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Solitary Human Endogenous Retroviruses-K LTRs Retain Transcriptional Activity *in Vivo,* the Mode of Which Is Different in Different Cell Types

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Solitary long terminal repeats (LTRs) of human endogenous retroviruses (HERVs), tens of thousands of which are spread all over the genome, contain a variety of potential transcription regulatory elements. Information on transcriptional behavior of individual solitary LTRs, however, is limited. We studied the transcriptional activity of several individual HERV-K LTRs in a variety of tissues and cell lines. The RT-PCR technique targeted at specific amplification of the U3 or U5 regions of individual LTRs together with their unique genomic flanks was used to estimate the content of each region in the transcripts. An unequal abundance of the U3 and U5 regions in the transcripts of the same LTR in different cells and tumors was observed. Each LTR is transcribed differently in different cells or tissues, and transcriptional behavior of different LTRs was different in the same cell line or tissue. The transcriptional status of LTRs varies in response to mitogenic and stress factors and in tumor tissues compared to normal counterparts. The LTRs thus seem to be the subjects of specific transcription regulation. The data obtained indicate that an appreciable fraction of the LTRs retained regulatory potential throughout millions of years of evolution and thus may contribute to the overall transcription regulatory network. © 2001 Academic Press

Key Words: human genome; retroelements; human endogenous retrovirus; testicular germ cell tumor.

INTRODUCTION

It is becoming increasingly clear that the gene regulation in mammalian genomes involves enormously complex networks of *cis*-regulatory elements interacting with *trans*-acting factors to achieve and maintain correct tissue and developmental specificity of gene expression. The *cis*-regulatory sequences can be physically very distant from the genes under their control. Sometimes the sequences responsible for correct spatial and temporal regulation of a particular gene locus are scattered over hundreds of kilobases of DNA (for a brief review see Bonifer (1999)). Despite a great many efforts to decipher the regulatory system, it is still very far from comprehensive understanding. To reach such an understanding it would be necessary to undertake a genome-wide investigation of all DNA segments with structural potential for gene regulation. Among the multitudes of candidate sequences that may be a part of the genome regulatory machinery, different types of human endogenous retrovirus (HERVs)[2] and their long terminal repeats (LTRs) are probably the most obvious. HERVs are composed of sequences related to viral *gag, env,* and *pol* genes and flanked by LTRs. Different families of HERV elements were identified in the human genome (Lower *et al.,* 1996; Steinhuber *et al.,* 1995). Most HERV elements (85%) are represented by solitary LTRs with the internal sequence lost by homologous recombination between the flanking LTRs. The HERVs are interspersed throughout the genome and likely represent footprints of ancient germ-cell infections occupying more than 8% of the human genome (Lander *et al.,* 2001). One of the most biologically active HERV families, HERV-K, comprises about 30–50 proviruses, some of them containing almost complete retroviral genomes (Barbulescu *et al.,* 1999; Berkhout *et al.,* 1999; Mayer *et al.,* 1997). The estimated number of HERV-K-related solitary LTRs is several thousand copies per haploid human genome (Leib-Mosch *et al.,* 1993).

Solitary LTRs of endogenous retroviruses contain a variety of potential transcription regulatory elements such as promoters, enhancers, hormone-responsive elements, and polyadenylation signals that are conserved among evolutionarily distant LTRs (Lavrentieva *et al.,* 1998). Promoter and enhancer activity of solitary HERV LTRs of different families was detected in transient transfection assays (Casau *et al.,* 1999; Domansky *et al.,* 2000; Feuchter and Mager, 1990; Sjottem *et al.,* 1996), and it was also shown that HERV LTRs could specifically interact with cellular regulatory proteins (Akopov *et al.,* 1998;

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[2] Abbreviations used: HERV, human endogenous retrovirus; LTR, long terminal repeat; TGCT, testicular germ cell tumor; Myr, million of years.

