THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 3, Issue of January 19, pp. 1896–1903, 2001

Printed in U.S.A.

Long Terminal Repeats Are Used as Alternative Promoters for the Endothelin B Receptor and Apolipoprotein C-I Genes in Humans*

Received for publication, July 24, 2000, and in revised form, September 28, 2000
Published, JBC Papers in Press, October 27, 2000, DOI 10.1074/jbc.M006557200

Patrik Medstrand‡, Josette-Renée Landry§, and Dixie L. Mager¶

From the Terry Fox Laboratory, British Columbia Cancer Agency and Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, V5Z 1L3, Canada

To examine the potential regulatory involvement of retroelements in the human genome, we screened the transcribed sequences of GenBank™ and expressed sequence tag data bases with long terminal repeat (LTR) elements derived from different human endogenous retroviruses. These screenings detected human transcripts containing LTRs belonging to the human endogenous retrovirus-E family fused to the apolipoprotein CI (apoC-I) and the endothelin B receptor (EBR) genes. However, both genes are known to have non-LTR (native) promoters. Initial reverse transcription-polymerase chain reaction experiments confirmed and authenticated the presence of transcripts from both the native and LTR promoters. Using a 5′-rapid amplification of cDNA ends protocol, we showed that the alternative transcripts of apoC-I and EBR are initiated and promoted by the LTRs. The LTR-apoC-I fusion and native apoC-I transcripts are present in many of the tissues tested. As expected, we found apoC-I preferentially expressed in liver, where about 15% of the transcripts are derived from the LTR promoter. Transient transfections suggest that the expression is not dependent on the LTR itself, but the presence of the LTR increases activity of the apoC-I promoter from both humans and baboons. The native EBR-driven transcripts were also detected in many tissues, whereas the LTR-driven transcripts appear limited to placenta. In contrast to the LTR of apoC-I, the EBR LTR promotes a significant proportion of the total EBR transcripts, and transient transfection results indicate that the LTR acts as a strong promoter and enhancer in a placental cell line. This investigation reports two examples where LTR sequences contribute to increased transcription of human genes and illustrates the impact of mobile elements on gene and genome evolution.

A very high proportion of mammalian DNA consists of retroelements that have arisen via RNA reverse transcription and reintegration into the genome (1). Retroelements are found in most, if not all, species, where they have amplified to high copy numbers during evolution (2). The sheer number of such mobile elements suggests that they affect the host genome, and several observations indicate that retroelements impact on the species in a number of ways by acting as insertional mutagens or contributing regulatory functions to genes (3). While transposable elements can be harmful to their host, the vast majority of transposable elements present in humans are derived from ancient transpositional events which are fixed in Old World primates. Potential long term effects of the majority of these elements must be either neutral or beneficial; otherwise they would be eliminated by selection.

Human DNA contains essentially two classes of retrosequences, (i) the non-long terminal repeat (non-LTR)[1] retroposons represented by LINE and Alu sequences, and (ii) the LTR retroelements in which the endogenous retroviruses (ERVs), solitary LTRs derived from ERVs, and other LTR-like sequences fall (4). Human ERVs (HERVs) are classified into different families based on sequence similarity and monophyletic clustering (5, 6). The thousands of ERVs and solitary LTRs that are present in human DNA are the result of infections and transposition events during primate evolution. Solitary LTRs are common features in the human genome, and they probably arose from a recombination event between the 5′ and 3′ LTR of a full-length provirus. Despite their evolutionary age, many ERVs are still transcriptionally active in human cells, where different ERV families show quite different sites and levels of transcription (7). The LTR and ERV elements are especially interesting in this regard, since they naturally possess enhancer, promoter, and polyadenylation functions within their LTRs, which probably accounts for differences in transcription of the various HERV families. Besides promoting transcription of retroviral genes, several studies have demonstrated that ERVs and LTRs can assume gene regulatory functions (8–10). For example, the paratoid-specific expression of amylase in humans is dependent and under control of an HERV-E element (11). HERV-E also appears to be involved in the expression of human pleiotrophin in placenta (12). It has also been demonstrated that a HERV-K LTR encodes the last 67 amino acids of one form of the leptin receptor OBR (13). These findings indicate that an LTR insertion adjacent to or within a gene could have a variety of effects without destroying gene function. Such new insertions may alter tissue specific gene expression or enhance the general transcription levels of the gene, which could be selectively advantageous.

* This work was supported by a grant from the Medical Research Council (MRC) of Canada with core support provided by the British Columbia Cancer Agency, by the Crafoord Foundation and the Royal Physiografic Foundation, Lund, Sweden. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a fellowship from the Swedish Cancer Foundation and Knut and Alice Wallenberg Foundation, Sweden.

§ Supported by a studentship from the MRC of Canada.

¶ To whom correspondence should be addressed: Terry Fox Laboratory, BC Cancer Agency, 601 West 10th Ave., Vancouver, British Columbia V5Z 1L3, Canada. Tel.: 604-877-6070 (ext. 3185); Fax: 604-877-0712; E-mail: dixie@interchange.ubc.ca.

[1] The abbreviations used are: LTR, long terminal repeat; ERV, endogenous retrovirus; HERV, human ERV; apoC-I, apolipoprotein C-I; EBR, endothelin B receptor; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends; kb, kilobase pair(s); bp, base pair(s); HCR, hepatic control region; SD, splice donor; ET, endothelin.

