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Adequate System for Studying Translation Initiation on the Human Retrotransposon L1 mRNA in Vitro

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Abstract—The L1 retrotransposon codes for a unique bicistronic mRNA, which serves as a transposition intermediate and as a template for the synthesis of two proteins. According to preliminary data, the translation of both cistrons is initiated by a noncanonical mechanism. The L1 mRNA was translated in rabbit reticulocyte lysate (RRL), a standard system widely used to study the eukaryotic mechanisms of protein synthesis. Translation yielded not only the expected products, but also several products of aberrant translation initiation on internal AUG codons. Such products are not generated during *in vivo* translation of the L1 mRNA. When RRL was supplemented with a cytoplasmic extract of HeLa cells, the aberrant products were not synthesized, while the first cistron was translated with the same efficiency. The efficiency of translation of the second cistron became substantially lower, corresponding to the situation *in vivo.* These and other experiments clearly demonstrated that the new combined system RRL + HeLa is far more adequate for studying the mechanisms of translation initiation than the standard RRL system.

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INTRODUCTION

Retrotransposon L1 is a mobile element that belongs to the LINE family and is extremely abundant in mammalian genomes [1]. Its copies have increased in number to over 500,000 during evolution and together account for about 17% of human chromosomal DNA. The L1 mRNA serves both as a transposition intermediate and as a template for synthesis of two proteins, an RNA-binding protein (p40) with an unknown function and a reverse transcriptase. The open reading frames of these two proteins do not overlap; they are separated by a 63-nt region. Thus, the L1 mRNA is a natural bicistronic template, which is absolutely unusual for eukaryotes. Another feature of the L1 mRNA is a surprisingly long (910 nt) 5'-untranslated region (5'-UTR) upstream of the first cistron. The 5'-UTR contains two short open reading frames and is GC-rich apart from the last 100 nt. The third unique feature is that the other parts of the mRNA have a high adenine content: almost every second base is adenine in some regions. Owing to these features, the L1 mRNA provides an interesting model for studying the noncanonical mechanisms of translation initiation in eukaryotes.

In vitro systems are most convenient for investigating the molecular mechanisms of translation initiation in mammals. We used a rabbit reticulocyte lysate (RRL), a classical example of such systems. RRL is a cytoplasmic extract of erythrocyte precursors; it is prepared by the standard method [2] and is commercially available from Promega. Although reticulocytes are specialized cells with intense protein synthesis from a few templates (mostly those of α- and β-globins), RRL is widely used to study translation of various mRNAs. However, some specific features of RRL must be taken into account when studying translation initiation mechanisms in order to avoid possible artifacts produced by this specialized system (see below).

The objective of this work was to develop a system for studying translation initiation of the L1 mRNA and other structurally intricate templates *in vitro* so that the translation conditions would be similar to those in the living cell and the results similar to those obtained *in vivo*.

EXPERIMENTAL

Molecular genetic constructs were obtained on the basis of pGL3-Control (Promega). Manipulations with plasmids and gene engineering procedures followed the standard protocols [3]; the enzymes were from Fermentas (Lithuania). L1 fragment 1–1993, which included the 5'-UTR, the first cistron, and the intercistronic region, was PCR-amplified from p3LZ [4] (kindly provided by G.D. Swergold). The primers

were 5'-GGCGGAGGAGCCAAGATGG-3' and 5'-CGTGGCCAGTGTCATTATGATGTTAGCTGG-3'. To construct pLL2, the amplification product was cloned into the *Sma*I–*Nco*I sites (the latter was blunted) of pGL3. To construct pLL2imp, the *Hind*III–*Bal*I fragment of pLL2 was replaced by the PCR product amplified from pLL2 with primers 5'-CCAGCCAAACTAAGCTTCATAAGTG-3' and 5'-CCAGATCCATGGCGGCTATGATGTTATCTGGTG ATTTTGC-3' and digested with *Hind*III. The structure of the constructs was verified by sequencing (Genom, Moscow).

RNA was obtained using T7 RNA polymerase as recommended by Fermentas. The reaction was carried out at 37°C for 2 h, always in the presence of the ribonuclease inhibitor RiboLock (Fermentas). As a DNA template, we used the PCR product amplified from the proper plasmid with primers T7UTR (5'-CGCCGTAATAC-GACTCACTATAGGCGGAGGAGCCAAGATGG-3') and FLA50 (5'-$(T)_{50}$AACTTGTT TATTGCAGCT-TATAATGG-3'), purified by agarose gel electrophoresis, and eluted from gel using an H-1602 kit (Helikon, Russia). After transcription, RNA was precipitated with LiCl. The reaction mixture was combined with an equal volume of 4 M LiCl, incubated in ice for 1 h, and centrifuged at 12,000 *g* at 4°C for 10 min. The RNA pellet was washed with 70% ethanol, 5 mM $MgCl_2$, 50 mM $NaCH_3COO$; it was then dried and dissolved in water.