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Knossl *et al.,* 1999; Sjottem *et al.,* 1996). It might therefore be supposed that at least some of the LTRs are active *in vivo* and involved in transcription regulation of cellular genes. Indeed, such an involvement has been demonstrated for a few LTRs of different HERV families (Britten, 1997; Di Cristofano *et al.,* 1995; Feuchter *et al.,* 1992; Feuchter-Murthy *et al.,* 1993; Kato *et al.,* 1990; Liu and Abraham, 1991). Recently it was reported that HERV insertion can change the tissue-specificity of the expression of the human growth factor pleiotropin (PTN) gene (Schulte and Wellstein, 1998). A HERV insertion has occurred after the divergence of apes and Old World monkeys (it exists in humans, chimpanzees, and gorillas but not in rhesus monkeys) and generated an additional promoter with trophoblast-specific activity (Schulte *et al.,* 1996). As a result, fusion HERV-PTN transcripts were formed in human trophoblasts but not in normal adult tissues.

Known genes and HERV-K LTRs are often located in the same loci of human chromosome 19 (Vinogradova *et al.,* 1997), suggesting that the influence of the LTRs on expression may be a rather common event. It should be noted that all the LTRs identified so far as components of gene regulatory systems were positioned just in the 5′-upstream regulatory areas of the genes. Clearly, the more distant the LTR is from the gene, the more difficult it is to identify the LTR as an element essential for the expression of that gene. An example of a long-distance ERV-9 LTR possibly involved in functioning of a locus control region of human beta-like globin genes in erythroid cells has been reported (Long *et al.,* 1998). No doubt, a great many LTRs with their enormous regulatory potential can be similarly involved in regulation. That is why the study of individual LTRs and, in particular, those located at long distances from the genes (we will call such LTRs "orphan" LTRs) would help to reveal many cryptic elements of expression regulation in different parts of the genome.

It should be stressed that all earlier data just indicate the functional activity of the LTRs *in vivo.* All these results were obtained using the *in vitro* transfection approach. At the same time, various LTRs, even those belonging to a single family, differ substantially in their primary structures (Lavrentieva *et al.,* 1998), indicating differences in their transcriptional activities. Recent reporter gene assays (Casau *et al.,* 1999; Domansky *et al.,* 2000) demonstrated an essential difference in enhancer and promoter activities of individual HERV-K LTRs. However, these *in vitro* experiments did not provide details of the *in vivo* characteristics of individual LTR behavior. To address this question, we compared here the modes of transcription of a set of individual HERV-K LTRs in different testicular germ cell tumors (TGCTs), in normal tissues (testicular parenchyma, placenta), and in cell lines.

RESULTS

Choice of LTRs used for the research

Previously we demonstrated that LTRs belonging to the HERV-K family can be assigned to several subfamilies (Lavrentieva *et al.,* 1998) that arose in the human genome 50 to about 2 Myr ago in the course of primate evolution. Here, to compare the *in vivo* transcriptional status of differently aged individual LTRs, we singled out four HERV-K LTRs located on different human chromosomes (Table 1) in sequenced human genome loci available in GenBank and other genome sequence databases. The choice was based on two criteria: (i) the LTRs should be full-sized to comprise all regulatory elements and (ii) they should not be flanked by repeated sequences to allow design of unique PCR primers (see Fig. 1). Two of the LTRs selected were present only in humans, whereas two others, apparently inserted much earlier, were detected in the genomes of apes. Detailed structural characteristics of these LTRs and an experimental estimation of their ages have been published previously (Lebedev *et al.,* 2000).

Experimental approach

HERV solitary LTRs are multifunctional elements potentially able to serve as promoters, enhancers, transcription terminators, etc. At this point it is very difficult to reveal long-distance regulatory effects of LTRs (such as enhancer, silencer, or insulator activity). Therefore we focused on studying the activity of the LTRs in cases where they could take part in regulation being at the same time parts of the transcripts containing adjacent genomic regions. The approach used in this study is outlined in Fig. 1. Three principal modes of involvement of individual LTRs in transcription can be envisaged: (i) an LTR can act as a promoter and initiate transcription; (ii) it can terminate transcription initiated from an external promoter; and (iii) it can be a part of a transcript initiated from an external promoter and terminated somewhere outside the LTR. Other, less obvious interpretations of the RT-PCR results are also possible, e.g., inefficient termination of transcription within the LTR will lead to "read-through" transcripts (see also below). The above-mentioned three modes can be distinguished by RT-PCR using two different pairs of primers. The first pair includes a primer targeted at a unique genomic region adjacent to the LTR U3 region and a primer targeted at the U3 region itself (primers P1 and P2 in Fig. 1). The second pair consists of a primer targeted at the unique genomic region flanking the U5 region of the LTR and a primer corresponding to the LTR U5 region itself (primers P3 and P4 in Fig. 1). If an LTR initiates transcription similarly to retroviruses at the border of the U3 and R regions, an amplification product will be observed only in RT-PCR with the primers P3 and P4, whereas the tran-