TABLE I
Primers used for RNA analysis

Primer	Sequence	Position[a]
APO-ex1	5′-AGCCGCATCAAACAGAGTGAACTT-3′	+157/180
APO-ex2	5′-TCCTCCTGCTACATTCTGAGTGG-3′	−477/−455
APO-ex3	5′-ACGTGCCTTGGATAAGCTGAAG-3′	+95/+114
APO-LTR1	5′-GTCTGAGGAATTTTGTCTGCGGCT-3′	−500/−477
APO-N	5′-CCAAGCCCTCCAGCAAGGATTC-3′	−182/−161
EBR-ex1	5′-AGTCTATGTGCTCTGAGTATTGAC-3′	+571/+594
EBR-ex2	5′-GACTGGCCATTTGGAGCTGAGAT-3′	+496/+518
EBR-ex3	5′-CTTCTGGAGCAGGTAGCAGCATG-3′	−20/+3
EBR-ex4	5′-GACGCCACCCACTAAGACCTTATG-3′	+138/+161
EBR-ex5	5′-GACGCCTTCTGGAGCAGGTAGCA-3′	−25/−3
EBR-L1	5′-CATGGAGGATCAACACAGTGGCT-3′	−21,500[b]
EBR-N	5′-TTACTTTTGAGCGTGGATACTGGC-3′	−166/−143

[a] Positions relative to the first nucleotide of the translational initiation; positions upstream (−) in the genomic DNA or downstream (+) in the cDNA.
[b] Approximate position (see "Experimental Procedures.").

We are using LTR sequences to study the involvement of retroelements in gene regulation. Specifically, we have searched the expressed sequence tag and transcribed subset of GenBank™ for chimeric retroviral gene sequences. Here, we report two human genes that are affected by ERV LTRs, the apolipoprotein C-I (apoC-I) gene and the endothelin B receptor (EBR) gene. We show that these two genes use a HERV-E LTR as an alternative promoter, demonstrate the presence of the chimeric transcripts in human tissues, and test the significance of the LTRs at the genomic loci of apoC-I and EBR.

EXPERIMENTAL PROCEDURES

Reverse Transcription and PCR Amplification—Reverse transcription was done with Superscript II (Life Technologies, Inc.) using the same reaction conditions as described previously (14). PCR was carried out using 0.1–0.5 volumes of each cDNA (0.1–0.5 μg of the initial RNA) per reaction. RNA samples were either obtained from CLONTECH or prepared from different sections of placenta, as described previously (15).

The following primers were used to detect the different transcript forms shown in Fig. 2 (see Table I for primer sequences): LTR-apoC-I fusion transcript, primers APO-LTR1/APO-Ex1; native apoC-I transcript, primers APO-N/APO-Ex1; LTR-EBR fusion transcript, primers EBR-L1/EBR-Ex1; native EBR transcript, primers EBR-N/EBR-Ex1. Amplification was done by using 0.5 volumes of cDNA (see above) with the following cycling profile: one initial incubation of 95 °C for 1 min followed by 35 cycles (for the apoC-I amplifications) or 30 cycles (for the EBR amplifications) of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s, and one final elongation at 72 °C for 5 min. In the semiquantitative RT-PCR of different EBR transcript forms (Fig. 5), the following primer combinations were used: LTR-EBR fusion transcript, primers EBR-L1/EBR-Ex2; native EBR transcript, primers EBR-N/EBR-Ex2; total EBR transcript, primers EBR-Ex3/EBR-Ex2. In these experiments, 0.15 volumes cDNA was used in the same PCR profile as described above but with lower cycling (25–28 cycles) to avoid saturation effects during the amplification. The intensity of the amplification products was measured after 25 cycles from ethidium bromide-stained gels using the 1D Image Analysis software (Eastman Kodak Co.).

5′-RACE—Placental and brain Marathon-ready cDNA libraries were purchased from CLONTECH, and a 5-μl cDNA library was used in 5′-RACE analysis as described in the protocol supplied with the library. The first PCR amplification was performed using EBR exon-specific primer (Table I) EBR-ex4 and the AP1 primer (provided by the supplier) and with the apoC-I primer APO-ex1 and primer AP1. The nested PCR was performed by EBR primer EBR-ex5 and the AP2 primer (provided by the supplier) and with the apoC-I primer APO-ex2 and AP2. The following temperature profile was used for all amplifications: one initial denaturing at 95 °C for 1 min followed by 35 cycles at 95 °C for 30 s and annealing and extension at 68 °C for 4 min. The 5′-RACE products were cloned using the pGEM-T vector system I (Promega). Clones were selected for sequencing after hybridization using retrovirus-specific oligonucleotides APO-LTR1 and EBR-L1 (Table I).

Primer Extension—The oligonucleotide primer APO-ex3, complementary to exon 3 of apoC-I, was radiolabeled with γ-^{32}P, and 1.2×10^6 dpm of the labeled primer was incubated with 5 μg of total RNA in a 10-μl solution containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin at 61 °C for 20 min. The samples were then transferred to ice, and 300 units of Superscript II and 15 units of RNase inhibitor were added to the reaction and adjusted to a volume of 20 μl with a final concentration of 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, and 0.5 mM dNTPs. The primer extension reaction was performed by incubating samples at 25 °C for 10 min, 42 °C for 50 min, and 95 °C for 10 min. The reaction products were separated on a 6% polyacrylamide gel containing 7 M urea and visualized by exposing to x-ray film at −70 °C. The intensity of the extension products were measured using the ImageQuant software after incubation on PhosphorImager plates (Molecular Dynamics, Inc., Sunnyvale, CA).

Locus-specific PCR—Locus specific PCR was performed essentially as described previously (16). Genomic DNA prepared from marmoset (New World Monkey), baboon (Old World Monkey), gibbon, orangutan, gorilla, chimpanzee, and human cell lines (17) was used in PCR. Primers APO-P1 (5′-GGTTTTTACAGTGTCATCCAGCT-3′)/APO-P2 (5′-GATTCAGGTTGGTGCTGAGTG-3′) were used to detect the presence or absence of the solitary LTR in the apoC-I locus of different primates. The LTR upstream of the EBR locus was amplified by primer EBR-F1 (5′-AACATCCTCTGTCTCTCTCC-3′; sequence flanking the LTR integration) and primer EBR-LTR1 (5′-GATCGACCCCTGACCTAACC-3′; sequence from the LTR). The apoC-I and EBR primers were specified from GenBank™ accession numbers AB012576 and AL139002, respectively.

