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Expression of a cytoplasmic LINE-1 transcript is regulated in a human teratocarcinoma cell line

(repeated DNA/transcription/differentiation)

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ABSTRACT **The major primate family of highly repeated, long interspersed DNA sequences (LINE-1, previously *Kpn* I, family) includes several thousand 6-kilobase-pair long units that terminate in an A-rich stretch. Recent evidence indicates that long open reading frames occur in at least some family members. These results suggested that one or more LINE-1 family members might be structural genes. Accordingly, a variety of human cell lines was analyzed for the presence of a cytoplasmic, polyadenylylated RNA homologous to LINE-1 sequences. Such a transcript was detected in a human pluripotent teratocarcinoma cell line (NTera2 clone D1). The RNA is ≈6.5 kilobases long and is homologous to the LINE-1 strand with the open reading frames. The abundance of the transcript varies markedly with previously described variations in the phenotype of these cells and is highest when the cells display the embryonal carcinoma morphology. This RNA may represent a mRNA transcribed from one or more functional genes in the LINE-1 family.**

The major primate family of highly repeated, long interspersed DNA sequences (LINE-1 family, previously *Kpn* I family) includes several thousand 6-kilobase-pair (kbp)-long units that terminate in an A-rich stretch at one end (designated 3′ end) (1, 2). Many other family members are truncated at the 5′ end and some contain deletions or rearrangements of internal segments (reviewed in ref. 3). Altogether there are $>10^4$ family members interspersed in primate genomes. A 6-kbp sequence compiled from independently cloned and sequenced human and monkey LINE-1 subsegments contains four open reading frames occupying about 3.5 kbp on the same strand that contains the 3′-terminal A-rich stretch (3); the stop codon that terminates the downstream open reading frame is about 220 base pairs from the A-rich stretch. The internal region containing the open reading frames is conserved between primate and mouse genomes (3–5). Several randomly cloned human (6, 7) and mouse (4) segments from within the conserved region contain open reading frames homologous to that seen in the compiled sequence (3). These results suggested that some LINE-1 family members in various mammals may encode proteins and we sought evidence in support of this hypothesis.

Previously, abundant transcripts that are heterogeneous in size were detected in the nuclei of human and monkey cells from several cell lines (8–12). In contrast, very little RNA homologous to LINE-1 DNA was detected in the cytoplasm and very little of that appeared to be polyadenylylated. We therefore surveyed various additional cell types for nuclear and cytoplasmic RNA that hybridizes to LINE-1 DNA probes. The results confirm the earlier reports except with the human teratocarcinoma cell line NTera2 clone D1 (13–15). In these cells a cytoplasmic polyadenylylated RNA approximately 6.5 kilobases (kb) long hybridized to cloned LINE-1 DNA probes.

MATERIALS AND METHODS

Cell Cultures. PA-1, JEG-3, 293, and HTG cell lines were obtained from American Type Culture Collection (Rockville, MD), CaMa was provided by Z. Steplewski, and NTera2D1 (13–15) was provided by P. W. Andrews (both at Wistar Institute, Philadelphia). MOLT-4 was from R. Gallo (National Institutes of Health) and the COS-1 (16) monkey cells were from this laboratory's collection. PA-1, 293, CaMa, and COS-1 were cultured in Dulbecco's minimal essential medium (DMEM), JEG-3 and HTG in Eagle's minimal essential medium (EMEM), HeLa S3 in EMEM with Earle's salts for suspension culture (SMEM), and MOLT-4 in RPMI 1640 medium (all from GIBCO), all supplemented with 10% fetal calf serum (GIBCO), glutamine, and penicillin. MOLT-4 and HeLa S3 cells were grown in spinner cultures and collected at a density of 4–6 × 10^5 cells per ml. All other cells were grown on plates and, except for NTera2D1, were usually harvested when the monolayers were about 50% confluent. In some experiments, PA-1 and 293 cells were collected after formation of multilayers (not contact inhibited). High-density cultures of NTera2D1 cells were maintained at high density by subculturing with glass beads (13) and a dilution of 1:3. Low-density cultures of NTera2D1 cells were prepared from high-density cultures by subculturing with trypsin/EDTA (GIBCO) and at a plating density of 1–2 × 10^6 cells in 150-cm^2 tissue culture flasks. For treatment with retinoic acid NTera2D1 cells were plated as for low-density cultures. Retinoic acid (10 mM solution in dimethyl sulfoxide, all-*trans*, Eastman Kodak) was added on the next day to a final concentration of 2 μM (14). After 4–5 days cells were harvested with trypsin/EDTA and replated at the initial density in the presence of the above concentration of retinoic acid. After such a treatment cells divided through at least five more passages. The efficiency of differentiation was checked by incubating a confluent culture of treated cells for 2 weeks without subculturing and counting the number of foci of non-contact-inhibited cells that did not differentiate in response to retinoic acid. This number was usually smaller than about 10–20 foci per 5 × 10^7 cells.

