Volume 15 Number 6 1987 Nucleic Acids Research

Discrete high molecular weight RNA transcribed from the long interspersed repetitive element L1Md

Jaquelin P.Dudley

Department of Microbiology, University of Texas at Austin, Austin, TX 78712-1095, USA

Received October 1, 1986; Revised January 30, 1987; Accepted February 24, 1987

ABSTRACT

The repetitive element LINE (L1) previously has been shown to contain two long open reading frames which are overlapping and out-of-frame similar to those found in retroviruses (1). In rodents and in human cells, these repeats appear to be transcribed into a heterogeneous population of RNAs in most cell types (2,3,4). No discrete transcript has been reported which is likely to be a mRNA for the open reading frames in rodent cells. In this paper, a discrete RNA species of approximately 8 kb has been identified in most murine lymphoid cells examined. This RNA is cytoplasmic and binds to oligo (dT) cellulose columns. Hybridization with labeled probes indicates that the transcript is of the same strandedness as the open reading frames. These results are consistent with proposals that L1Md is a retroposon with protein-encoding function.

INTRODUCTION

Repetitive sequences constitute a large proportion of the eukaryotic genome. The postulated function of these sequences range from an extreme parasitic role (i.e., selfish DNA whose sole purpose is self-replication) to roles in gene regulation (5,6,7). Strategies for defining possible functions for repeated DNA vary, but the structures of a number of repeated elements have been elucidated because they lie in close proximity to unique genes (8). This approach seems likely to yield useful information if repeated elements are involved in the regulation of gene expression. Structural features of these elements also may provide clues for L1 function. At least one repeated family known as LINE 1 or L1 [also called the KpnI family in primates or BamHI family, Bam5, or MIF-1 in mice (6,7,8)] has been studied because of its presence in the murine and human β-globin loci (9,10), the immunoglobulin heavy chain region of mice and rats, in the murine V_κ and C_λ loci (12,13), and in the insulin 1 gene and a Moloney leukemia virus integration site of rats (11). Interesting features of the L1 elements of Mus domesticus (L1Md) (14) include an A-rich region at one end, truncation of many repeats at varying distances from this end, and direct repeats of cellular DNA which surround both the truncated and full-length copies (5-8). The full-length

copies of L1 are approximately 7 kb in length and some contain at least two long open reading frames whose length and arrangement are similar to the gag and pol genes of retroviruses and the comparable genes of retrotransposons (1; see Fig. 1).

Retrotransposons, exemplified by Ty of yeast and copia of Drosophila, have particle intermediates which contain reverse transcriptase activity and a single RNA transcript (15-17) but unlike L1 elements (retroposons), they contain long terminal repeats (LTRs). Replication-competent retroviruses differ from L1, copia, and Ty by the presence of at least one other gene, envelope, which is produced using a transcript distinct from the gag/pol mRNA (18). Therefore, retroviruses can be distinguished from retrotransposons and retroposons by their gene arrangement as well as by their pattern of transcription.

The presence of discrete poly (A)-containing transcripts of L1 have not been documented well. In the mouse system, a number of reports suggest that there are heterogeneous mixtures of L1Md transcripts in several mouse tissues, but no discrete RNAs have been observed (2,3). Moreover these studies did not employ probes which include the less abundant 5' end and the first open reading frame. Several discrete L1 transcripts have been reported in the human system (4,19,20). In a human lymphoblastoid cell line, L1 nuclear transcripts were 10-100 times more abundant as well as more heterogeneous than cytoplasmic transcripts (19). The discrete transcripts observed were less than 5 kb in length, a result observed by others with peripheral blood cells (20). Recently, a discrete poly (A)-containing transcript of 6.4 kb has been described in a human teratocarcinoma cell line (4). Thus in only one system with a single cell type have full-length transcripts of L1 been identified.