Translation of mRNA was carried out using nuclease-treated RRL (Promega). The reaction mixture (7 μl) contained 5 μl of RRL supplemented with a mixture of amino acids and [^{35}S]methionine (Amersham) as recommended by the manufacturer, 0.5 μl of mRNA (an aqueous solution), and 1.5 μl of water or a HeLa cell extract treated with micrococcal nuclease. The protein product was resolved by PAGE in 12% gel according to Laemmli [3]. Autoradiographs were obtained using a PhosphorImager (Molecular Dynamics) and analyzed using the Image Quant 5.0 program.

RESULTS AND DISCUSSION

To select the conditions of *in vitro* translation, we used the artificial LL2 mRNA as a model template (Fig. 1a). The LL2 mRNA was similar to the full-length L1 mRNA but the second cistron was substituted with the firefly luciferase (Fluc) reporter gene. This substitution was necessary because the second open reading frame of the natural L1 mRNA is too large to allow its efficient translation in RRL. The nucleotide context of the start codon of the second reading frame was preserved. The mRNA was obtained by T7 transcription *in vitro*. Since the L1 mRNA is polyadenylated in the cell [1], the 3' end of the mRNA was tagged with $(A)_{50}$. For this purpose, the mRNA was synthesized not from the plasmid

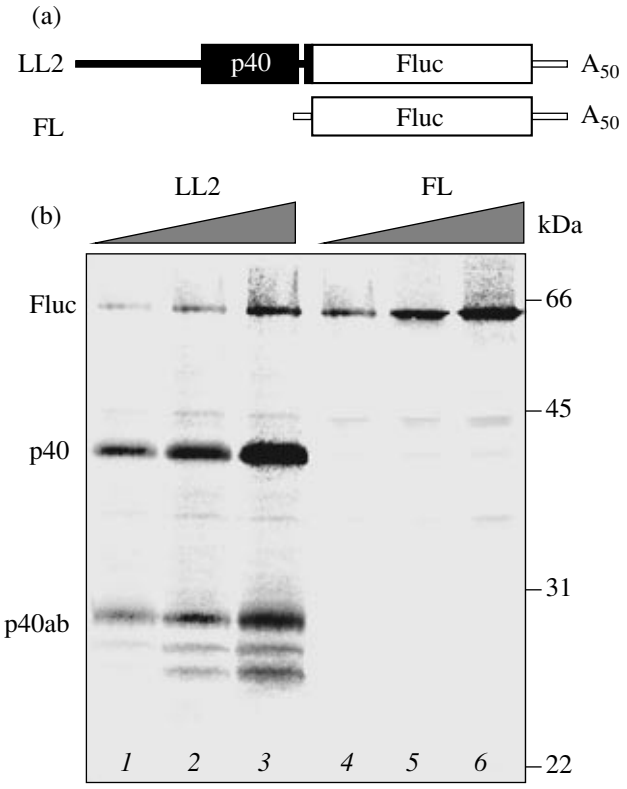

Fig. 1. Translation of the LL2 and FL mRNAs in RRL. (a) Structures of the LL2 and FL mRNAs. Bars, open reading frames. Retrotransposon sequences are shown in black. (b) Autoradiography of the products synthesized from (*1–3*) the LL2 mRNA and (*4–6*) the FL mRNA in RRL. The mRNAs were used at (*1, 4*) 12.5, (*2, 5*) 25, or (*3, 6*) 50 nM. Here and in Fig. 2, p40ab are aberrant products. Molecular weights (kDa) of marker proteins are indicated.

DNA, but from the PCR product amplified with pLL2 and primers T7UTR and FLA50. Primer T7UTR corresponded to the start of the 5'-UTR and contained the T7 promoter. Primer FLA50 was complementary to the plasmid region 200 bp downstream of the Fluc gene and contained $(T)_{50}$. This method of introducing a homonucleotide tract obviates the need for cloning the plasmids containing such regions. Homonucleotide tracts are prone to recombination in the bacterial cell, and the resulting plasmid population is heterogeneous.

The LL2 mRNA was translated in RRL supplemented with [^{35}S]methionine and the products were resolved by PAGE according to Laemmli. As a control, we used the standard monocistronic polyadenylated FL mRNA from a Promega translation kit. This mRNA also codes for luciferase and has a simple short 5'-UTR (Fig. 1a).