TABLE 1

Transcription of Individual LTRs in Various Tissues and Cell Lines

	LTR-containing sequence Accession No., location (LTR age)							
	L47334 19q13.2 (<5.6 Myr)		AC002508 7q31.3 (<5.6 Myr)		BC52374 19q13.1 (13–18 Myr)		AB006684 21q22.3 (<18 Myr)	
Tissues and cell lines	U3	U5	U3	U5	U3	U5	U3	U5
Tissues								
Placenta	−	−	−	−	−	−	−	+
Normal testicular parenchyma	−	−	−	−	+	−	−	−
Embryonal carcinoma	−	+	−	−	+	−	+	−
Seminoma	−	−	+	−	+	−	−	+
Teratoma	−	−	−	−	+	−	−	+
Mixed tumor (teratoma, yolk sac tumor)	−	+	−	−	−	−	−	+
Cell lines								
Lymphoma cell line, Jurkat	+	−	+	−	+	+	+	−
Jurkat, treated with PHA/TPA	−	−	+	−	+	+	+	−
Jurkat, heat-shocked	−	+	+	+	+	+	−	−
Embryonal carcinoma Tera 1	+	−	−	−	−	+	+	−
Rhabdomyosarcoma, RMS-13	−	−	−	+	+	+	−	−

Note. "+," transcription detected; "−," no transcription (for quantitative definitions see the text).

script will be amplified with the primers P1 and P2 when the LTR terminates transcription. Both pairs will work, in particular, when the LTR is embedded in a longer transcript. To discriminate between various individual LTRs different unique P1 and P4 primers corresponding to different LTR flanks can be used.

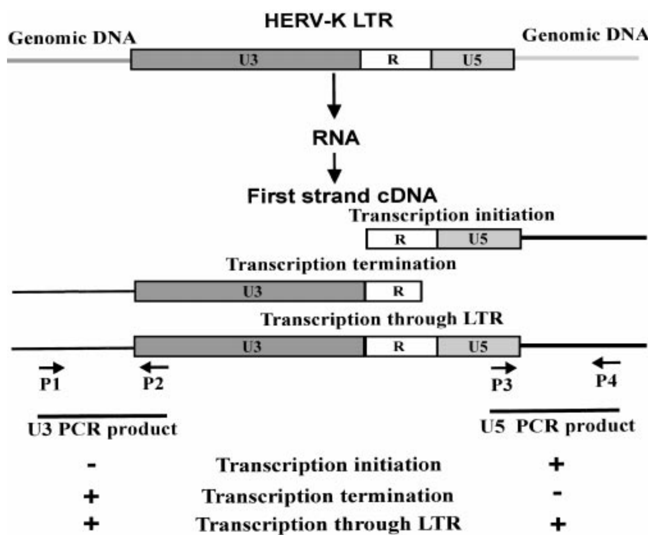

FIG. 1. Design of the differential RT-PCR experiments. P1/P2 and P3/P4 PCR primer pairs were used to amplify the U3 or U5 regions of the HERV-K LTR, respectively. Putative LTR activities and the corresponding RT-PCR products are indicated in the bottom part of the figure. Note that RT-PCR using primers P1/P4 will amplify both the LTR-containing genome locus and a product of the read-through transcription.

When comparing transcription levels of U3 and U5 regions, we considered the region transcribed ("+" in Table 1) when at least 100 ng of the PCR product was accumulated after 37 PCR cycles. In most cases, however, we detected 100 ng of the PCR product after 35 PCR cycles or even less. Amounts of the RT-PCR products in corresponding "−" (not transcribed) samples were beyond the sensitivity of detection or less than 5% of the amounts of the faintest "+" samples. Though only semiquantitative, this approach does reveal qualitative differences in the transcriptional status of LTRs.