Since discrete full-length transcripts of L1 have not been identified in rodent cells and since the distribution of these transcripts in different cell types is likely to provide clues to the function(s) of L1 open reading frames, a number of murine cell lines were examined for the presence of discrete RNAs homologous to L1Md. Several lymphoid cell lines were found to contain an 8 kb cytoplasmic poly (A)-containing RNA which is consistent with the retroposon nature of L1Md.

MATERIALS AND METHODS

Cells and culture conditions

Cell lines E102P4C, E102, GR, C6XL, ERLD, RBL-5B, 18-48, and S49 have been described previously (21,22). The 3T6 mouse line was obtained from Dr.

W. Folk, University of Texas at Austin. Lymphoid cells were grown in RPMI medium containing 10% fetal calf serum, 2 X 10^{-5} M 2-mercaptoethanol, and penicillin-streptomycin. GR and 3T6 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin-streptomycin.

Molecular clones

The Bam840, RI1350, 540, and R fragments (1; see Fig. 1) from L1Md in M13 phage replicative form DNA were provided by M. Comer, C. Hutchison, and M. Edgell, University of North Carolina, Chapel Hill, and were subcloned into pUC9 (23). The plasmid 8-29 was obtained from J. Majors, Washington University School of Medicine and contains a 1.4 kb PstI fragment of the C3H mouse mammary tumor virus (MMTV) LTR inserted in pBR322 (22). The clone pDHFR 11 containing all of the dihydrofolate reductase (DHFR) coding region and approximately 1 kb of non-coding sequence was provided by S. Sherwood and R. Schimke, Stanford University (24).

Cell fractionation, RNA extraction, and Northern blotting

Cells were fractionated into nuclear and cytoplasmic portions according to Berger and Birkenmeier (25) in the presence of 10 mM vanadyl ribonucleoside complexes. The nuclei were removed by centrifugation at 900 X g for 10 min at 4°C. The supernatant was precipitated with ethanol, and the precipitate was resuspended in 20 mM Tris-Cl, pH 7.4, 75 mM NaCl, 25 mM EDTA, 0.5% SDS, and 200 μg/ml proteinase K. The pelleted nuclei were resuspended in an identical solution and lysed by homogenization with a Virtis Ultrashear homogenizer. Nuclear and cytoplasmic fractions were extracted for RNA as previously described (26). DNA was removed by high salt extraction (27), and RNA was fractionated by oligo (dT) chromatography as reported previously (26). The RNA was subjected to electrophoresis on 1.2% formaldehyde-agarose

