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Key Role of the Internal 5'-UTR Segment in the Transcription Activity of the Human L1 Retrotransposon

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Abstract—The long 5'-untranslated region (5'-UTR) of the human L1 retrotransposon contains a unique internal promoter, allowing new L1 copies to be less dependent on the integration site at the transcriptional level. The mechanism of action of this promoter still remains unclear; however, some early studies have build up an opinion that the first 5'-UTR segment of 100–150 nt (known as the minimal promoter) is most crucial for the functioning of the full-length promoter. This study shows that the activity of the minimal promoter is rather low in comparison with the activity of the full-length 5'-UTR. Instead, 5'-UTR internal segment 390–662, containing numerous binding sites for various transcription factors, is indispensable for effective L1 transcription and can be considered as a transcriptional enhancer. Deletion of this segment dramatically reduces the level of transcription irrespective of the cell type, whereas deletion of the first 100 nt decreases the transcription level only by a factor of 1.5–2. Thus, the L1 regulatory region remains to be structurally similar to that of well-studied invertebrate LINEs. It is also possible that the internal 5'-UTR segment of L1 contains an alternative promoter, driving synthesis of a 5'-truncated L1 mRNA.

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Key words: L1 retrotransposon, LINE, internal TATA-less promoter of RNA polymerase II, deletion analysis, enhancer

INTRODUCTION

Human L1 retrotransposon is the only active autonomous mobile element in the modern human population [1]. L1 retrotranspositions are able to induce hereditary diseases and malignancies. To generate a new L1 copy in the genome, the full-length RNA of the mobile element should be synthesized to function both as a template for synthesis of two retrotransposon proteins and as an intermediate of retrotransposition. Transcription of the L1 mRNA is controlled by the internal TATA-less promoter, located within the long 5'-untranslated region (5'-UTR) [2]. In spite of the fact that the unusual properties of this promoter have been studied in twenty or more investigations, the mechanism of its action is still unclear. The prevailing opinion is that the full-length L1 mRNA is synthesized by RNA polymerase II and that the main transcription determinants are within the 668-nt 5'-proximal region belonging to the 5'-UTR. This region contains binding sites for some transcription factors (TFs) (Fig. 1). In his classic work of 1990, Swergold [2] showed by deletion analysis of the full-length 5'-UTR that the removal of the first 100 nt causes a total loss of promoter activity. Although deletion of some internal segments of the 5'-UTR also significantly decreases the transcription activity, Swergold's paper promoted the opinion that the internal L1 promoter is confined entirely within the first 100 nt of the 5'-UTR. Shortly after, a study of the promoter activity of the 287-nt 5'-proximal region of the 5'-UTR strengthened this opinion [3]. The determinants affecting the transcription efficiency were localized within the first 40 nt of this fragment, whereas segment 155–187 did not demonstrate an intrinsic promoter activity and even inhibited transcription when contained in the full 287-nt 5'-proximal region. More recently, functional binding sites were revealed for YY1 and Sp1 in segment 13–21 and for RUNX3 in segment 83–101 [4, 5, 6], and it became clear that the most important determinants of L1 transcription are within the first 100 nt; this region was called the minimal promoter of the L1 retrotransposon. Later, functional binding sites for TFs of the SOX group (472–477 and 572–577) were revealed in the internal 5'-UTR region [7], but they were assumed to determine the tissue specificity of L1 transcription. A functional binding site for RUNX