First-strand cDNAs prepared using total RNAs isolated from different human tissues or cells were used as templates for PCR with pairs of primers schematically shown in Fig. 1. To prevent missing non-polyadenylated LTR-containing regulatory transcripts or intron-embedded LTRs, cDNA was synthesized with a mixture of oligo(dT) and random primers. However, the RT-PCR results were qualitatively the same when only random hexamers were used for priming the cDNA synthesis (not shown). In parallel, control samples prepared as above but in the absence of reverse transcriptase were analyzed to demonstrate the absence of residual genomic DNA.

Genomic DNAs of all cell lines and tissues were controlled by PCR amplification with primer pairs P1 and P4 to ensure that the loci under study are present and not rearranged (data not shown). No PCR products were detected in the same experiments when rodent genomic DNA or rodent cDNA not containing any HERV sequences was used as template.

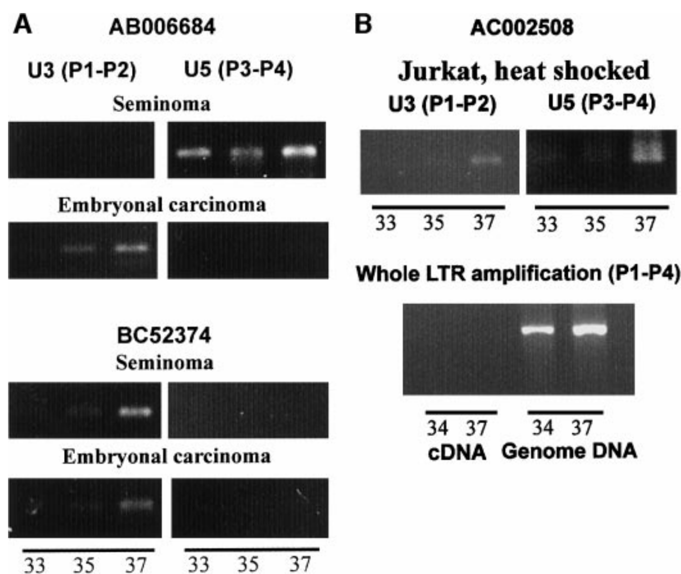

FIG. 2. Examples of the differential RT-PCR. (A) Different activities of individual LTRs in different tumors. U3 and U5 correspond to the amplification of the LTR U3 and U5 regions. GenBank accession numbers of the sequences harboring the corresponding solitary LTR are indicated; figures labeled 33–37 indicate the number of amplification cycles. (B) Simultaneous amplification of the U3 and U5 regions of AC002508 LTR in the heat-shocked Jurkat cells. The absence of the read-through PT-PCR product is confirmed using primers P1 and P4. For details see the text.

The transcriptional activity of all LTRs tested was compared to that of actin. The level of LTR transcripts was about 0.1–1% of the actin transcripts in the same tissue/cell line (data not shown). Although in low abundance, these transcripts could be reliably identified by distinct PCR products after 33–37 cycles of PCR.

Transcription of different LTRs in different tissues

As seen in Fig. 2A, for the LTR from a genomic sequence with GenBank Accession No. AB006684, selective transcription of its U5 region was characteristic of seminoma, whereas only the U3 region was transcribed in embryonal carcinoma. Consequently, one and the same LTR can act differently in different cell lines. In contrast, for the LTR from the BC52374 sequence only transcripts of its U3 region were found both in seminoma and embryonal carcinoma, with transcription of the U5 region not detected in any of the tumor or normal tissues tested.

The transcription data for all four LTRs and a panel of cDNAs from six tissues and three cell lines are summarized in Table 1, which clearly demonstrates that, generally, the transcription pattern of individual LTRs depends on the particular tumor tissue or cell line. For instance, the transcription of the LTR located within the AC002508 sequence was not observed in placenta and embryonal carcinoma tissues, while its U3 and U5 regions were transcribed in seminoma and rhabdomyosarcoma cells, respectively. Moreover, transcription patterns of individual LTRs can be different even in a tumor and its normal tissue counterpart, or in closely related tumors (Table 1).