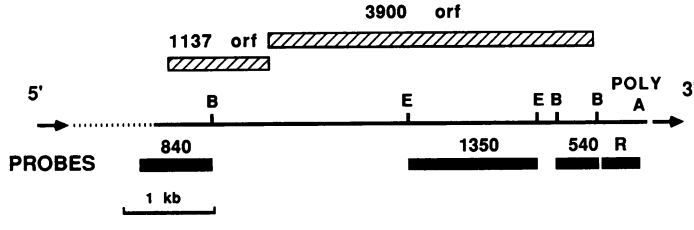
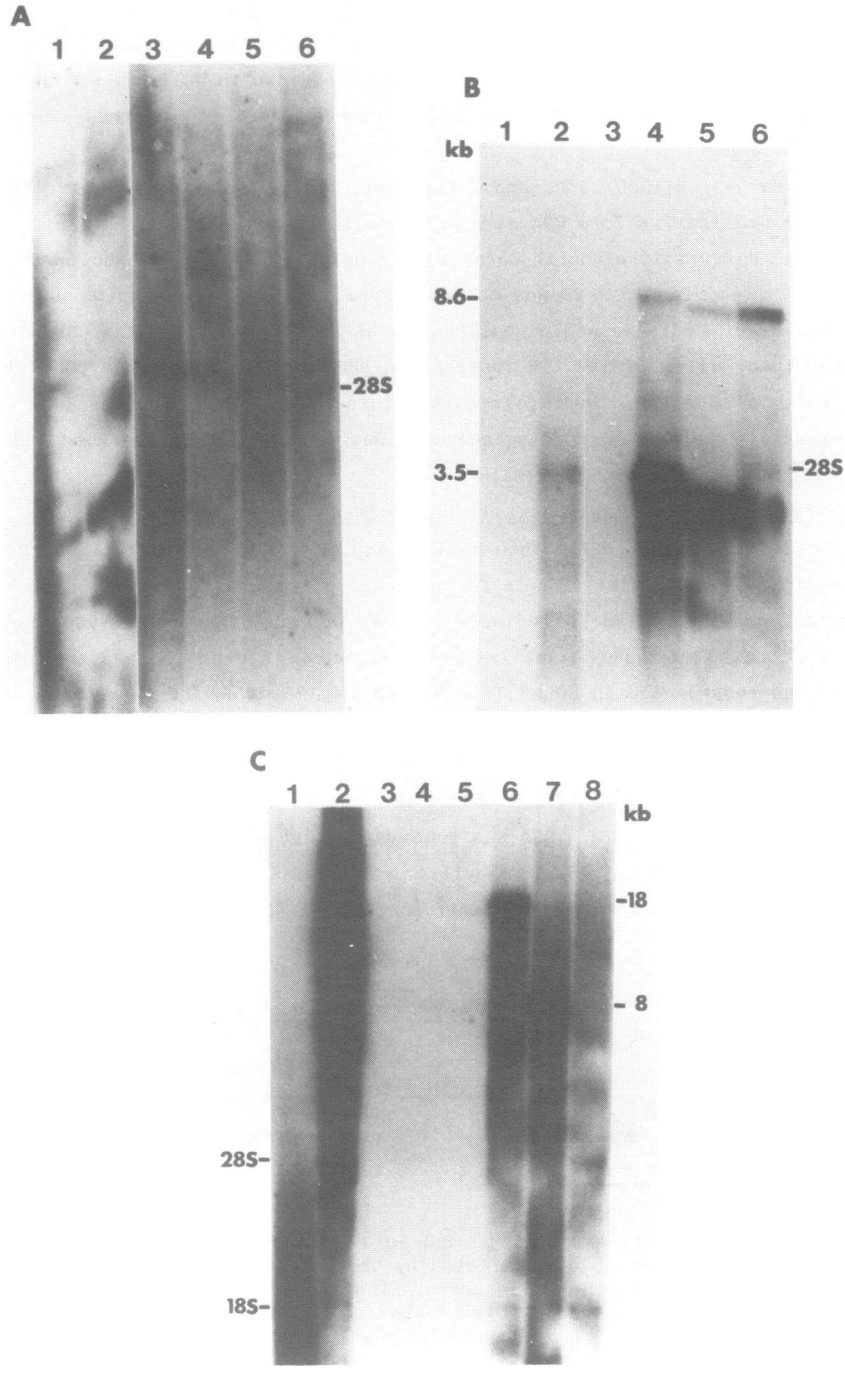


Fig. 1. Consensus restriction enzyme map of L1Md and location of probes. The open reading frames (orf) are indicated by the hatched boxes. The location of probes used is shown by the solid boxes. The arrows indicate the direct repeats of cell DNA surrounding L1Md. The consensus restriction enzyme sites are BamHI (B) and EcoRI (E). The dashed line indicates a region of direct repeats found at the 5' end of L1Md. The figure is from data by Loeb et al. (1).


A
1 2 3 4 5 6
-28S
B
kb
1 2 3 4 5 6
8.6-
3.5-
-28S
C
1 2 3 4 5 6 7 8
kb
-18
- 8
28S-
18S-

gels and transferred to nitrocellulose membranes (Schleicher and Schuell) by the method of Thomas (28). The size of RNA transcripts was estimated using denatured lambda DNA cut with HindIII, 18S ribosomal RNA (1.9 kb), and MMTV mRNA.