Plasmid Constructs—The 5′-flanking regions of apoC-I and EBR were amplified from human genomic DNA and subcloned upstream of the luciferase gene in the promoterless luciferase reporter plasmid pGL3B (Promega). To facilitate directional cloning into pGL3B, primers were designed with terminal sequences specific for restriction enzyme recognition. The restriction enzyme adaptor is indicated after each primer (see below), where the following suffixes are used: K, *Kpn*I; B, *Bgl*II; Ba, *Bam*HI; X, *Xba*I; Xh, *Xho*I. The numbers in parenthesis after each primer indicate the start and end positions of the primer upstream in the flanking DNA sequence, relative to the first nucleotide of the initiation codon of the two genes. The primer sequences are available upon request. Because the exact distances of the EBR LTR and the hepatic control region (HCR) to EBR and apoC-I are uncertain, the primer sequences used to amplify the EBR LTR and the HCR are shown below, where primer sequence in lowercase type indicates the restriction enzyme recognition sequence.

The following EBR constructs were made. For pEBR-NP, the 5′-flanking region of the native EBR transcription initiation site was isolated using primers EBR-NP1K (−1259/−1239) and EBR-NP2B (−208/−187). Digested and purified amplification products were inserted into *Kpn*I/*Bgl*II-digested pGL3B. For pEBR-LTR, the complete LTR was amplified with primers EBR-LTR1K (5′-ggggtaccTAAGGGAGGATACCACC-3′)/EBR-LTR2B (5′-GCAGCTTCTCCTGCTACAagatcttc-3′) and inserted into *Kpn*I/*Bgl*II-digested pGL3B. pEBR-NP+LTR-S and pEBR-NP+LTR-A were made by introducing the LTR at a distance of the native promoter region of construct pEBR-NP. The full LTR was amplified with LTR-specific primers, EBR-LTR1Ba (5′-cgggatccTAAGGGAGGATACCACC-3′)/EBR-LTR2Ba (5′-GCAGCTTCTCCTGCTACAggatcccg-3′). Purified and *Bam*HI-digested amplification products were introduced into the *Bam*HI site of construct pEBR-NP, which is located 2 kb from the *Kpn*I/*Bgl*II site on pGL3B. Constructs introduced either in sense (LTR-S) or antisense (LTR-A)

with respect to the native EBR promoter region were isolated. The location of the primers was based on the initiation codon at position 1260 of GenBank™ accession number D13162. The LTR primers were derived from GenBank™ accession number AL139002.

The following apoC-I constructs were made. For pAPO-P, the 5′-flanking region of the apoC-I transcription initiation site was isolated using primers APO-P1K (−1271/−1249)/APO-P2B (−165/−145) and inserted into *Kpn*I/*Bgl*II-digested pGL3B. This construct contains both the native and LTR promoter regions. For pAPO-LTR, the complete LTR of the apoC-I locus was amplified with primers APO-LTR1K (−920/−901)/APO-LTR2B (−484/−465) and introduced in the *Kpn*I/*Bgl*II site of pGL3B. For pAPO-P-noLTR, the LTR was removed from the apoC-I locus by amplifying the non-LTR parts of pAPO-P with primers APO-P1K (−1271/−1249)/APO-P3X (−941/−924) and APO-P4X (−455/−439)/APO-P2B (−165/−145). The two amplification products were digested with *Xba*I, ligated together, and introduced after *Kpn*I/*Bgl*II digestion into pGL3B. This construct has the same structure as the pAPO-P except that the LTR is absent. pAPO-P-noLTR+LTR-S and pAPO-P-noLTR+LTR-A were made by introducing the LTR at a distance of the native apoC-I promoter region lacking the LTR (construct pAPO-P-noLTR). The full LTR was amplified with LTR-specific primers, APO-LTR1Ba (−920/−901)/APO-LTR2Ba (−484/−465). Purified and *Bam*HI-digested amplification products were introduced into the *Bam*HI site of construct pAPO-P-noLTR. Constructs introduced either in sense (LTR-S) or antisense (LTR-A) with respect to the apoC-I promoter region were isolated. The location of the primers is with respect to the apoC-I initiation codon at position 27457 of GenBank™ accession number AB012576.

The following baboon apoC-I constructs were made. For pBAPO-P, the baboon apoC-I locus was amplified from baboon genomic DNA with primers APO-P1K (−782/−760)/APO-P2B (−160/−140) and inserted into *Kpn*I/*Bgl*II-digested pGL3B. For pBAPO-P+LTR, construct pBAPO-P was amplified with primers APO-P1K (−782/−760)/APO-P5Ba (−463/−444) and APO-P6Ba (−435/−414)/APO-P2B (−160/−140). The two amplification products were digested with *Bam*HI and ligated together. This religated fragment was inserted into pGEM-T (construct pBAPO-GEM). The LTR that was amplified with primers APO-LTR1Ba/APO-LTR2Ba (see above) was introduced into the *Bam*HI site of pBAPO-GEM. After selection of LTR-positive clones, the *Kpn*I/*Bgl*II cassette (containing the LTR in the baboon apoC-I at the same orthologous site as in humans) was subcloned into pGL3B. Positions of the primers are from GenBank™ accession number L13176 and with respect to the initiation codon of the baboon apoC-I at position 1017.

The HCR was isolated from human DNA using PCR and primers HCR1Xh (5′-ccgctcgagTTAGAGAACAGAGCTGCAGGCT-3′) and HCR2Xh (5′-ATGCCCCGACCCCGAAGCctcgagcgg-3′). The primer sequences were derived from positions 36815–36836, and positions 37201–37218 of GenBank™ accession number AF050154, respectively. The PCR product was digested with *Xho*I, and the purified fragment was introduced into the pGL3B *Sal*I site of all apoC-I constructs, which is 3′ to the luciferase gene.