RNA Isolation and Analysis. Total RNA was isolated by lysing cells with 1% $NaDodSO_4$/10 mM Tris·HCl, pH 8.0/1 mM EDTA, 200 μg of proteinase K (Merck) per ml followed by extraction with phenol at pH 5.2 and 65°C (17). Residual contamination with genomic DNA was removed by a 30-min digestion at 37°C with 500 units of DNase I [Worthington; treated with proteinase K (18) in the presence of calcium] per ml. The effectiveness of this treatment was monitored by the disappearance of added nanogram quantities of pUC8 DNA

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Abbreviations: kb, kilobase(s); kbp, kilobase pair(s).

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using hybridization with a pUC8 probe. The size distribution of LINE-1 transcripts remained unchanged. For preparation of cytoplasmic and nuclear fractions, cells were swollen in RSB buffer (19) supplemented with 10 mM vanadyl–adenosine complex and were lysed in 0.45% Nonidet P-40. Nuclei and nuclear RNA were isolated as described (19), but 10 mM vanadyl–adenosine complex was added to all solutions. Cytoplasmic RNA was purified with phenol at pH 5.2 and 65°C (17). Electrophoresis of RNA was in a formaldehyde/1.0% agarose gel system (20). The gels were electroblotted onto GeneScreenPlus membrane (New England Nuclear) and the membrane was baked for 1 hr at 80°C to reverse the formylation. Filters were prehybridized for 2–4 hr in 0.6 M NaCl/0.06 M sodium citrate, 45% formamide, 5 × Denhardt's concentrated solution (21), and 1% $NaDodSO_4$ at 45°C.

Hybridization to the DNA probes labeled with ^{32}P by nick-translation (22) was carried out under the same conditions. After hybridization, filters were washed twice in 50% formamide, 0.3 M NaCl/0.03 M sodium citrate, and 0.5% $NaDodSO_4$ at 45°C and washed overnight in 30 mM NaCl/3 mM sodium citrate and 0.1% $NaDodSO_4$ at 55°C. After autoradiography filters were rehybridized directly with pR1-7.4, which is a human ribosomal DNA clone containing sequences of both 18S and 28S rRNAs (provided by N. Arnheim, Stony Brook, NY) to visualize the positions of rRNAs and their precursors.

Quantitative Dot Blots (23). A set of nitrocellulose filters (BA85, Schleicher & Schuell) containing immobilized 1:2 serial dilutions of known amounts of nuclear or total RNA isolated from different cell lines was prepared by using a dot blot minifold (Schleicher & Schuell). Dilutions of recombinant phage λB3 and λF2 DNAs, which contain one copy each of monkey 6-kbp LINE-1 segments as well as genomic monkey DNA (1), were included in each panel as standards. Filters were prehybridized and were hybridized to ^{32}P-labeled probes under conditions specified above but sonicated *Escherichia coli* DNA was included at a concentration of 100 μg/ml. After autoradiographic exposure the Cerenkov radiation was determined on individual cut-out dots and the amount was normalized against the standards.

Hybridization Probes. Recombinant plasmids P1–P7, containing different nonoverlapping regions from within long LINE-1 family members have been described (1) (see Fig. 1 *Right* for the location of the regions on the 6-kbp-long LINE-1 segment). A human β-actin cDNA probe (pHFβA-3'UT)) (24) was provided by P. Gunning (Stanford University, Palo Alto, CA). For preparation of strand-specific RNA probes the 1.2-kbp insert in P5 that is in the vector pUC8c2 was transferred into pSP64 and pSP65 (25) vectors using *Eco*RI and *Hin*dIII sites present in the pUC8c2 polylinker. The orientation of inserts was confirmed by restriction analysis. RNA probes were synthesized by using [^{32}P]UTP (600 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and phage SP6 RNA polymerase (Promega Biotec, Madison, WI).