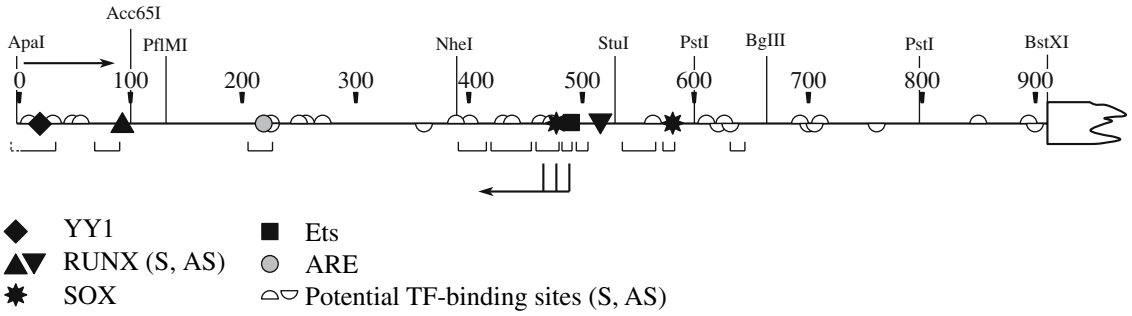

Fig. 1. Arrangement of the TF-binding sites (in the forward (S) and reverse (AS) orientations) in the 5'-UTR of the human L1 retrotransposon. The functional sites are indicated according to [4, 6, 7, 13]. The potential recognition sites were determined using the MatInspector 7.4 (Genomatix) software package with the following parameters: C.S. = 1.0, M.S. > 0.8. The L1 segments inaccessible for DNAse I cleavage in the nuclear extract are shown with brackets at the bottom [3, 5, 13]. Nucleotide coordinates are indicated according to the standard L1 numbering. The start sites and direction of transcription from the forward and reverse promoters are indicated with arrows. The restriction sites used for construction are shown.

(508–526) [6] proved to be important for the functioning of the antisense promoter, which directs transcription of the noncoding L1 strand from the internal 5'-UTR segment in the opposite direction [8].

Nevertheless, some studies suggested that the internal 5'-UTR segment is also important for retrotransposon transcription [9, 10]. Moreover, Athanikar et al. [11] cast doubt on the necessity of the 5'-terminal YY1 binding site for the effective functioning of the promoter. Finally, two studies revealed that an internal 5'-UTR segment (386–659) of one of the genomic copies of the L1 transposon functions as an enhancer towards the promoter of the apolipoprotein (a) gene, located 20 kb away [12, 13]. Although the influence of the enhancer on the expression of the retroelement itself was not studied, the presence of functional binding sites for Ets and Sp1 in the mentioned region makes it possible to suppose that this segment plays an important role in L1 transcription.

We systematically studied the contribution of different segments of the full-length 5'-UTR of the human L1 retrotransposon into the promoter activity and showed that deletion of its internal region 400–580 sharply decreases the level of reporter gene expression in various cells, whereas deletion of the first 100 or even 386 nt has a significantly lower effect, depending on the cell type. The minimal promoter (1–130) itself provides a low level of transcription, whereas the internal segment resembles in many respects a transcriptional enhancer located within the transcribed sequence.

EXPERIMENTAL

Molecular genetic constructs were developed on the basis of pRF [14]. All operations with plasmids and genetic engineering manipulations were carried out according to [15], using enzymes manufactured by Fermentas (Lithuania). L1 fragment 1–952, including the 5'-UTR and the first 45 nt from ORF1, was amplified from p1LZ [2] (kindly provided by G. Swergold) in PCR with primers CTGGGCGGAGGAGCCAAGATGG and CTGCGTTTTAGAGTTTCCAG. The fragment was ligated into pRF treated with *Pvu*II and *Bal*I. To obtain L1wt, the *Sma*I–*Eco*RV fragment, containing the SV40 promoter, was removed from the plasmid. Other constructs were developed on the basis of L1wt, which was digested with *Acc*65I (L1Δ(1–98)'), *Pvu*II–*Acc*65I (L1Δ(1–98)), *Pvu*II–*Nhe*I (L1Δ(1–386)), *Pvu*II–*Bgl*II (L1Δ(1–664)), *Acc*65I–*Nhe*I (L1Δ(103–386)), *Nhe*I–*Stu*I (L1Δ(390–526)), *Stu*I–*Bgl*II (L1Δ(527–662)), *Nhe*I–*Bgl*II (L1Δ(390–662)), *Nhe*I–*Pst*I (L1Δ(400–580)), *Bgl*II–*Bst*XI (L1Δ(667–887)), and *Pfl*MI–*Bst*XI (L1Δ(133–887)) with the subsequent end blunting and ligation. In the last three variants, we used the following adapters instead of end blunting: CTAGCACAGCGGTNGACAGCAGTAACCTCTGCA and GAGGTTACTGCTGTCGACCGCTGTG (L1Δ(400–580)), GATCAAGTAGATAAAACCACAAAG and GTGGTTTTATCTACTT (L1Δ(667–887)), and AAGTAGATAAAACCACAAAG and GTGGTTTTATCTACTTTGT (L1Δ(133–887). To obtain constructs of a ΔE series, the *Bam*HI–*Ava*III fragment was deleted from the plasmids; in the case of L1Δ(390–662)ΔEdir(390–662) and L1Δ(390–662)ΔErev(662–390), the blunted *Nhe*I–*Bgl*II fragment was inserted in place of the deleted one (in the forward or reverse orientation, respectively). To obtain pRluc, the *Xba*I–*Xba*I fragment was deleted from pRF.