Cell Lines and Transient Transfections—Plasmid DNA was purified by using the Qiagen plasmid midi kit (Qiagen) prior to transfections. HepG2 (human hepatoblastoma cells; ATCC HB-8065) cells were cultured in Dulbeccos's minimal essential medium supplemented with 10% fetal calf serum. Cells were seeded 24 h prior to transfections in six-well plates at a density of 3×10^5 cells/well. Transient transfections of HepG2 were done by cotransfecting 1.5 μg of plasmid DNA and 50 ng of pRL-TK vector (Promega) using calcium phosphate (Cellphect; Amersham Pharmacia Biotech) as described in the protocol supplied with the reagent. JEG-3 cells (human choriocarcinoma; ATCC HTB-36) were maintained in RPMI supplemented with 5% fetal calf serum. JEG-3 cells were seeded in six-well plates at a density of 2×10^5 cells/well and cotransfected 24 h later with 1.0 μg of plasmid DNA and 200 ng of pRL-TK using 7 μl of LipofectAMINE (Life Technologies, Inc.), as described in the protocol from the supplier. After 24 h, the cells were lysed, and the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the internal control. Transfections were performed in triplicates and repeated at least twice.

DNA Sequencing—Double-stranded plasmid DNAs were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI 373 sequencer (PerkinElmer Life Sciences).

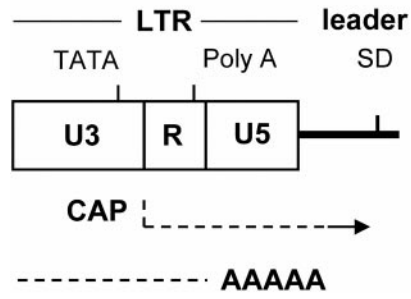

FIG. 1. **General structure and transcriptional regulation mediated by LTRs.** Regulatory regions are located within the U3 region, and the transcription initiation site defines the U3/R boundary. Polyadenylation signals are located within R, where the polyadenylation site defines the R/U5 boundary. An SD that is used for subgenomic splicing is located in the leader region downstream of the 5′ LTR of a provirus.

RESULTS

Identification and Characterization of Chimeric Transcripts—Using the strategy outlined in Fig. 1, we searched GenBank™ and the human expressed sequence tag data bases using the LTR and the leader region of published HERV elements (7). Transcripts with only the U3-R part of the LTR and no other HERV sequence probably represent mRNA polyadenylated by the LTR, whereas transcripts with R-U5 or R-U5-leader probably represent promotion by an LTR. During data base screenings, we encountered two transcripts where HERV-E (18) sequences were fused to the EBR gene (accession number D90402) and the apoC-I gene (accession number W79313). The structure of these transcripts suggests that the EBR utilizes the splice donor (SD) in the leader region of the HERV element, which is located downstream of the 5′ LTR (Fig. 1) of an integrated provirus. The same SD is used in subgenomic splicing of HERV-E envelope transcripts, suggesting that this represents the original SD of HERV-E (19). The apoC-I fusion transcript represents another possible LTR-driven transcript type, which is derived from a solitary LTR and reads into the flanking non-HERV region.

To authenticate the presence of fusion transcripts, we synthesized primers corresponding to the retroviral and the gene-specific regions of the identified transcripts. By using this primer combination in RT-PCR, it is possible to detect the presence and the relative abundance of the fusion transcripts in human tissues. Both of the genes were previously reported as having a different promoter region, separated from the retroviral LTR (20–22). We will refer to these two regions as the "native" apoC-I and EBR promoter. To detect any biases of the LTR and native transcripts, we also used a primer unique to transcripts of the native promoters of the two genes. Results of the RT-PCR on a panel of RNAs derived from different human tissues are shown in Fig. 2. The LTR-promoted EBR transcript is restricted to placenta, where its levels appear comparable with that of the widely expressed native transcript (Fig. 2*B*). In the case of apoC-I, transcripts from the native promoter in liver were high as expected (20) but are also detectable by PCR in many of the other RNAs tested (Fig. 2*A*). Transcripts from the solitary LTR were detected in two distinct forms (see Fig. 3*A*), both of which were also detected in many tissues. The result of this experiment clearly demonstrates the presence of fusion transcripts between LTRs of HERV-E and the genes for EBR and apoC-I. The LTRs at the EBR and apoC-I loci vary in their tissue specificity, with the EBR LTR being much more restricted in activity. Sequencing of the PCR products verified the nature of the fusion transcripts, where the two fusion transcript forms of apoC-I are the result of differential splicing in the 5′ UTR (Fig. 3).

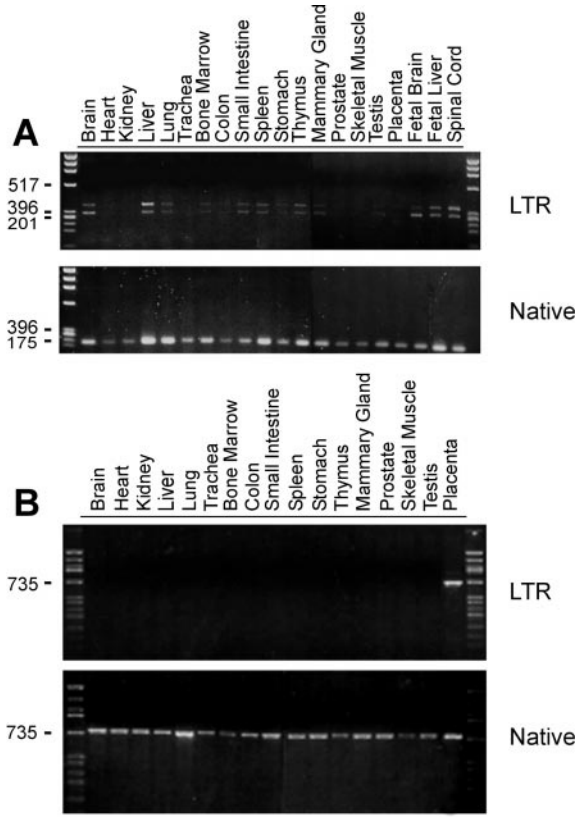

FIG. 2. **Detection of fusion transcripts by RT-PCR of apoC-I and EBR in different human tissues.** *A*, *upper panel*, LTR-apoC-I fusion transcripts were detected by using LTR and apoC-I exon-specific primers in RT-PCR. *Lower panel*, amplification products derived from primers detecting transcripts of the native apoC-I promoter. *B*, *upper panel*, detection of LTR-EBR fusion transcripts by using leader (derived from the provirus) and EBR exon primers. *Lower panel*, result of amplification using primers specific for transcripts derived from the native EBR promoter. Primer sequences are shown in Table I. Expected amplification product sizes were obtained for the different primer combinations. The *numbers* to the *left* are sizes of the DNA markers.