Preparation of probes and hybridization conditions

Double-stranded probes for L1Md, MMTV, and DHFR were prepared by nick-translation as described by Maniatis (29). Specific activities were 1-4 X 10^8 cpm/μg. Strand-specific probes were prepared as follows. The pUC9 plasmid containing the Bam840 insert was cleaved with either HindIII or EcoRI, heated at 100°C for 5 min, quickly cooled in ice water, and used in synthetic reactions containing M13 universal primer or reverse primer (Bethesda Research Laboratories), respectively. The reactions contained 1 μg template DNA, 50 ng primer, 10 mM DTT, 10 mM Tris-Cl, pH 7.9, 60 mM NaCl, 6.6 mM $MgCl_2$, 0.2 mM each TTP, dATP, dGTP, 150 μCi ^{32}P-dCTP (3000 Ci/mmole; New England Nuclear), and 1.5 units Klenow fragment of DNA polymerase I (Bethesda Research Laboratories). Reactions were incubated at 30°C for 1 h prior to separation of the unincorporated triphosphates on Sephadex G-50 mini-spin columns (Cooper Biomedical). The orientation of the Bam840 insert in pUC9 was deduced from restriction enzyme mapping. Minor amounts of hybridization seen using a probe complementary to the anti-sense strand may be due to small regions of homology in the anti-sense probe capable of base-pairing with sense RNA (as determined from computer-based homology programs). Alternatively, the anti-sense probe may contain small amounts of sense RNA probe. Both probes had specific activities of approximately 4 X 10^8 cpm/μg and had similar levels of hybridization to double-stranded Bam840 DNA. Hybridi-

Fig. 2. Northern blot analysis of nuclear and cytoplasmic RNA hybridizing to an L1 3' probe. RNA from cytoplasmic or nuclear fractions was subjected to electrophoresis on 1.2% formaldehyde-agarose gels and transferred to nitrocellulose membranes by the method of Thomas (28). Transfers were hybridized to 1 X 10^7 cpm/ml of nick-translated double-stranded Bam540 plasmid (Panels A and C) or MMTV LTR (Panel B) (see MATERIALS AND METHODS). Panels A and B show the same RNA transfer hybridized to different probes; each lane contains 10 μg. The lanes in Panel C contain 20 μg of RNA. The samples in Panels A and B are as follows: lane 1, 3T6 nuclear RNA; lane 2, GR nuclear RNA; lane 3, 3T6 cytoplasmic poly (A)-containing RNA; lane 4, GR cytoplasmic poly (A)-containing RNA; lane 5, E102P4C cytoplasmic poly (A)-containing RNA; lane 6, S49 cytoplasmic poly (A)-containing RNA. The samples in Panel C are: lane 1, 18-48 nuclear RNA; lane 2, S49 nuclear RNA; lane 3, E102 cytoplasmic poly (A)-minus RNA; lane 4, 18-48 cytoplasmic poly (A)-minus RNA; lane 5, S49 cytoplasmic poly (A)-minus RNA; lane 6, E102 cytoplasmic poly (A)-containing RNA; lane 7, 18-48 cytoplasmic poly (A)-containing RNA; lane 8, S49 cytoplasmic poly (A)-containing RNA.