Cell culture. Cells were cultured in DMEM with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (PanEco). HEK293T cells were cultured and replated by the standard technique. NTera2/D1 cells (kindly provided by S. Akopov) were maintained in a high-density culture, since such conditions provide for the maximal L1 expression in these cells [16].

Cell transfection was carried out in 24-well plates at a cell density corresponding to 60–80% of the monolayer, using Unifectin-56 as recommended by Rusbiolink. We used 0.25 μg of a reporter plasmid and 0.25 μg of the normalization vector (pRluc or pCMV-lacZ (Clontech)) per well. After 48 h incubation, cells were lysed and the activities of Fluc and Rluc luciferases and β-galactosidase were measured using DLA and BGEAS kits (Promega), respectively.

RESULTS

Studying the mechanism of translation initiation on L1 ORF1, we have found that the 5'-UTR devoid of the first 100 nt and located between two reporter genes in bicistronic construct pRF ensures high-level expression of the second cistron in vivo [17]. However, further experiments have unambiguously shown that such expression is determined by the high promoter activity, typical for this DNA fragment, rather than by the presence of an internal ribosome binding site within the fragment [17]. Since this result was quite unexpected in the light of Swergold's data [2], we decided to study in detail the causes of this discrepancy, using DNA transfection of cultured cells.

We obtained a set of molecular genetic constructs based on pRF [14] with the deleted SV40 promoter. The resulting plasmid is no longer bicistronic and is equivalent to the standard pGL3EV vector (Promega); it contains the firefly luciferase gene (*Fluc*) as a reporter and the downstream SV40 enhancer, which is often used as a nonspecific enhancer in studies of moderate promoters. Upstream of *Fluc*, we placed the full-length 5'-UTR and the first 45 nt of the L1 coding region, which were fused in frame with the luciferase ORF (Fig. 2a). The promoter activity was analyzed in vivo in two cell cultures: Ntera2/D1 teratocarcinoma cells, which express endogenous L1 [16], including the copy we used as a basis for our constructs [18]), and HEK293T cells, in which expression of endogenous L1 copies is practically absent [7, 16].

First, we studied the contribution of the minimal promoter, located within the first 100 nt of the 5'-UTR, into L1 transcription. We compared the levels of reporter gene expression directed by the full-length 5'-UTR (L1wt), the 5'-UTR with the deletion of the first 100 nt (L1Δ(1–98)), and the 5'-terminal segment of about 130 nucleotides (L1Δ(133–887)). To assess the efficiency of transfection, we used the normalizing pRluc vector, containing the *Renilla* luciferase gene under the SV40 promoter. The comparison showed that, contrary to the current opinion, deletion of the 5'-proximal L1 segment did not greatly affect the promoter activity, while the minimal promoter provided a rather low level of transcription (Fig. 2b). This was especially discernible in the case of NTera2/D1 cells:

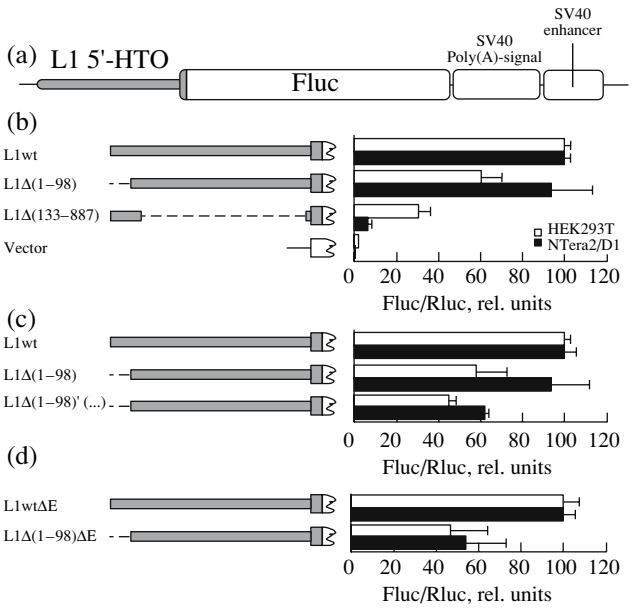

Fig. 2. Contribution of the minimal promoter into the total L1 transcription efficiency. (a) Structure of the L1wt construct. (b) Structures of the 5'-UTR constructs L1wt, L1Δ(1–98), and L1Δ(133–887) and pRFΔSV40 used as a negative control (on the left) and relative luciferase activity during the expression of the constructs in the cell culture (on the right). The pRluc plasmid, expressing the *Renilla* luciferase gene (*Rluc*) under the SV40 promoter, was taken as a normalizing vector. For each construct, the Fluc/Rluc values were normalized by the L1wt values. Each experiment was repeated at least three times. (c) The result of deletion of the 100 5'-terminal nucleotides does not depend on the adjacent sequence; the designations are as in Fig. 2b. (d) The result of deletion of the 100 5'-terminal nucleotides does not depend on the presence of the SV40 enhancer in the plasmid; Fluc/Rluc values were normalized by the values obtained for L1wtΔE.

deletion of the 5'-terminal fragment practically did not influence the expression, and the activity of the minimal promoter was close to that of the negative control. Similar results were obtained with pCMV-lacZ used as a normalizing vector (data not shown). It is interesting that, in cells transfected with L1Δ(1–98), the relative *Fluc* activity greatly varied in different experiments and the error was rather high in spite of many repetitions (Fig. 2b). We did not register such a variation with other constructs. Hence, we concluded that the contribution of the 5'-terminal fragment to the total transcription activity strongly depends on the state of a cell culture, serum preparation, and other hardly identifiable factors influencing the cell state. It is possible that this observation partly explains the inconsistency between our results and Swergold's data [2]. Nevertheless, in all our experiments, the level of L1Δ(1–98) expression was no less than 50% of the L1wt expression, whereas the activity of an analogous construct (pD1) was less than 1% of the activity of the full-length 5'-UTR in [2].

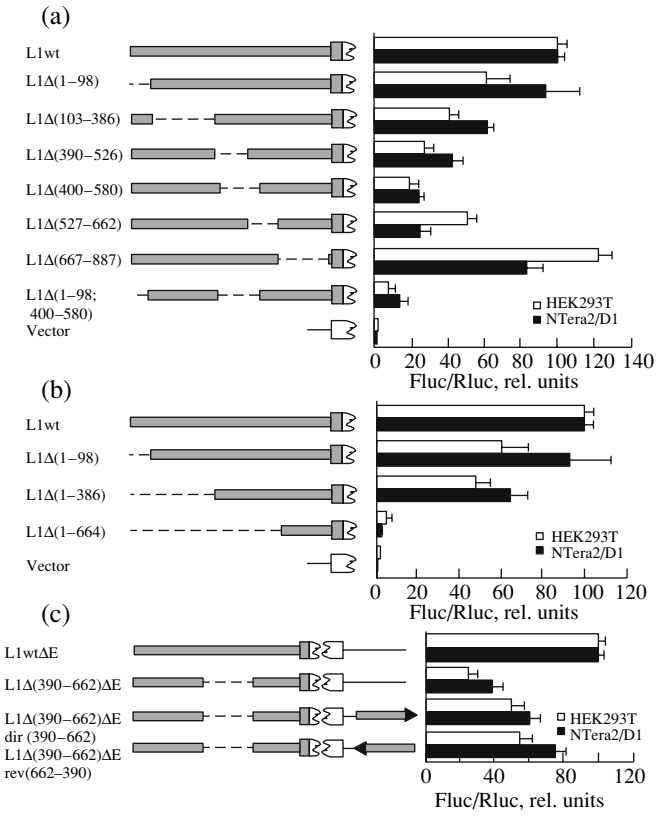

Fig. 3. Contribution of the internal 5'-UTR segment into the transcription activity of the human L1 retrotransposon. (a) Result of stepwise deletions of 5'-UTR segments. The values were calculated as in Fig. 2b. (b) Result of 5'-terminal deletions from the 5'-UTR. (c) Effect of the removal of the internal 5'-UTR segment out of the gene; Fluc/Rluc values were normalized by the values obtained for L1wtΔE.