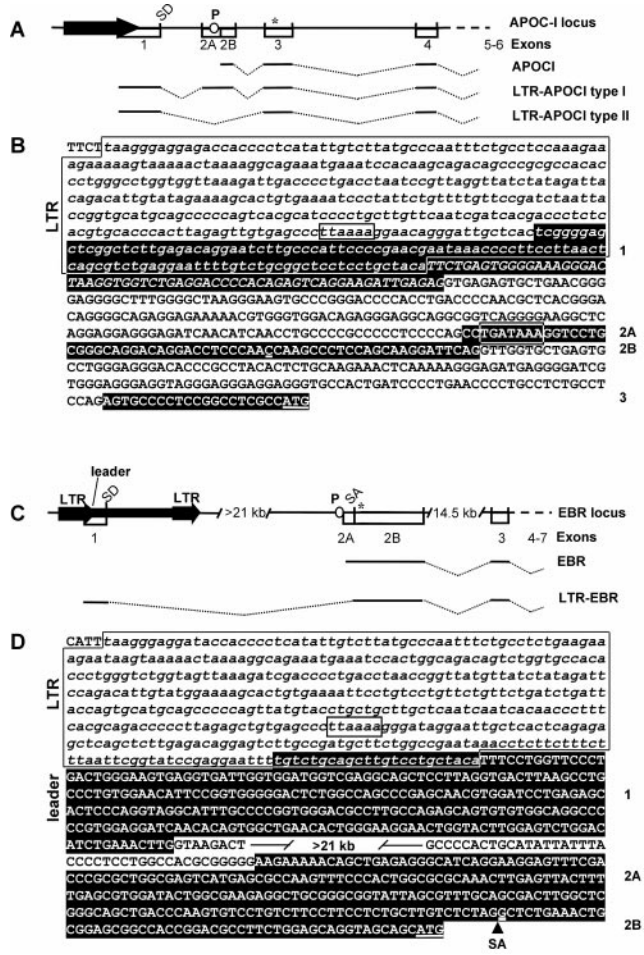

FIG. 3. **Genomic structure and transcripts forms of apoC-I and EBR.** *A*, the structure of genomic DNA of apoC-I, where the position of the solitary LTR (*arrow*) is shown with respect to apoC-I exons (*rectangles*). The native promoter (*P*) is indicated upstream of exon 2B. An *asterisk* indicates the start of the protein-coding region in exon 3. *Below* this is a schematic illustration of three different forms of apoC-I transcripts. The two LTR-apoC-I forms were determined by RT-PCR and 5′-RACE. The apoC-I form derived from the native promoter was reported previously (20). Distances are not drawn to scale. *B*, DNA sequence of the promoter regions upstream of apoC-I. The solitary LTR sequence is shown in *lowercase letters* and *framed*. Putative TATA regions are *boxed* (located upstream of exon 1 and exon 2B). Nonintronic transcribed sequence is shown on a *black background*, where exon 1 initiates in the LTR, and exon 2B is the start site derived from the native promoter. The first nucleotide of exon 2B and the translational start in exon 3 is *underlined*. The *numbers* to the *right* refer to exons shown in Fig. 3*A*. *C*, genomic structure of the EBR locus. The proviral element is shown as *filled rectangles* and *arrows*, where the 5′ and 3′ LTRs and the SD in the leader are shown. The proviral element is located about 21 kb upstream of the native promoter (*P*). The translational start of EBR is indicated with an *asterisk*. A schematic representation of the different forms of EBR transcripts are shown *below*. The LTR-EBR fusion transcript was determined by RT-PCR and 5′-RACE. The EBR transcript of the native promoter was reported before (21, 22). Distances are not drawn to scale. *D*, genomic sequence upstream of the initiation sites of the two EBR transcripts. The LTR sequence is *framed* and shown in *lowercase type*. A putative TATA present in the LTR is *boxed*. Nonintronic transcribed sequence is highlighted on a *black background*. The first part of exon 1 is located in the LTR. Exon 1 is joined to exon 2B by splicing. The splice donor is located in the proviral leader region, and the splice acceptor (*SA*), defining the start of exon 2B, is located >21 kb downstream. Transcripts derived from the native promoter define the start of exon 2A. The first nucleotide of exon 2B and the translational start is *underlined*. The *numbers* to the *right* refer to exons shown in *C*.

Genomic Structure and Transcript Forms—To confirm that the apoC-I and EBR fusion transcripts initiate within the LTRs and do not represent transcripts from a promoter located upstream of the LTRs, we isolated the 5′-ends of both LTR fusion gene transcripts. Using a 5′-RACE protocol, we established that both the apoC-I and EBR fusion transcript initiate within their LTRs (see below and Fig. 3). Sequencing of several 5′-RACE clones showed that the apoC-I and EBR initiation site is located downstream of a previously reported TATA box of HERV-E (18, 19). This is the TATA also used by other HERV-E proviruses because a full-length transcribed HERV-E element (GenBank™ accession number M74509) starts 2 bp downstream of the initiation site of the apoC-I LTR. In the case of the EBR fusion transcript, the sequence representing the longest 5′-UTR also began within the LTR, but at a position 3′ (90 bp) to the apoC-I initiation site.