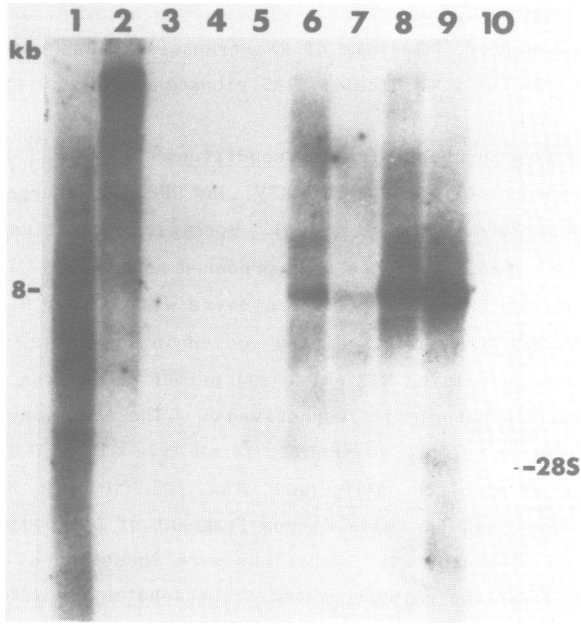


Fig. 3. Northern blot analysis of cytoplasmic and nuclear RNA hybridizing to an L1 5' probe. Transfers were hybridized to the double-stranded Bam840 probe shown in Fig. 1. Lanes 1 and 2 show nuclear RNA, lanes 3-5 show poly (A) depleted fractions, and lanes 6-10 show poly (A)+ RNA. The RNA is lane 10 was treated with boiled RNase A for 15 min prior to gel electrophoresis. The lanes with nuclear RNA contained 10 μg; all other lanes contained 20 μg. Lanes 1, 4, and 7 contain RNA from ERLD (C57BL/6) lymphoma cells; lanes 2, 5, and 8 contain RNA from E102P4C (C57BL/6) lymphoma cells; lanes 3 and 6 were from C6XL (C57BL/6) lymphoma cells; lanes 9 and 10 were from RBL-5B lymphoma cells. All tumor cells were positive for the Thy1 antigen.

zations were performed in 50% formamide at 42°C as described previously (22,26).

RESULTS

Nuclear and cytoplasmic RNA from several murine cell lines was subjected to Northern blot analysis as described in MATERIALS AND METHODS. Both nuclear and cytoplasmic RNA fractions from these mouse cells contained a population of RNA molecules ranging in size from greater than 10 kb to less than 1 kb when hybridized to a Bam540 probe specific for the 3' end of L1Md (Fig. 1; Fig. 2A, lanes 1-6). This result was not due to degraded RNA since discrete MMTV RNAs (26) could be detected on the same RNA transfer (Fig. 2B). Two cell lines also contained discrete RNAs of 18 or 8 kb with this probe

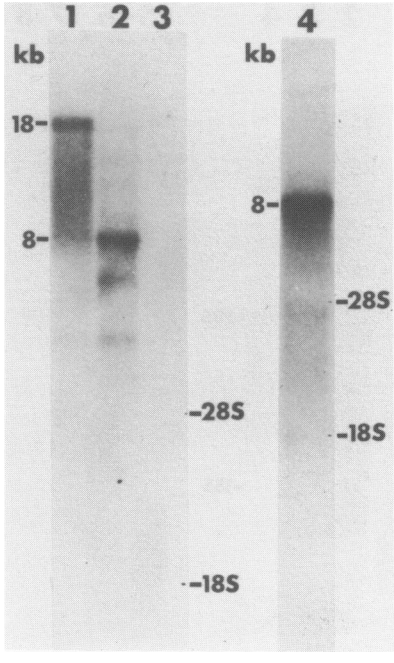


Fig. 4. Northern blot analysis of cytoplasmic poly (A)-containing RNA from various lymphoid tumor cells. RNA was prepared as described in MATERIALS AND METHODS. Twenty micrograms of poly (A)-containing RNA was used in each lane and hybridized to the double-stranded Bam840 probe. RNA from the following sources was used: lane 1, E102 Abelson-transformed (C57BL/6) tumor cells; lane 2, 18-48 Abelson-transformed (Balb/c) tumor cells; lane 3, S49 (BALB/c) T-cell lymphoma; lane 4, RBL-5B (C57BL/6) T-cell lymphoma. The E102 and 18-48 cell lines lack the Thy1 antigen.

(Fig. 2C, lanes 6 and 7,respectively). Comparable RNA transfers were prepared and hybridized to a probe (Bam840) (Fig. 1) which encompasses the most 5' portion of the 1.1 kb open reading frame(1). As observed with the 3' probe, nuclear RNA from several lymphoma lines was heterogeneously sized with no detectable discrete species (Fig. 3, lanes 1 and 2). Cytoplasmic poly (A)-containing RNA from four C57BL/6 (B6) T-cell lymphomas contained a discrete RNA of approximately 8 kb (lanes 6-9). This RNA comigrated with the 8 kb transcript observed with the L1 3' probe. An RNA species of this size was not observed in poly (A)-depleted fractions derived from these cell lines (Fig. 3, lanes 3-5) nor was the hybridization due to contamination by cellular DNA (lane 10). Several other lymphoid lines were examined for the presence of the 8 kb RNA. Northern blot analysis of cytoplasmic poly (A)-