RESULTS

RNA Homologous to LINE-1 Sequences in Various Human Cell Lines. We analyzed both total and cytoplasmic RNA from various human cell lines by RNA blotting using as probes subcloned regions from within the 6-kbp long African green monkey LINE-1 sequence. In agreement with previous findings (8–12) total RNA in all cells examined contains heterogeneously sized LINE-1 transcripts (Fig. 1 *Left*). The sizes range from 0.5 to >14 kb and several bands of discrete size are evident. The explanation for the discrete bands that are visible at about 5 and 2 kb in most samples is unknown. They have been observed previously (11) and may be an artefact caused by the very large amounts of rRNA on the gels. The relative abundance of HeLa cell nuclear transcripts homologous to seven different and nonoverlapping subcloned regions from within the 6-kbp LINE-1 segment was measured by using the quantitative dot blot hybridization

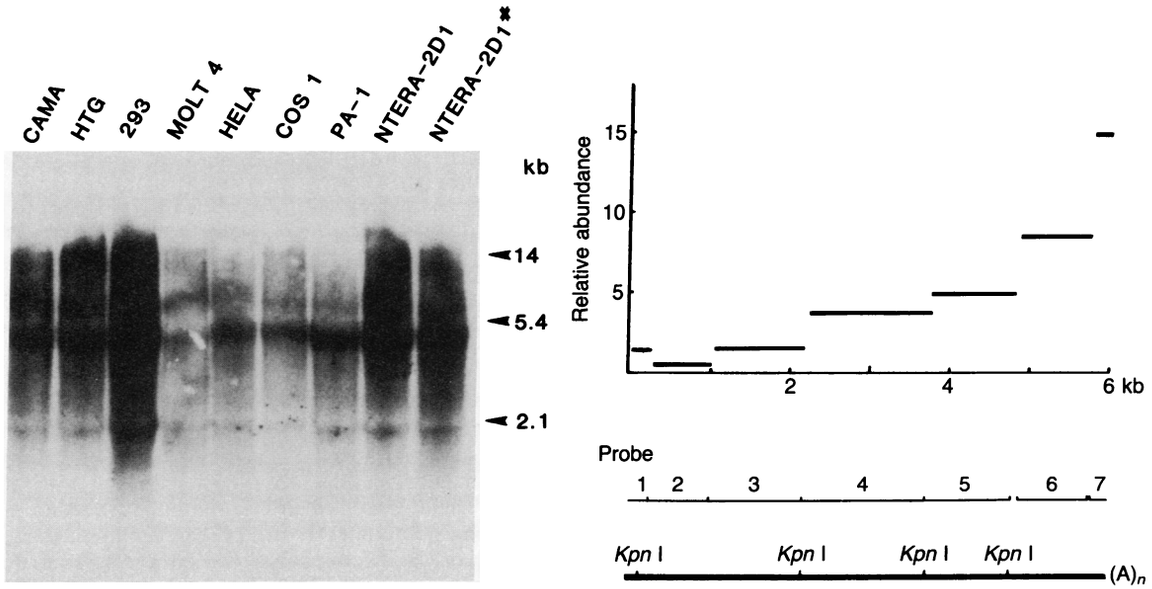

FIG. 1. LINE-1 sequences are transcribed in a variety of human cell lines. (*Left*) Total RNA (10 μg) isolated from indicated human or African green monkey cell lines was electrophoresed, blotted, and hybridized to ^{32}P-labeled probe P5 (see below). The sizes shown on the right give the location of 18S (2.1 kb) and 28S (5.4 kb) rRNAs and 45S (14 kb) rRNA precursor, which were used as size markers. NTERA-2D1* designates NTera2D1 cells differentiated by exposure to retinoic acid. (*Right*) Relative abundance of transcripts present in HeLa S3 cells that are homologous to different regions of the 6-kbp monkey LINE unit. The abscissa represents the 6 kbp of a LINE-1 element with the 3'-terminal A-rich stretch at the right. The ordinate gives the relative abundance of transcripts hybridizing to each probe, as measured with the quantitative dot blot hybridization method (23) and appropriate subcloned probes (1). The diagram underneath shows the location of subcloned fragments in the 6-kbp monkey LINE unit (1).