The main distinction of L1Δ(1–98) from pD1 consisted in the use of another vector (pGL3EV). We examined whether the high level of L1Δ(1–98) transcription was connected with the 5'-flanking region of the plasmid. It is known that the nucleotide sequence flanking the 5' terminus of L1 can significantly influence its expression [19]. Moreover, segment 83–101 of the L1 5'-UTR contains the RUNX3-binding site [6]. Deletion partly destroyed this site but part of it was preserved. Therefore, the nucleotides located upstream of the L1 sequence in the vector can be important. We constructed L1Δ(1–98)', in which the L1 region was downstream of another vector sequence, and observed that the result remained qualitatively the same (Fig. 2c). Another significant distinction of L1Δ(1–98) from pD1 was the presence of the SV40 enhancer. However, the pair of L1wtΔE and L1Δ(1–98)ΔE, similar to the L1wt and L1Δ(1–98) pair but lacking the SV40 enhancer, demonstrated a similar ratio of the levels of reporter gene expression (Fig. 2d). Other differences between L1Δ(1–98) and pD1 were inessential in our opinion. For example, the lack of the 3'-UTR of L1 could hardly determine the revealed difference, since it is known that this region does not significantly influence the L1 expression [20].

The next step was to determine the contribution of other 5'-UTR segments of L1 into its promoter activity. Based on L1wt, we developed a set of constructs serial deletions of 100–250 nt and analyzed their expression in cells of both types. It is important that such deletions practically do not influence the level of translation of the corresponding mRNAs [17]; therefore, we could judge the efficiency of transcription by the activity of the reporter protein. The lowest expression level was registered in the case of deletion of internal 5'-UTR segment 400–580 (Fig. 3a). Simultaneous deletion of the internal segment and about 100 nt from the 5' end (L1Δ(1–98; 400–580)) dramatically reduced the level of transcription. Similar results were obtained with constructs lacking the SV40 enhancer (data not shown).

In light of these data, we decided to study the influence of different 5'-terminal deletions on the promoter activity of the L1 retrotransposon. An increase in deletion size firstly caused a smooth fall of the promoter activity, but deletion of the middle 5'-UTR fragment (387–663, L1Δ(1–664)) totally prevented the expression of the reporter gene (Fig. 3b). This result confirms the importance of the internal 5'-UTR segment for the L1 transcription activity.

Segment (400–580) contains functional binding sites for RUNX3 [6] and SRY-family TFs [7] and the antisence L1 promoter [8] (Fig. 1). Moreover, at least in one L1 copy, this region contains a binding site for Ets-1 [13] and fragment 386–659 harbors an enhancer of the *apo(a)* gene, located 20 kb away [12, 13]. To examine whether this fragment is able to stimulate L1 transcription when located at a large distance from the 5' terminus, we obtained four constructs lacking the SV40 enhancer. In one construct (L1wtΔE), Fluc synthesis was directed by the full-length 5'-UTR, and the other three constructs had 5'-UTR lacking fragment 390–662. In L1Δ(390–662)ΔEdir(390–662) and L1Δ(390–662)ΔErev(390–662), this fragment was inserted immediately downstream of the *Fluc* coding region and the poly(A) signal, i.e., instead of the deleted SV40 enhancer. Located in a remote position, the internal 5'-UTR segment retained the ability to stimulate L1 transcription, although this stimulation was lower than in the case of the normal position of the fragment (Fig. 3c).

DISCUSSION

Until now, internal promoters of RNA polymerase II were found only in LINE retrotransposons. Because of the small number of such internal promoters, their organization and structure still remain unclear. Early structural analysis of the L1 promoter denoted a major

role of the 5'-proximal segment of the UTR in its function. Since transcription starts from this region, this assumption seemed to be quite consistent. In spite of the insufficient volume of these data, the concept of the "minimal promoter" was commonly accepted. Even clear evidences that only a low level of transcription is provided by short 5'-terminal segments compared with the full-length 5'-UTR [11, 21] and that internal 5'-UTR segments are potentially important [2, 7, 10] were unable to withstand this concept.