The results of translation of the LL2 mRNA used at three different concentrations are shown in Fig. 1b (lanes *1–3*). The reaction mixture contained the products translated from the first (~40 kDa) and the second

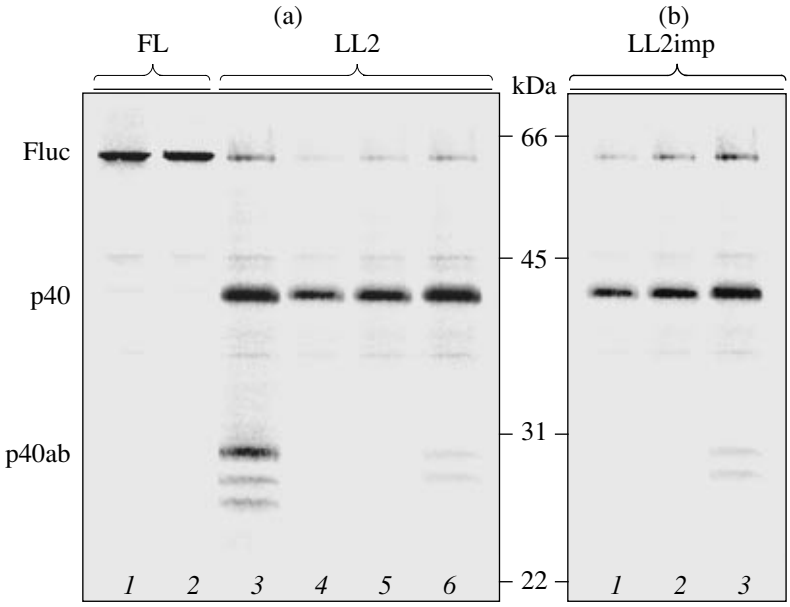

Fig. 2. Translation of different mRNAs in the RRL and RRL + HeLa systems. (a) Translation of the LL2 and FL mRNAs. Lane *1*, 25 nM FL in RRL; *2*, 25 nM FL in RRL + HeLa; *3*, 25 nM LL2 in RRL; and *4–6*, LL2 (12.5, 25, and 50 nM, respectively) in RRL + HeLa. (b) Translation of the LL2imp mRNA in the RRL + HeLa system. The mRNA was used at (*1*) 12.5, (*2*) 25, and (*3*) 50 nM.

(~60 kDa) cistrons: p40 and Fluc, respectively. In addition to these two proteins, bands of several other polypeptides of less than 30 kDa were detected on the autoradiograph (Fig. 1b, lanes *1–3*). Protease and RNase inhibitors did not prevent their synthesis (data not shown). These products corresponded in size to polypeptides translated from the same frame as p40 but starting from internal AUG codons. Translation of the FL mRNA did not yield such products (Fig. 1b, lanes *4–6*). Thus, several products of aberrant translation initiation from the internal AUG codons of the first open reading frame were synthesized during translation of the LL2 mRNA in RRL. Similar products have already been reported for L1 mRNA translation in RRL [4], but their origin has not been discussed. However, such polypeptides have not been detected in the living cell [4, 5]. This finding indicates that the classical variant of the RRL system fails to reproduce the conditions existing in the living cell, which is most probably explained by the above specialization of reticulocytes used to prepare RRL. It is known, for instance, that mRNA-binding proteins essential for efficient translation of some viral mRNAs are scarce in RRL [6–8]. Aberrant initiation has also been observed with picornaviral mRNAs translated in the RRL system [9–12] but not in extracts of other (e.g., HeLa) cells [9, 10, 12].

At the same time, RRL has several advantages over other cell extracts: it is available, allows a high efficiency of translation, and yields reproducible results. We tested a combined RRL-based system supplemented with a HeLa cell extract treated with micrococcal nuclease S30 (20% v/v). A similar system has been used to study translation of the poliovirus mRNA [10] but has not received wide acceptance.

The results of translation in the combined system are shown in Fig. 2a. The HeLa cell extract had virtually no effect on the efficiency of translation of the control FL mRNA (compare lanes *1* and *2*). Yet the pattern of LL2 mRNA translation considerably changed in the presence of the HeLa cell extract (lanes *3–6*). The products of aberrant translation initiation were undetectable. Weak bands in the corresponding zone were detected only at the highest (50 nM) mRNA concentration examined. Hence it is possible to assume that mRNA-binding proteins are responsible for masking the aberrant initiation sites: at a higher mRNA concentration, the protein–RNA ratio is lower, the mRNA is naked, and inaccessible internal regions of the mRNA molecule become accessible to ribosomes. Such an effect of mRNA-binding proteins has already been observed [13]: added to RRL, mRNA-binding proteins decrease the likelihood of translation initiation on an internal ribosome entry site (IRES) of a bicistronic mRNA and increase the efficiency of translation of the 5'-proximal cistron. To explain the generation of the aberrant translation products in the case of the LL2 mRNA, it is possible to assume that the entire region of the first cistron is predominantly single-stranded because of its abnormally high adenine content; it can also be assumed that this region is capable of binding with the ribosome in the absence of mRNA-binding proteins, as is characteristic of the single-stranded U-rich IRES of RhPV [14] or the