method (Fig. 1 *Right*). Transcripts homologous to the 5′ end of the unit are less abundant by a factor of ≈10 than those homologous to the 3′ end that contains the A-rich stretch. This distribution is similar to the relative genomic abundance of the two ends themselves (1, 8). The monkey genome, for example, contains an approximately 5-fold excess of truncated LINE-1 family members compared to 6-kbp-long members (1); truncated members typically lack varying amounts of sequence from the 5′ end of the 6-kbp element (1, 10, 27). Analysis of total HeLa cell RNA gave similar results to those shown for nuclear RNA. The relative abundance of transcripts hybridizing to P3 and P6 probes in nuclear RNA isolated from JEG-3, MOLT-4, NTera2D1, PA1, 293, and HTG human cell lines did not vary by >10% from that obtained for HeLa cells.

Additional characterization of the nuclear transcripts with strand-specific probes corresponding to subcloned probes P3 and P7 from near the 5′ and 3′ ends of the 6 kbp, respectively (see Fig. 1 *Right* for location of the probes), indicated that both strands are represented (not shown). With the P7 probe (3′ end) both strands are represented in nuclear RNA with approximately equal abundance, a result that is consistent with a previous report (12). In RNA homologous to the P3 probe, strands with a polarity opposite to that of the open reading frames are most abundant.

Although all of the cell lines examined contained abundant LINE-1 sequences in nuclear RNA, very little, if any, homologous RNA was detected in the corresponding cytoplasms (not shown), with the one exception noted below. The small amounts of hybridization detected on RNA blots with cytoplasmic RNA from HeLa, MOLT4, and 293 cells had a pattern similar to that of nuclear RNA and showed the same relative abundance pattern with probes P3 and P7 and the same lack of strand specificity when analyzed by the dot blot technique as did nuclear RNA. This RNA probably originates from contamination of the cytoplasmic RNA by nuclear RNA. Rare but discrete-sized transcripts were detected previously in a human lymphoblastoid cell line (8) but their significance was not clarified.

Cytoplasmic Poly(A)$^+$ RNA Homologous to LINE-1 Sequences in NTera2D1 Cells. The one exception to the typical finding with cytoplasmic RNA was with the NTera2D1 cell line, which is a human pluripotent teratocarcinoma line (13). Here we detected a discrete RNA about 6.5 kb long (Fig. 2). This RNA binds to an oligo(dT)-cellulose column and hybridizes with probes derived from different regions of the monkey LINE-1 DNA unit that altogether span >5 of the total 6 kbp (Fig. 2 *Upper*). This RNA species also was stably bound to poly(U)-Sepharose under conditions that remove RNA molecules containing short oligoadenylate stretches (30, 31) (Fig. 3, lanes 1 and 2), indicating that it is polyadenylylated. The ≈6.5-kb RNA hybridizes to only one of the two strands of a LINE-1 sequence, the strand complementary to that containing the open reading frames (antisense RNA) (Fig. 2 *Lower*). Thus, the features displayed by this transcript are consistent with those expected for transcripts of putative LINE-1 functional genes. The opposite strand probe (sense RNA) shows a band coinciding with the largest RNA on the blot in the experiment shown. This band was variable in intensity from one experiment to another and its significance is unknown.

The Level of Cytoplasmic Poly(A)$^+$ LINE-1 RNA in NTera2D1 Cells Varies with Phenotypes. The morphological phenotype of NTera2D1 cells depends on the culture conditions (13, 15). In low-density cultures, large flat cells predominate. Cells that have reached confluence and have begun to pile up are mainly small, with a high ratio of nucleus to cytoplasm and the characteristic appearance of embryonal carcinoma cells. The morphological differences between low- and high-density cultures are associated with the different