Both the apoC-I and EBR genomic loci were partially characterized at the time of our initial studies. The only retroviral remnant of the original proviral insertion at the apoC-I locus is a solitary LTR, which is located 300 bp upstream of the native apoC-I promoter. The two initiation sites are separated by 390 bp, where the initiation sites of the native and LTR promoters are located 180 and 575 bp upstream of the apoC-I initiation codon, respectively (Fig. 3). The EBR LTR was not present in the reported 2-kb sequence upstream of the EBR native promoter (GenBank™ accession D13162), which is located ~250 bp upstream of the EBR initiation codon (21, 22). A genomic clone containing both the HERV-E proviral element and the EBR genomic locus was recently deposited in GenBank™ (accession number AL139002). The sequence of this clone is in a

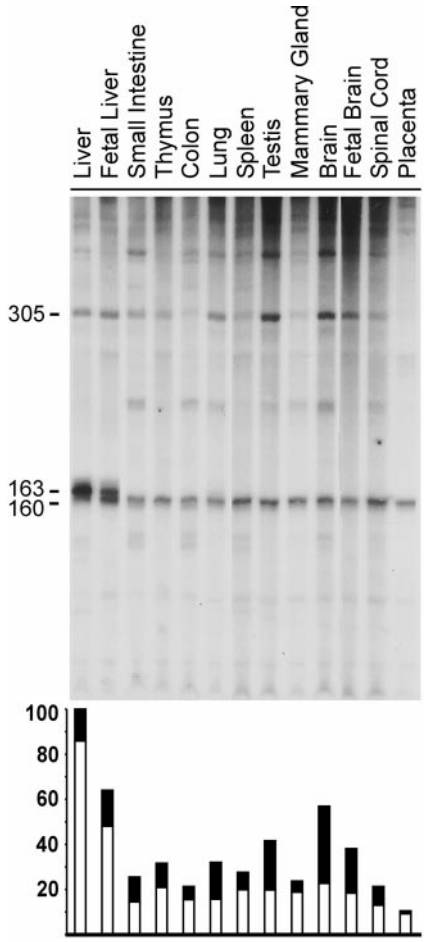

FIG. 4. **ApoC-I primer extension analysis.** An oligonucleotide complementary to exon 3 of apoC-I (see Fig. 3) was used to analyze the relative abundance of the different transcript forms. Extension products of about 160 and 305 bp, corresponding to expected sizes of the native (163-bp) and type II LTR fusion (304-bp) transcript were detected. Sizes were estimated by comigrating DNA markers. Shown *below* the gel is the relative abundance of the transcript forms in the different tissues. All values were adjusted to the total observed in liver (as percentages). The amounts estimated from the LTR-apoC-I fusion transcript and the native transcript form are shown as *filled* and *open rectangles*, respectively.

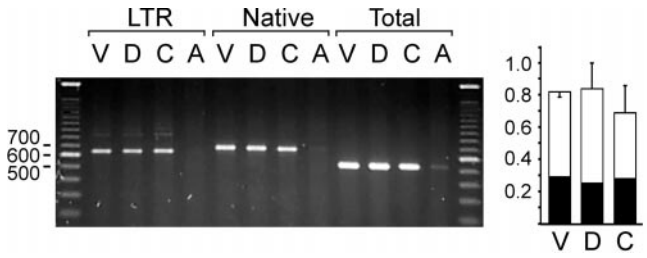

FIG. 5. **RT-PCR of EBR transcripts in placenta.** The relative abundance of the LTR and native transcript forms was estimated by low cycle RT-PCR. The LTR-driven form was detected by using primers derived from exon1/exon3, the native form with primers from exon 2A/exon3, and the total EBR was amplified using primers derived from exon 2B/exon3 (see Fig. 3). RT-PCR was done on cDNA of different section of placenta: villi (*V*), decidua (*D*), chorion (*C*), amnion (*A*). Sizes of the DNA markers are shown to the *left*. Expected amplification product sizes were obtained using the different primer combinations. The relative abundance of the different transcript forms is shown to the *right* of the gel. Total EBR levels of the different samples are indicated with a *bar*. *Filled* and *open rectangles* show the values of the native and LTR-driven forms, respectively. All values were adjusted to the total seen in decidua.

preliminary state of annotation and contains unordered pieces of DNA. The parts of the LTR and leader region that were identified in EBR 5′-RACE (see above) are identical to the proviral element of AL139002. It has been previously reported that several other alternative transcripts (named EDNRBΔ) are created by initiation 560 and 940 bp upstream of the ATG codon and alternative splicing of the 5′-UTR (23). The 5′ LTR leader of the HERV-E element is located over 20 kb from the EBR gene and is joined by splicing to the same splice acceptor in the 5′-UTR as are the spliced EDNRBΔ transcripts. The genomic organization and the structures of the different transcripts at these two loci are shown in Fig. 3. Due to the retroviral sequence, the fusion transcripts have partially different 5′-UTRs compared with the native forms, but all maintain the same apoC-I and EBR coding regions.

Evolutionary Age of the LTRs—Using primers flanking the integration sites of the LTRs in PCR of different primate DNAs, we earlier assigned the time of integration of various HERV-K elements during primate evolution (16). Using the same approach, we were able to determine when the two HERV-E LTRs integrated in the primate lineage. The apoC-I LTR was detected in all hominoids, whereas Old and New World monkeys did not have this LTR integrated in the apoC-I locus, suggesting that the integration took place after the divergence of hominoids and the Old World monkeys, about 20–30 million years ago (Ref. 24). Since we could detect the presence of the EBR LTR both in baboons and hominoids, but not in New World monkey, we conclude that the LTR at the EBR locus is older than the apoC-I LTR, because it integrated after the split between the New and Old World monkeys, ~30–40 million years ago (24). Sequence comparison of the 5′ and 3′ LTRs of the EBR HERV-E revealed that they are 12% divergent. The same time estimate of 30–40 million years is obtained by assuming that the two LTRs diverged an average of 6% since integrating in the primate lineage, taking a pseudogene divergence rate of 0.15–0.21% per million years into account (6, 25).