Transcription patterns of different LTRs in the same tumor tissue or cell line also differ; e.g., the LTRs from AC002508 and BC52374 loci were represented in seminoma transcripts by their U3 parts and the LTR from AB006684 was represented by its U5 part, whereas no transcripts containing LTR from L47334 were detected. Hence, the LTR-specific transcription does not follow changes in the total transcription level of HERV genes known to be increased in seminomas and other testicular germ cell tumors (Roelofs *et al.,* 1998) and placenta (Medstrand and Blomberg, 1993).

The mode of a given LTR transcription in the same cell line could be changed after heat shock or treatment with mitogenic agents like phytohemagglutinin (PHA) and phorbol-12-myristate 13-acetate (PMA). As seen from Table 1, when Jurkat cells were subjected to heat shock, qualitative changes in the mode of functioning were detected for three of four LTRs under study. After treatment of Jurkat cells with PHA/PMA the transcription of the U3 region of the LTR from L47334 locus ceased.

When an LTR is transcribed in a given tissue or cell line, in most cases either U3 or U5 transcripts are present, alternatively. However, there are exceptions when both U3 and U5 regions of the LTR are transcribed in the same cell type (mostly in lymphoid cells) with similar efficiency, as for the BC52374 LTR transcription in Jurkat and rhabdomyosarcoma cells (Table 1). In all such cases we performed additional experiments to discriminate between two possible explanations of this effect: (i) it is due to the presence of all primer binding sites within the same read-through transcript as shown in Fig. 1 or (ii) the effect results from simultaneous independent transcription of the U3 and U5 parts of the LTR. For this purpose RT-PCR with primer pairs corresponding to genomic flanks of LTRs has been carried out. The results obtained for the LTR within the AC002508 locus in Jurkat cells after heat shock are presented in Fig. 2B. As can be seen, no PCR product of the expected length (~1 kb) was detected, despite the presence of this locus in the genomic DNA. Similar results were obtained for four other LTR/cell line combinations (see Table 1). Therefore, the pattern observed is due to the generation of two independent types of transcripts rather than to the formation of read-through transcripts. This, in turn, suggests that a single solitary LTR may function in the same cell either as a promoter and transcription terminator or as a direct and reverse promoter (see Discussion). This dualism can be also due to the heterogeneity of the cell cultures used, e.g., if LTRs are transcribed differently at different stages of the cell cycle.

There is no straightforward correlation between the activity and the age of LTRs

One of the questions of interest was whether the transcriptional activity of the LTRs analyzed correlates with their experimentally determined evolutionary ages (Lebedev *et al.,* 2000). Surprisingly, older LTRs (BC52374 and AB006684), which can also be found in the genome of gibbon, are transcribed in a greater number of cell lines than younger, human-specific LTRs L47334 and AC002508 (see Table 1). There is no easy explanation for this phenomenon, and the results obtained are insufficient to consider it a general rule. The question is discussed in some more detail below.

DISCUSSION

In this research we, for the first time, investigated the functional *in vivo* repertoire of individual solitary HERV-K LTRs with known positions in the genome. There are three main conclusions from the results obtained:

All four LTRs retained some transcriptional activity *in vivo*

This finding does not endorse the widespread opinion that a great majority of the retroelements are inactivated in the host genome either by mutations or epigenetically by means of, for instance, methylation (Yoder *et al.,* 1997). We addressed the question of whether the transcripts we observed are significant or represent just a background transcription that can take place from any promoter. There are two lines of evidence in favor of significance of the transcription. First, the transcriptional activity of all LTRs tested was not less than 0.1–1% of the actin transcripts in the same tissue/cell line (data not shown). Actin mRNA is known to be an abundant transcript, represented by 1000–3000 copies per cell (Femino *et al.,* 1998). We tested the presence of the actin mRNA in the different tissues that we used and found that it was approximately equal in all of them. Therefore we can estimate the transcription level of LTRs as 1–30 copies per cell. This level corresponds to low-abundance mRNAs produced by a great majority of cellular genes (Nelson *et al.,* 1999). Second, the observation that one and the same LTR can be either transcribed or not depending on the tissue is, in itself, evidence of specific and not background transcription.