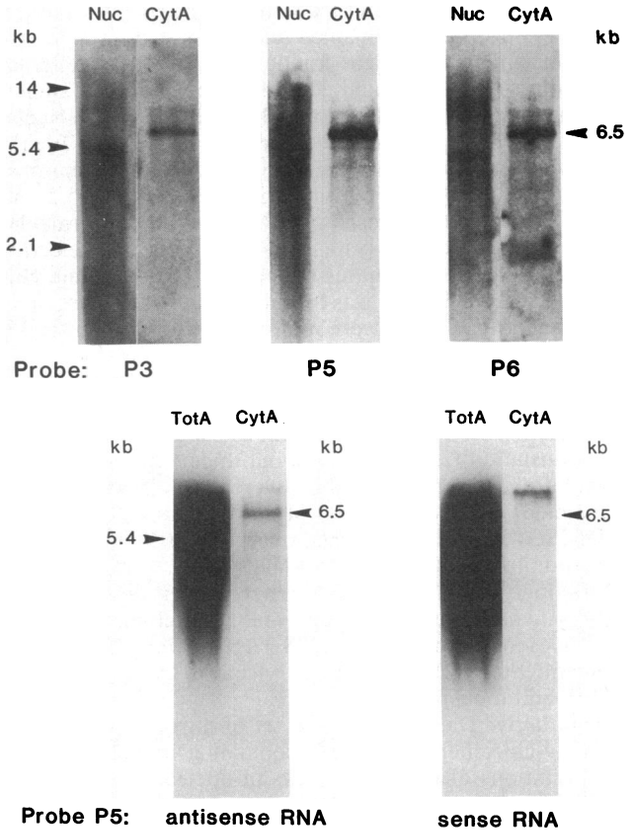

FIG. 2. A cytoplasmic polyadenylylated LINE-1 transcript in NTera2D1 cells corresponds to the LINE-1 strand that has long open reading frames. RNA was prepared from high-density NTera2D1 cells 2 days after they became confluent. The total cellular RNA for this experiment was isolated by the guanidinum thiocyanate method (28). The total and cytoplasmic RNA preparations were enriched for polyadenylylated RNA by two consecutive rounds of chromatography on oligo(dT)-cellulose columns (29). (*Upper*) One microgram of nuclear RNA (Nuc) or 5 μg of cytoplasmic polyadenylylated RNA (CytA) was separated on a formaldehyde/agarose gel, electroeluted onto GeneScreenPlus membrane (New England Nuclear), and hybridized to ^{32}P-labeled probe P3, P5, or P6. rRNA and its nuclear precursors served as size markers. The apparent band seen at about 2 kb with P6 did not coincide precisely with the lane and was not reproducible. (*Lower*) Five micrograms of total (TotA) or 5 μg of cytoplasmic (CytA) polyadenylylated RNA was electrophoresed as in *Upper* and hybridized to ^{32}P-labeled RNA probes representing each of the complementary strands of the LINE-1 segment. The filters were hybridized with RNA probes in 0.5 M NaCl/50 mM phosphate buffer, pH 7.0/5 mM EDTA/50% formamide/5× concentrated Denhardt's solution/1% NaDodSO$_4$ at 50°C, washed in 50% formamide/0.5 M NaCl/50 mM phosphate buffer at 60°C, and incubated with 10 μg of RNase A per ml in 0.3 M NaCl/0.03 M sodium citrate for 10 min at 37°C.

expressions of surface markers (13, 15). A third set of morphological features occurs when the cells are treated with retinoic acid; there is a differentiation into specific cell types, including neurons (14). We prepared cytoplasmic polyadenylylated RNA from each of these three types of NTera2D1 cells (Fig. 3). The 6.5-kb LINE-1 transcript is present in high levels only in RNA isolated from high-density cultures. It is present in low but detectable amounts in low-density cultures, which, in our hands, always contain a small proportion of cells with the embryonal carcinoma-type morphology typical of high-density cultures. It is undetectable in cells differentiated with retinoic acid, both logarithmically growing cells and cells from confluent cultures. Thus, expression of the putative LINE-1 mRNA correlates with only one