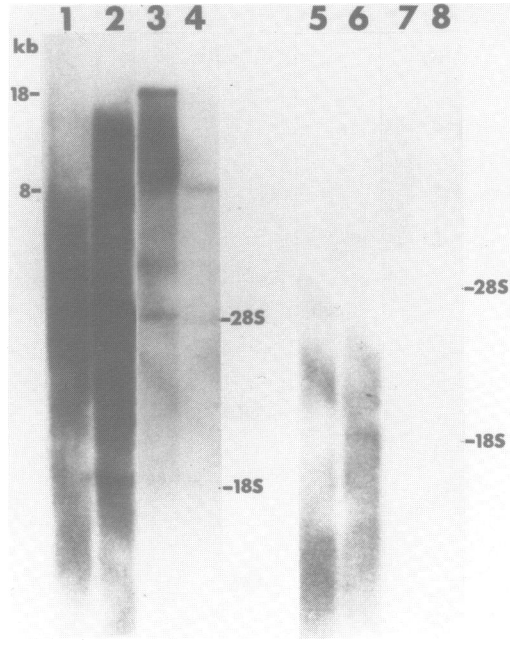


Fig. 5. Hybridization of Northern transfers to single-stranded probes for the Bam840 region. Lanes 1-4 were annealed to a probe which detected sense RNA and lanes 5-8 were annealed to the probe for the opposite strand (anti-sense RNA). All lanes contained 15 μg of RNA. Both probes had specific activities of approximately 4 X 10^8 cpm/μg and had similar levels of hybridization to double-stranded Bam840 DNA. Lanes 1 and 5, E102 nuclear RNA; lanes 2 and 6, E102P4C nuclear RNA; lanes 3 and 7, E102 poly (A)-containing RNA; lanes 4 and 8, E102P4C poly (A)-containing RNA. Lanes 5-8 were found to contain hybridizable RNA by annealing the same blot to a nick-translated probe for the mouse dihydrofolate reductase gene.

containing RNA from an Abelson virus-transformed Balb/c pre-B cell line (30) showed a transcript similar to that seen in the B6 T-cell lines (Fig. 4, lane 2) whereas a T-cell lymphoma line also derived from Balb/c mice (31) did not contain detectable cytoplasmic transcripts. Nuclear RNA from both cell lines was comparable to profiles shown in Fig. 3 (data not shown); this was not due to RNA degradation since intact MMTV mRNA (26) was detected in some cell lines. However, analysis of polyadenylated cytoplasmic RNA from E102 cells, an early B6 lymphoid cell line (30), showed a discrete transcript of approximately 18 kb (Fig. 4, lane 1) which comigrated with an RNA species detected with the L1 3' probe. This transcript was not detected in poly (A)-depleted cytoplasmic fractions from this cell line (data not shown). Both the 8 and

18 kb RNAs could be detected by probes for the RI1350 and R regions (see Fig. 1; data not shown).

An RNA species encoding the open reading frames of the full-length L1 elements would be expected to hybridize to minus-strand probes but not to plus strand probes for Bam840. Single-stranded probes were annealed to Northern transfers of nuclear and cytoplasmic RNA (Fig. 5). Both the 18 kb and the 8 kb transcripts were detected with probe complementary to the coding strand (lanes 1-4). Minor amounts of hybridization also were seen using a probe complementary to the anti-sense strand (lanes 5-8; see MATERIALS AND METHODS). Both probes showed similar hybridization to double-stranded plasmid DNA containing the Bam840 insert (not shown).