The LTRs used in this work were singled out practically randomly without any preselection for biological activity. One of the criteria for the choice was structural—the chance of full-size LTRs being transcriptionally active seems to be higher because they retain all features of the retroviral LTRs. Another criterion was purely technical and required LTRs with unique genomic flanks at least 150 bp in length to allow PCR primer design. According to our estimation based on sequences flanking 234 LTRs available in GenBank, this fraction constitutes 37% of all full-length HERV-K solitary LTRs. Therefore we can conclude that a significant fraction of HERV-K solitary LTRs retained some transcriptional activity *in vivo.*

Though we do not know whether the LTR-mediated transcription is essential for genome functioning, the results suggest that there is a multitude of active LTRs that can affect genome activity in many ways through intrinsic regulatory motifs, and the LTRs might be considered components of the regulatory genomic network.

The LTR transcription activity and its pattern (U3 or U5) apparently do not correlate with the LTR integration age

In the reporter gene assays using constructs with LTRs lacking their genomic flanks the most active LTRs fell into rather young group integrated just before or even after the divergence of the human and chimpanzee lineages (Casau *et al.,* 1999; Domansky *et al.,* 2000). The situation *in vivo* is more complicated since the LTR regulatory activity in this case can depend on the genomic and/or chromatin context and on cellular transcription factors. As a result, highly active *in vitro* LTRs may have low activity in their native environment. The LTR activity–age correlation can also be obscured by different evolutionary pressures: the LTRs essential for the cell can mutate at lower rates than their "indifferent" counterparts.

Each of the LTRs has its own peculiar pattern of transcription in various cell lines and tissues

This is the case even for closely related cells, such as embryonal carcinoma and Tera1. This finding is not quite unexpected in view of the data on transcription behavior of LTRs in transient reporter gene expression assays (Casau *et al.,* 1999; Domansky *et al.,* 2000), although these data could not be directly extrapolated to the *in vivo* functional properties of LTRs.

It is surprising that each LTR can be transcribed in varying manners, depending on cells and conditions: only U3, only U5, or both parts simultaneously. It is known that LTRs are able not only to initiate transcription of some human genes but also to terminate it (Baust *et al.,* 2000; Mager *et al.,* 1999). The U5-containing transcripts can be interpreted as a result of transcription initiation within LTRs usually believed to occur at the border of the U3 and R regions. In this case the resulting transcripts are supposed to comprise the whole U5 region as observed in some of our experiments. The U3-containing transcripts could be a result of transcription termination within LTRs. However, as we demonstrated previously (Domansky *et al.,* 2000), HERV-K LTRs, including the LTR from the L47334 locus used in this work, retain significant promoter activity when placed in re-

TABLE 2

Primers Used for LTR Sequence Amplification

Primer location	Corresponding Sequence GenBank Accession No.	Flank	Primer sequence, 5′-3′
Primers targeted at unique sequences flanking specific LTRs (P1 and P4, see Fig. 1A)	L47334	U3	CAGTCTTATCTCCTTTACTGACC
		U5	CCAAGCACAAACACGAGGC
	AC002508	U3	CTCCCATTTTAATTTAGCACCG
		U5	CATCTCTGGGCTAAGGCATC
	BC52374	U3	GGCTGGCTTTTCAGGTCG
		U5	GTCAGTGGCTGCCTGCTGATTTG
	AB006684	U3	TTGGGATGACCAGTAACCG
		U5	CATCTCTGGGCTAAGGCATC
Internal LTR primers (P2 and P3, see Fig. 1A)	All LTRs except BC52374	U3	TGTTCCAGAGAGCACGGGGTGGG
	BC52374		AACCCTGATTCAATACAACACATG
	All LTRs	U5	TCCTCCGTATGCCTGAACGCTGGTTCC

verse (U5-R-U3-reporter gene) orientation in transient expression assays. A similar effect was observed previously for LTRs of another ERV family, HERV-H (Feuchter and Mager, 1990). Therefore, the presence of the U3-containing transcripts can be alternatively explained by the assumption that the LTRs can function *in vivo* as “reverse” promoters initiating transcription directed toward the U3 region. The approach used here cannot distinguish between these two possibilities. However, our results indicate that whatever function of the LTR was in a given tissue or cell line, it can be completely different in other tissues or cell lines, or even in the same cells after exposure to some external factors. For instance, an LTR functioning as a direct promoter in one tissue can serve as a transcription terminator/reverse promoter in another tissue, and so on.