A-rich IRES of crTMV [15]. When a HeLa cell extract is added, its mRNA-binding proteins form ribonucleoprotein complexes with the mRNA, thereby blocking aberrant translation initiation on the internal sites and directing ribosomes to the proper start codons, whose selection for translation initiation is a result of specific mechanisms. The fact that the efficiency of translation did not appreciably change in the presence of a HeLa cell extract in the cases of the FL mRNA and the first cistron of the LL2 mRNA supports the existence of such a mechanism, which is probably classical 5'-end-dependent initiation in the former case and the same or another, still unknown mechanism in the latter.

The situation with the second cistron of the LL2 mRNA is different. In the presence of a HeLa cell extract, the efficiency of its translation decreased dramatically, although to a lesser extent than that of the aberrant products (compare the intensities of the Fluc and p40ab bands, Fig. 2a, lanes *3, 5*). Hence, it is possible that translation initiation on the second cistron in the original RRL mostly followed the mechanism of aberrant internal ribosome binding, as is the case with other internal AUG codons. The fact that Fluc was still synthesized when RRL was supplemented with a HeLa cell extract suggests an additional mechanism for translation initiation on the second cistron, although the efficiency of this mechanism is rather low. It should be noted that low-level translation of the second cistron was not at all unexpected. The second cistron of the original L1 mRNA codes for reverse transcriptase, which is necessary in minor amounts for the retrotransposon. Reverse transcriptase occurs at an extremely low concentration in living cells, and its presence in tissues and cultured cells was demonstrated only recently [16]. The L1 mRNA exists in the cytoplasm in the form of a ribonucleoprotein particle which contains mostly p40 (the product of the first cistron) [17]. Reverse transcriptase is probably also contained in this mRNP. Its activity is present in mRNP particles [19], but the protein itself has not yet been detected in particles, even with antibodies. It is thought that the mRNP particle contains only two reverse transcriptase molecules, if not a single one [19], while p40 molecules are quite abundant. A disproportion in the contents of the structural protein and polymerase is characteristic of many virus particles and is often caused by a difference in the efficiency of translation initiation. Although retrotransposons are not true viruses, several aspects of their life cycle demonstrate their similarity, in particular, to Retroviridae [1]. In line with the above assumptions, the activity of the second exon was extremely low in cultured cells transfected with the LL2 mRNA (S.E. Dmitriev, unpublished data).

To check whether the second cistron is indeed subject to low-efficient translation via a specific mechanism differing from that of aberrant initiation on other internal AUG codons, we tried to increase its expression in the RRL + HeLa system. The low level of translation initiation could be determined by the non-optimal nucleotide context of the AUG start codon (atcataATGaca). We constructed pLL2imp, wherein the context was changed to optimal (gccgccATGgat) [20, 21]. The LL2imp mRNA obtained with this construct was translated in the RRL + HeLa system (Fig. 2b). The change in context considerably increased the efficiency of translation initiation on the second cistron (compare Fig. 2b, lanes *1–3* and Fig. 2a, lanes *4–6*). At a medium LL2imp mRNA concentration, the reaction yielded two major polypeptides, Fluc and p40, while the aberrant products of translation initiation on the other internal AUG codons (many of which were originally in nearly optimal contexts) were almost undetectable (lanes *1, 2*). Thus, specific mechanisms initiating translation of the L1 mRNA were efficient in our system, while aberrant translation initiation was virtually undetectable. The accuracy of translation initiation and the ratio of the translation products obtained with our system matched the *in vivo* situation far better than with RRL alone.

We think that studies of the mechanisms of protein biosynthesis should take into account the fact that the RRL *in vitro* translation system yields artifacts at a high probability, as observed with the L1 mRNA and reported for picornaviral mRNAs [9–12]. RRL is suitable for checking the length and integrity of open reading frames, producing labeled recombinant proteins, and for some other technical applications. Yet other approaches are necessary for studying the mechanisms of translation initiation, especially in the case of mRNAs with extended single-stranded regions. One such approach was proposed in this work.