Nevertheless, several LINEs from other organisms were found to have two key transcription-regulating regions: a 5'-terminal segment, where mRNA synthesis starts, and an internal 5'-UTR segment, located at a certain distance from the first segment. For example, the first 40 nt from the *Drosophila F* element form a basal promoter, containing classic Inr and DPE: its activity is stimulated many times by an internal enhancer, located in region 165–269 (see [22] and references therein). It is interesting that this internal segment also contains the antisense promoter of the *F* element, which, like the antisense L1 promoter, directs synthesis of a noncoding RNA from within 5'-UTR to the 5'-adjacent segment [23]. The *I* element, another *Drosophila* LINE, has a similar structure of the basal promoter, and its internal 5'-UTR segment also contains an enhancer, providing a tissue-specific expression of this retrotransposon [24]. It has been shown that the internal segment is important for the functioning of the promoter of TRAS1, a silkworm telomeric retrotransposon [25]. In this case, the central segment of the 5'-UTR not only significantly stimulates 5'-terminal transcription, but is also able to function as a promoter in the absence of the first 40 nt, containing the basal promoter elements.

In spite of the lack of homology between the nucleotide sequences of the noncoding regions of the L1 and these three retrotransposons, we should not ignore a certain similarity of the functional organization of their regulatory segments. In a strict sense, the concept of the internal enhancer (i.e., a regulatory segment located within the transcribed region) is not revolutionary: classic internal enhancers were detected in the immunoglobulin genes as early as the 1980s (for a review, see [26]). However, the application of this principle to the regulatory segment of the L1 retrotransposon would be a new and unexpected confirmation of such a possibility. We showed that changes in the distance between the putative enhancer and the transcription start only slightly affect the enhancer effect (Fig. 3a, L1Δ(103–386)). Moreover, the effect is partly preserved even when this 5'-UTR segment is transferred far from its initial position (Fig. 3c). The structure of the internal 5'-UTR segment strongly resembles certain enhancers: this relatively short DNA segment contains a large number of TF-binding sites (Fig. 1). For example, this segment contains two binding sites for the TFs belonging to the SRY (SOX) group, which are able to bend the DNA strand and, thereby, bring together the adjacent recognition sites for the activator proteins RUNX/AML/CBFα, Ets-1, CREB, Sp1, etc. Following the same scheme, the same TFs form the activator complexes known as enhanceosomes [27].

Nevertheless, one should note that deletion of 100 or even 386 5'-terminal nucleotides from the retrotransposon did not dramatically reduce the expression of the reporter gene in our experiments (Fig. 3b; L1Δ(1–98), L1Δ(1–386)). It is difficult to explain this result in terms of the "basal promoter + internal enhancer" scheme. Actually, classic enhancers alone (without a promoter) are hardly able to ensure gene transcription. It is possible that, when the natural transcription start has been deleted, the protein factors that form the activator complex at the internal 5'-UTR segment induce transcription from earlier inactive (cryptic) transcription start sites, as is the case when a mutation affects the YY1-binding site in region 13–21 of the 5'-UTR [11]. Alternatively, our data can be explained by the presence of a second forward promoter with a transcription start site within region 400–662 of the 5'-UTR, in addition to the well-characterized reverse promoter lying within this region. A similar situation has been described for a plant LINE [28]: the internal 5'-UTR segment of the ATLN39 retrotransposon contains an additional forward promoter, directing synthesis of truncated mRNAs. The 5' terminus of L1 transcripts has been identified by primer extension [3, 18, 19]; however, in most cases, the primers were close to the 5' terminus of the UTR. In addition, the L1 mRNA has been analyzed using Northern hybridization [2, 16], but the resolution of this method might be insufficient for uncovering truncated transcripts. Although synthesis of such truncated RNA copies seems senseless in view of the data on the *cys*-action of L1 proteins [29], there is some indirect evidence of the existence of the alternative promoter. For example, hybridization has shown that distal and proximal segments of the L1 5'-UTR are present among cell RNAs in unequal proportions (with a significant predominance of 5'-distal segments) [8], and the EST database contains many truncated transcripts starting in region 570–630. To more thoroughly study the contribution of the internal 5'-UTR segment into transcription of the human L1 retrotransposon and to clarify the question of the additional internal promoter, it is necessary to analyze the expression of the reporter constructs at the RNA level. This work is in progress in our lab.

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