DISCUSSION

Two species of RNA (8 and 18 kb) homologous to the repetitive element L1Md have been identified in murine lymphoid cells. Both RNAs are (1) polyadenylated, (2) cytoplasmic in location, and (3) of the same strandedness as the long open reading frames described by Loeb et al (1) (Figs. 2-5). Probes which span the L1 element hybridize to both the 8 and 18 kb species, suggesting that these RNAs are transcribed from full-length repeats. Although the data do not exclude the 18 kb RNA from an mRNA role, the 8 kb species is a more attractive candidate since it closely resembles full-length elements in size and is found in several different cell lines from at least two mouse strains (BALB/c and C57BL/6).

The potential role of LINE 1 elements in the mammalian genome is unknown. Studies of the L1 copies found near unique genes, such as immunoglobulin or β-globin, have clarified the organization of the repeats (9-13) and may reveal whether L1 has a role in gene regulation. Recent results in the rat system suggest that the target sites for L1 are non-random and that their presence or absence may cause allelic variation in several single copy loci (11). Thus it is possible that the transcriptional activity of a given region may influence the ability of an L1 element to integrate and, subsequently, L1 may modify transcription from this region. Surveys of RNA homologous to the 3' end of L1 suggest that a wide variety of transcripts can be initiated from truncated L1 copies (Fig. 2). Cellular genes into which L1 has integrated may supply promoter activity. Readthrough transcription has been reported between a LINE 1 element of human DNA and an integrated human T-cell leukemia virus (32), and it is possible that the 18 kb transcript shown in Fig. 4 represents a similar situation.

L1 also may function through its open reading frames (1,14). The structure of L1Md is similar to retrotransposons such as Ty and copia which use a single mRNA transcript for two overlapping and out-of-frame genes (15-17). Therefore, the presence of a single 8 kb L1 transcript in most lymphoid cells is consistent with the transcriptional strategy used by these retrotransposons and the capacity for L1 protein expression.

A functional role for L1 protein expresssion is likely to benefit from a survey of tissues in which the full-length cytoplasmic transcripts are found. In the mouse system no discrete cytoplasmic transcripts of L1 RNA have been documented previously (2,3). Several discrete cytoplasmic transcripts of 5 kb or less have been reported for the human LINE family (19,20). It is unclear whether this is a fundamental difference in the expression of the mouse and human LINE families or whether this is due to the technical difficulties of isolating intact RNA from lymphoid cells (25). Skowronski and Singer have described a 6.5 kb transcript of the LINE 1 family in teratocarcinoma cells but not in other human or monkey cell lines including several of lymphoid origin (4). The other cell lines did contain heterogeneously sized nuclear RNAs homologous to the LINE 1 repeat (4) and similar results have been obtained using a GR mouse mammary tumor cell line and a mouse embryo 3T6 cell line (Fig. 2).

Given the apparent tissue distribution of full-length transcripts in the human and mouse systems and the reported low level homology of an L1 open reading frame to reverse transcriptase (1), it is possible that L1 is packaged into an intracellular particle in which L1 RNA is reverse-transcribed. The reverse transcripts then might be integrated into the cellular genome in regions of active transcription by RNA polymerase II; other regions also might be used for integration but at a lower frequency. The effect of these integrations would be to create diversity in these areas. In early stage cells, some of these integrations will be beneficial while others will be neutral, lethal or potentially carcinogenic (19). It is interesting to note that a retrotransposon of mice, the intracisternal A particle (IAP), often is expressed in embryonic and lymphoid tumor cells (33,34). Like other transposons, IAPs have been shown to participate in gene activation (e.g., the oncogene c-mos) (35,36). Whether the L1 read-through transcript reported by Okamoto et al. (32) or the 18 kb transcript found in E102 cells (Fig. 4) is involved in tumorigenesis remains to be determined.

ACKNOWLEDGEMENTS

I am indebted to M. Edgell and M. Comer for cloned DNA from L1Md and for helpful discussions. W. Folk, H. Bose, W. Sugden, and P. Gottlieb provided useful suggestions on the manuscript. This work was supported by Grant F-956 from the Robert Welch Foundation and Grant R01 CA34780 from the National Institutes of Health.

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