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REFERENCES

1. Ostertag E.M., Kazazian H.H., Jr. 2001. Biology of mammalian L1 retrotransposons. *Annu. Rev. Genet*. **35**, 501–538.
2. Pelham H.R., Jackson R.J. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem*. **67**, 247–256.
3. Sambrook J., Russell D. 2001. *Molecular Cloning: A Laboratory Manual*. 3d ed. N.Y.: Cold Spring Harbor Lab. Press.

4. Leibold D.M., Swergold G.D., Singer M.F., Thayer R.E., Dombroski B.A., Fanning T.G. 1990. Translation of LINE-1 DNA elements *in vitro* and in human cells. *Proc. Natl. Acad. Sci. USA*. **87**, 6990–6994.
5. McMillan J.P., Singer M.F. 1993. Translation of the human LINE-1 element, L1Hs. *Proc. Natl. Acad. Sci. USA*. **90**, 11533–11537.
6. Meerovitch K., Svitkin Y.V., Lee H.S., Lejbkowicz F., Kenan D.J., Chan E.K., Agol V.I., Keene J.D., Sonenberg N. 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *J. Virol*. **67**, 3798–3807.
7. Hunt S.L., Hsuan J.J., Totty N., Jackson R.J. 1999. unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes Dev.* **13**, 437–448.
8. Jia X.Y., Scheper G., Brown D., Updike W., Harmon S., Richards O., Summers D., Ehrenfeld E. 1991. Translation of hepatitis A virus RNA *in vitro*: Aberrant internal initiations influenced by 5' noncoding region. *Virology.* **182**, 712–722.
9. Brown B.A., Ehrenfeld E. 1979. Translation of poliovirus RNA *in vitro*: Changes in cleavage pattern and initiation sites by ribosomal salt wash. *Virology.* **97**, 396–405.
10. Dorner A.J., Semler B.L., Jackson R.J., Hanecak R., Duprey E., Wimmer E. 1984. *In vitro* translation of poliovirus RNA: Utilization of internal initiation sites in reticulocyte lysate. *J. Virol*. **50**, 507–514.
11. Dasso M.C., Jackson R.J. 1989. On the fidelity of mRNA translation in the nuclease-treated rabbit reticulocyte lysate system. *Nucleic Acids Res*. **17**, 3129–3144.
12. Jackson R.J. 1991. Potassium salts influence the fidelity of mRNA translation initiation in rabbit reticulocyte lysates: Unique features of encephalomyocarditis virus RNA translation. *Biochim. Biophys. Acta*. **1088**, 345–358.
13. Svitkin Y.V., Ovchinnikov L.P., Dreyfuss G., Sonenberg N. 1996. General RNA binding proteins render translation cap-dependent. *EMBO J*. **15**, 7147–7155.
14. Terenin I.M., Dmitriev S.E., Andreev D.E., Royall E., Belsham G.J., Roberts L.O., Shatsky I.N. 2005. A cross-kingdom internal ribosome entry site reveals a simplified mode of internal ribosome entry. *Mol. Cell. Biol*. **25**, 7879–7888.
15. Dorokhov Y.L., Skulachev M.V., Ivanov P.A., Zvereva S.D., Tjulkina L.G., Merits A., Gleba Y.Y., Hohn T., Atabekov J.G. 2002. Polypurine (A)-rich sequences promote cross-kingdom conservation of internal ribosome entry. *Proc. Natl. Acad. Sci. USA*. **99**, 5301–5306
16. Ergun S., Buschmann C., Heukeshoven J., Dammann K., Schnieders F., Lauke H., Chalajour F., Kilic N., Stratling W.H., Schumann G.G. 2004. Cell type-specific expression of LINE-1 open reading frames 1 and 2 in fetal and adult human tissues. *J. Biol. Chem*. **279**, 27753–27763.
17. Hohjoh H., Singer M.F. 1996. Cytoplasmic ribonucleoprotein complexes containing human LINE-1 protein and RNA. *EMBO J*. **15**, 630–639.
18. Deragon J.M., Sinnett D., Labuda D. 1990. Reverse transcriptase activity from human embryonal carcinoma cells NTera2D1. *EMBO J*. **9**, 3363–3368.
19. Ostertag E.M., Kazazian H.H., Jr. 2001. Twin priming: A proposed mechanism for the creation of inversions in L1 retrotransposition. *Genome Res*. **11**, 2059–2065.
20. Kozak M. 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol*. **196**, 947–950.
21. Grunert S., Jackson R.J. 1994. The immediate downstream codon strongly influences the efficiency of utilization of eukaryotic translation initiation codons. *EMBO J*. **13**, 3618–3630.