro* when placed immediately upstream of a *lacZ* reporter gene, as expected if translation initiation follows ribosome scanning. There are many examples of cellular transcripts that, like L1Hs mRNA, have highly structured 5′ UTRs with at least one AUG codon upstream of the translation initiation site. It has been suggested that their poor translation may be essential to their restricted expression (28).

The insertion of a stable hairpin ($\Delta G = -74.8$ kcal/mol) at nt 661 of the 5′ UTR of L1Hs results in a 3- to 8-fold inhibition (a 70–87.5% decrease) of the translation of either a *lacZ* reporter gene or p40 from ORF1 both *in vitro* and *in vivo*. Stable secondary structures are known to inhibit the migration of scanning 40S ribosomes, so these results again suggest that scanning precedes the initiation of ORF1 translation. However, stem–loop structures of similar stability ($\Delta G = -61$ kcal/mol) to our insert are apparently too stable to be unwound by 40S ribosomes and have been shown to inhibit translation by 85–90% (26), somewhat more than the inhibition we observed. By contrast, 80S ribosomal complexes were shown to be able to disrupt very stable secondary structures (26). We have noted that at nt 16 there is an AUG in good context, which starts a three-codon reading frame. Another reading frame of 20 codons commences at nt 607 in some elements, including that used in the construction of the plasmids p1LZ and p3LZ used in this study. It has been suggested that upstream AUGs may actually facilitate the translation of mRNAs with highly structured leader sequences (26, 28). Thus, 80S complexes may form at one or both of the AUGs in the 5′ UTR and continue to scan after termination of the short ORFs, thereby melting stable secondary structures.

Our results, however, do not preclude the possibility that ribosomes bind internally in addition to, or rather than, at a methylated cap structure at the 5′ extremity of the mRNA as occurs, for example, with polio virus mRNA (reviewed in refs. 29 and 30). Both capped and uncapped L1Hs RNAs transcribed *in vitro* are translated with equal efficiency in rabbit reticulocyte lysates and wheat germ extracts (J.P.M., unpublished results), even though translation in wheat germ extracts is known to be highly cap-dependent (31). This result suggests that L1Hs RNA may direct at least some ribosome binding in a cap-independent manner at least *in vitro*. The direct chemical detection of a 5′-methylated cap is difficult due to the very small amount of full-length L1Hs mRNA in teratocarcinoma cells. We have attempted to determine whether p40 is translated in polio virus-infected cells, where cap-dependent translation is inhibited, but have been unsuccessful for technical reasons.

Cytoplasmic, polyadenylylated, full-length L1Hs RNA has been detected in teratocarcinoma cells with the embryonal carcinoma phenotype (9, 10), and at least some of these can function as mRNAs (8, 12). No evidence has been found, in this study or elsewhere, for spliced RNAs. Several pieces of evidence presented here indicate that the translation of L1Hs ORF2 is most likely to be initiated internally. Thus, the level of translation of ORF2 in transfected NTera2D1 cells is not directly proportional to that of ORF1 translation. The decrease in ORF1 expression effected by the hairpin insertion was accompanied by an increase in the number of cells staining positively for β-gal encoded in ORF2. However, in the complete absence of ORF1 translation, as with pL1.1Z transfections, ORF2 translation was decreased but was not totally abolished. Similar results were obtained in *in vitro* experiments (this paper and J.P.M., unpublished results) with the exception that the elimination of ORF1 translation did not affect the efficiency of ORF2 translation. In addition, *in vitro* experiments provided no evidence of an ORF1–ORF2 fusion protein.

A number of mechanisms have been described whereby the 3′ ORFs of bicistronic or polycistronic mRNAs may be translated in eukaryotic cells. These include (*i*) ribosomal frameshifting to produce a fusion polypeptide from two cistrons that overlap but are in different frames and (*ii*) suppression of an in-frame termination codon to produce a fusion polypeptide (reviewed in ref. 32). Because the two ORFs of L1Hs are in the same frame, we can assume that frameshifting is not required for the translation of ORF2. The L1Hs inter-ORF region contains at least two termination codons, and we have found no evidence for a fusion polypeptide. Thus we conclude that the translation of ORF2 is not the result of read-through by suppression of the in-frame termination codons. Other possible mechanisms include reinitiation by attached ribosomes after termination of ORF1

translation or independent internal initiation. Ilves *et al.* (33) recently provided evidence that the second open reading frame of the rodent L1 is translated by reinitiation or internal initiation rather than frameshifting, even though in this case the two ORFs overlap and are out of frame. In their experiments, they were unable to distinguish between reinitiation and internal initiation. If translation of L1Hs ORF2 were the result of reinitiation, we would expect ORF2 translation to be dependent on, and proportional to, ORF1 translation. This was not observed. On the basis of these results, we conclude that ORF2 translation is most likely initiated internally. Internal initiation at the AUG of a 3′ ORF has been demonstrated for the *pol* genes of the pararetroviruses (34–36).

Why should a decrease in the translation of p40 from ORF1 result in an increase in the expression of ORF2? Two possibilities are (*i*) the p40 product itself can inhibit ORF2 translation and (*ii*) the process of ORF1 translation interferes with the recognition of signals required for the initiation of ORF2 translation. We think that the first possibility is unlikely because the amount of ORF2 translation *in vitro* was not increased in the total absence of p40 translation nor was there an increase in β-gal expression in cells transfected with pL1.1Z, which cannot direct the translation of p40. We suggest that there are sequences within ORF1 or the inter-ORF region (or both) that are recognition signals for the internal binding of ribosomes prior to initiation of ORF2 translation. A low, but detectable, level of ORF1 translation, as observed in cells transfected with the loop constructs, may be required to expose these recognition signals through melting of secondary structure by translating ribosomes. However, when ORF1 is being efficiently translated, the translation apparatus may cover these sequences and lead to an inhibition of ORF2 translation. In the complete absence of ORF1 translation, as in cells transfected with pL1.1Z, ORF2 translation would also then be predicted by this model to be inefficient as we have observed.

What are the implications of independent translation of ORF1 and ORF2? The L1Hs product, p40, contains no homologies to gag polypeptides and there are as yet no clues to its function (ref. 12; unpublished observations). On the other hand, the L1Hs ORF2 predicts a polypeptide with homologies to both the structural and catalytic proteins of other retroelements (37). Therefore, all of the proteins required for transposition may be encoded by ORF2. Independent translation of ORF2 would then yield, as a primary product, a single polypeptide, analogous to the fusion proteins translated from other retroelements.

We would like to thank Dr. G. D. Swergold for providing the plasmids p1LZ and p3LZ and Dr. H. H. Kazazian, Jr., for providing the plasmids pL1.1 and pL1.2.

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