CONCLUSIONS

The LTRs studied here are characterized by completely different positions in the human genome (Table 1). The LTRs within BC52374 and AB006684 are located in proximity to known transcribed sequences. Downstream of the BC52374 LTR, several actively transcribed human ESTs were found. The LTR from the AB006684 locus is located ~1 kb upstream of the transcription start of the *AIRE* gene on chromosome 21 (Aaltonen and Bjorses, 1999) and could take part in its regulation. However, no genes or even significant open reading frames have been identified thus far within 5 kb upstream and downstream of the LTRs within L47334 and AC002508. What function can be assigned to these orphan LTRs? They could serve, for example, as promoters for not yet identified noncoding RNAs (Eddy, 1999). Moreover, they could be good candidates for the production of regulatory antisense and/or RNAi transcripts (Bosher and Labouesse, 2000) and/or initiate and terminate intergenic transcripts playing a role in chromatin remodeling (Ashe *et al.,* 1997; Gribnau *et al.,* 2000).

All the LTRs studied here are transcriptionally functional regardless of their location. The multitude of other similar LTRs dispersed throughout the genome may also retain their transcriptional potential, and their role in the genome regulation network should be taken into account.

MATERIALS AND METHODS

Cell lines

The cell lines used in this work were obtained from the collection of the Institute of Cytology, St. Petersburg, Russia, except rhabdomyosarcoma cell line RMS13, which was kindly provided by Dr. P. S. Meltzer, National Center for Human Genome Research, NIH. Cells were grown in RPMI 1640 medium with 10% fetal calf serum, 2 mM glutamine, and 10 μg/ml gentamicin. Jurkat cells were stimulated with PHA (2 μg/ml) and PMA (50 ng/ml) for 24 h. For heat shock, a suspension of Jurkat cells at about 600,000 cells/ml was centrifuged, resuspended in growth medium (1/10 of initial volume), and incubated for 15 min in a water bath at 45°C. After the heat shock the cells were diluted to the initial volume with fresh medium and grown for 6–8 h before RNA isolation.

Tissue samples

Human term placenta was obtained from the Cancer Research Center. Tumors and parenchyma were sampled from orchidectomy specimens with TGCTs under nonneoplastic conditions. Representative samples were divided into two parts; one was immediately frozen in liquid nitrogen, and the other was formalin-fixed and paraffin-embedded for histology analysis.

Oligonucleotide primers

Oligonucleotide primers for PCR amplification (Table 2) were synthesized using an ASM-102U DNA synthesizer (Biosan, Novosibirsk, Russia).

RNA isolation and RT-PCR

Total RNA was isolated from frozen tissues pulverized in liquid nitrogen and from cell lines using an RNeasy Mini RNA purification kit (Qiagen). All RNA samples were further treated with DNase I to remove residual DNA. cDNA synthesis was performed according to a standard protocol using mixed oligo(dT) and random hexamer primers with (+RT) or without (−RT) addition of AMV reverse transcriptase. The efficiency of cDNA synthesis was equal in all preparations, as it was verified using RT-PCR with primers specific for the beta-actin gene (Gene Checker Kit, Invitrogen).

A cDNA equivalent of 20 ng total RNA was used as template in each PCR. PCR was performed in a final volume of 50 μl using unique genomic primers and the corresponding (U3 or U5) LTR primer (Table 2). Ten microliters of the reaction mixture after 33, 35, and 37 cycles of amplification was analyzed by electrophoresis in 2% agarose gels. Gel images obtained by charge-coupled device camera systems were quantitated using Gel-Pro analysis software (Media Cybernetics). No transcription detected ("−" in Table 1) meant that less than 5 ng of the product was formed after 37 cycles of PCR (detection sensitivity). In contrast, the transcription level was considered significant if at least 100 ng of the corresponding product was accumulated after 33–37 cycles of PCR ("+" in Table 1). All RT-PCR experiments were reproduced at least twice using different cDNA preparations.

Genomic PCR

Ten nanograms of genomic DNA purified from tissues and cell cultures was PCR-amplified with primers specific for all four studied LTR-containing genomic loci (Table 2) under the same conditions as RT-PCR but using 27–32 cycles.

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