Estimation of the Proportion of Transcripts Contributed by the LTR Promoters—We could not discriminate between the LTR-driven and native transcripts using Northern blot analysis for either the apoC-I and EBR genes. For the apoC-I transcripts, all are in sizes ranging from 400 to 600 bases, and the resolution in this area of agarose gels is poor. For EBR, the only unique sequence of the LTR-driven transcript is from the retroviral part (Fig. 3), and it is not feasible to use this region as a probe because of the repetitive nature of the LTR sequences in human DNA.

We instead performed a primer extension protocol using RNAs from several tissues and an oligonucleotide derived from exon 3 of apoC-I. Using this strategy, we detected transcripts of sizes corresponding to the native and the LTR-driven transcripts. We only detected transcripts corresponding to the shorter, double-spliced apoC-I LTR fusion transcript. As expected, the relative level of transcription was highest in liver (Fig. 4), which is the major site of apoC-I transcription (20). By densiotometry, we estimated that the short transcript derived from the LTR promoter represents ~15% of the total in liver. Other sites of transcription (*e.g.* testis, lung, and brain) were also detected using this analysis. However, in these tissues the level of transcription was lower than in liver. We also used a fragment spanning the coding region of apoC-I in Northern hybridization (not shown). The signal in liver was at least 40–50 times stronger than for any of the other tissues, indicating that the primer extension analysis underestimated the level of apoC-I mRNA in liver. A possible explanation of this could be saturation effects in the primer annealing step of the extension protocol or poor quality of the liver RNA.

To estimate the relative level of the LTR-EBR fusion transcript, we performed a low cycle PCR protocol. This was done by

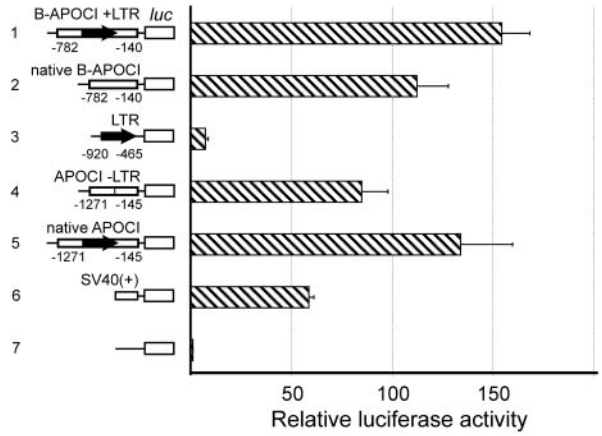

FIG. 6. **Effect of the LTR on apoC-I promoter activity in human and baboon.** The native human apoC-I, baboon (*B-APOCI*), and LTR fragments were inserted upstream of the luciferase (*luc*) vector pGL3B. Constructs where the LTR was removed from the human or added to the baboon apoC-I promoter region were used as a comparison with the native constructs. *Numbers 1–7* shown on the *left* refer to constructs pBAPO-P+LTR, pBAPO-P, pAPO-LTR, pAPO-P-noLTR, pAPO-P, pGL3P, and pGL3B, respectively. On the *right* are the results of the luciferase activities obtained from the different constructs after transient transfection in HepG2. All values are normalized to -fold activity in respect to pGL3B.

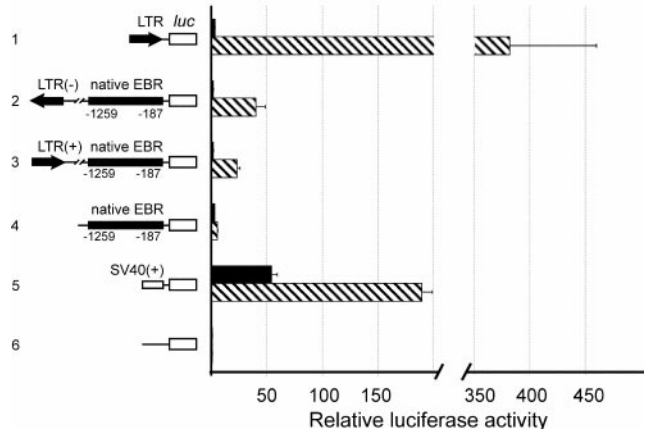

FIG. 7. **Effect of the LTR on EBR promoter activity.** The native promoter region and the LTR fragments were inserted upstream of the promoterless luciferase (*luc*) reporter vector pGL3B, as shown on the *left*, where the *numbers 1–6* refer to constructs pEBR-LTR, pEBR-NP+LTR-S, pEBR-NP+LTR-A, pEBR-NP, pGL3P, and pGL3B, respectively. Luciferase activity after transient transfection in HepG2 (*black rectangles*) or JEG-3 (*striped rectangles*) cells, normalized to -fold activity relative to pGL3B is shown to the *right*.

serial dilution of the input reverse transcribed RNA to avoid saturation effects during amplification. Because the LTR-driven EBR fusion transcript was detected in placenta, we tested RNA prepared from different parts of the placenta in amplifications with primers specific for the native and the LTR-driven transcripts. We also used primers specific for the exons only, which would allow estimation of the total level of EBR mRNA in the different samples (Fig. 5). Depending on the origin of the cDNA used, ~50–65% of the total amount of EBR transcripts were estimated by densitometry to be derived from the native promoter, and 25–30% of the total was derived from the LTR promoter. As has been reported previously, we saw no evidence for EBR expression in amnion (26).

Significance of the HERV-E LTRs in Expression of ApoC-I and EBR—To investigate the significance of the LTR in expression regulation of the apoC-I gene, we inserted the native promoter region, which naturally contains the LTR and the native promoter, upstream of a promoterless luciferase reporter plasmid (pGL3B). We also tested the activity of the LTR by itself and the native construct where the LTR was removed. We then performed transient transfections to test the relative levels of promoter activity of the different constructs. The LTR was also inserted at a distance (see "Experimental Procedures") in constructs with the apoC-I promoter where the LTR was removed, to test for the possibility that the LTR acts as an enhancer of the native promoter. The expression in liver is completely dependent on a distal HCR (27), and we saw no promoter activity of the apoC-I constructs without the presence of this HCR. The results of the transfections of HepG2 (liver) cells with a variety of apoC-I constructs are shown in Fig. 6 and suggest that the LTR by itself is not contributing significantly to the overall expression levels of apoC-I in liver. However, when the LTR is removed from the apoC-I locus, the promoting activity of the region drops about 40% in HepG2, suggesting that the presence of the LTR in the apoC-I locus contributes to the overall activity of the native promoter region. However, we found no evidence that the LTR alone acts as an enhancer in liver cells when positioned at a greater distance from the native promoter.