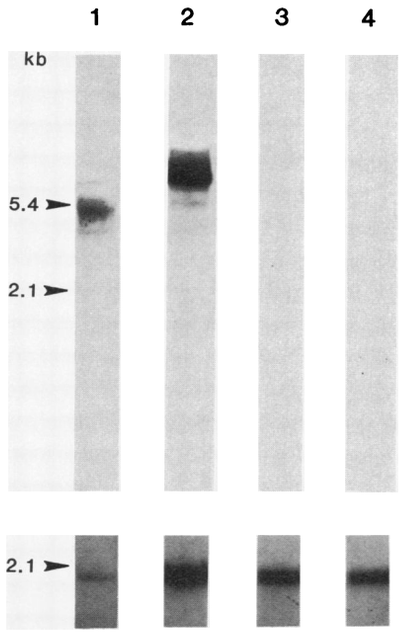

FIG. 3. The LINE-1 transcript is most abundant in NTera2D1 cells having the embryonal carcinoma morphology. (*Upper*) Cytoplasmic RNA isolated from high-density (lanes 1 and 2) and low-density (lane 3) cultures of NTera2D1 or cells differentiated in the presence of retinoic acid (lane 4) was bound to and eluted from poly(U)-Sepharose columns (Pharmacia). RNA eluted with 10% formamide (lane 1) and 90% formamide (lanes 2–4) was hybridized to the P3 probe labeled with ^{32}P by nick-translation. The 6.5-kb transcript, although not visible in lane 3, was detectable on the original autoradiogram. The sizes shown correspond to the positions of residual 18S and 28S rRNA bands. (*Lower*) After autoradiography the blot was hybridized with human β-actin cDNA probe (24).

particular NTera2D1 phenotype—namely, the stem cell, embryonal carcinoma morphology.

DISCUSSION

Several observations are consistent with a model assuming that the LINE-1 sequences abundantly represented in the nuclear RNA of all tested primate cells arise from passive transcription of LINE-1 units that are contained within unrelated transcription units. Therefore, these transcripts are not likely to be related to the productive expression of the putative functional genes within the LINE-1 family. As shown here the overall abundance in the RNA of different regions from within the 6-kbp unit reflects the genomic abundance of the respective LINE-1 segments. Nuclear LINE-1 transcripts are linked to unrelated sequences, as evidenced by their presence in the heterogeneously sized transcripts whose lengths frequently exceed that of the 6-kbp LINE-1 unit. In fact, human cDNA clones containing LINE-1 sequences, including one with an inverted LINE-1 segment, linked to unrelated sequences have been described (32). Finally, both strands of the LINE-1 unit are represented in nuclear transcripts, only a small proportion of which is polyadenylylated. Although LINE-1 sequences have not been found in the introns of primate genes yet, they are found in introns of mouse genes (33, 34).

The cytoplasms of a variety of primate cells have been analyzed for the presence of LINE-1 transcripts, generally with negative results. The few discrete-size cytoplasmic RNAs that were observed were neither polyadenylylated nor associated with polyribosomes (8). The only identified exception to these negative findings is the cytoplasmic polyadenylylated RNA detected in NTera2D1 cells. This RNA is about 6.5 kb long and hybridizes to probes covering at least 5 of the 6 kbp of the typical long LINE-1 unit. Moreover, the RNA represents the LINE-1 strand that contains long open reading frames. We suggest that this RNA may be a mRNA transcribed from one or more functional genes in the LINE-1 family.

NTera2D1 is a human teratocarcinoma cell line that expresses the stage-specific embryonic antigen 3 (SSEA-3) (13–15). SSEA-3 is also expressed in murine zygotes and early cleavage-stage embryos (35) as well as visceral extraembryonic endoderm (36). Although the relation between human teratocarcinoma cells and embryonic cells is not known, experience with the extensively analyzed murine system suggests that NTera2D1 cells may correspond to cells in cleavage-stage human embryos (15, 26). If so, then our results suggest that the proposed functional genes in the LINE-1 family are active in cleavage-stage primate embryos and the same may be true of other mammals. This suggestion has interest apart from its implications for the functional role of the putative LINE-1 genes. The extraordinary abundance of LINE-1 sequences in mammalian genomes indicates that an active dispersal process must take (or have taken) place in germ-line cells or in their progenitors in early embryos. An appropriate model for the dispersal mechanism is provided by current ideas concerning the formation of processed pseudogenes: reverse transcription of a mRNA and insertion of the cDNA into staggered breaks in genomic DNA (37). The structural analogy between characterized LINE-1 sequences and processed pseudogenes has already been pointed out (1, 4, 10, 38, 39). In addition to features such as the polyadenylylation signals and 3′-terminal A-rich stretches, both 6-kbp and truncated LINE-1 sequences are often flanked by short direct repeats that appear to be target site duplications (27, 40). The presence of a cytoplasmic, polyadenylylated LINE-1 transcript in pluripotential embryonic cells meets a formal requirement for the above model.

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