A test of the effect the LTR had at the time of integration in the primate lineage would be to insert the LTR into the apoC-I locus of a species that naturally lacks the LTR. Our analysis showed that all hominoids have the LTR integrated in the apoC-I locus but that it is absent in the baboon. The sequence of apoC-I baboon locus has been determined (28), and sequence alignments of the human and baboon loci verified the absence of the LTR in the baboon (not shown). We inserted the LTR into the baboon apoC-I locus at the orthologous site, and compared the relative promoting activity between the constructs with and without the LTR. The LTR insertion into the baboon locus resulted in increased expression, similar to that seen in the human locus, suggesting that the LTR had a similar effect when it first integrated in the primate lineage (Fig. 6).

To investigate the effect of the LTR in EBR expression, the native EBR promoter region or the LTR alone were inserted upstream of the luciferase gene of pGL3B. We also inserted the LTR at a distance, in direct and opposite orientation with respect to the native EBR promoter region, to test for potential enhancing effect of the LTR on the EBR native promoter region. The choriocarcinoma cell line JEG-3 and the liver cell line HepG2 were transiently transfected with these constructs, and the results are shown in Fig. 7. In both JEG-3 and HepG2, the activity of the native EBR promoter segment alone is low, and it is evident that the native EBR promoter is dependent on an enhancer element not present in the constructs or on a factor that is absent in the cell lines. However, when the LTR was inserted in either direction at a distance with respect to the native promoter (see "Experimental Procedures"), a significant increase in activity was observed, indicating that the LTR can act as an enhancer of the native promoter region extrachromosomally. When constructs containing only the LTR upstream of the luciferase gene were transfected into JEG-3, a very high activity was observed compared with that seen in HepG2 and in comparison with the other constructs in JEG-3 or the SV40 promoter control plasmid pGL3P. The high activity of the LTR in JEG-3 and absent activity in HepG2 agrees with the RT-PCR results, where the LTR-EBR fusion transcripts were detected only in placenta. As an independent control of enhancing activity of the LTR, constructs with the LTR upstream of the SV40 promoter (pGL3P) were transfected into JEG-3. Independent of the orientation of the LTR with respect to the SV40 promoter, a 7–10-fold increase in activity was seen relative to constructs with the SV40 promoter alone (data not shown), suggesting that the LTR also enhances the SV40 promoter in placental cells.

DISCUSSION

In this study, we detected and characterized alternative transcripts of the apoC-I and EBR genes with HERV-E sequences at their 5′ termini. Both fusion transcripts are expressed in a variety of human tissues and were shown by 5′-RACE to initiate downstream of a putative TATA box within HERV-E LTRs, demonstrating that the LTRs are alternative promoters for these genes in humans. For apoC-I, we found that only a minor fraction of transcripts is derived from the LTR promoter in liver. The significance of apoC-I in other tissues is not known, and the general transcription levels are lower than observed in liver. However, the LTR and native promoters appear to be equally active in many of the other tissues tested.

In the case of EBR, it should be noted that the LTR-EBR fusion transcript was first isolated from a placental library by Arai *et al.* (29) but was considered to be a gene rearrangement or artifact due to the LTR in the 5′-UTR, which differed from the originally described UTR region of EBR (21, 22). In our 5′-RACE of placental cDNA, the major form corresponded to transcript sizes derived from the native promoter, demonstrating that this is the most abundant transcript form in placenta. However, the semiquantitative RT-PCR analysis using cDNA derived from different parts of the placenta indicated that 25–30% of the total was derived from the LTR promoter, depending on the placental cDNA used. In decidua and chorion, the total level of amplified EBR was estimated to be higher than was seen for the LTR and native derived amplification products combined, which could be accounted for by the minor EDNRBΔ forms (see "Results"), because these transcripts are also expressed in placenta (23).

Although the LTRs of the apoC-I and EBR locus are 88% identical in sequence, the expression pattern of the two fusion transcripts is different, where activity of the LTR-EBR is restricted to placenta and the apoC-I LTR-derived transcripts are detected in many tissues. It is possible that restrictive expression of the LTR-EBR transcript is due to methylation of the HERV locus in adult tissues. Methylation is a widely used mechanism employed by mammalian cells to restrict the expression of unwanted gene products and retroelements (30, 31). The apoC-I LTR may be protected from methylation and thereby expressed in adult tissue, due to its close proximity to the native apoC-I promoter region. Another explanation for the different transcription pattern, although less likely, is that acquired nucleotide substitutions have specifically destroyed or created transcription factor binding sites in the two LTRs. The nucleotide divergence of the LTRs is probably a direct effect of substitutions acquired after their integration into the genome. We estimated that the LTR integrated into the EBR locus about 30–40 million years ago and that the LTR integrated into the apoC-I locus 20–30 million years ago. It is likely that HERV-E elements were actively transposing in the primate lineage during this time period, because the previously characterized HERV-E element 4-14 and the HERV-E of the pleiotrophin locus are of similar age (32, 33). As is the case for many other HERV families, no recent integrations involving this endogenous family have been observed, indicating that the HERV-E elements are deeply fixed in the primate lineage.

Transient transfections were performed to test the significance of the LTRs in the genomic loci of apoC-I and EBR. Although these experiments only monitor the extrachromosomal interactions, the results using the apoC-I constructs supported the *in vivo* results, where the LTR alone was shown not to contribute significantly to the overall expression levels of apoC-I in liver. However, when the LTR was removed from the apoC-I locus, the promoting activity of the apoC-I locus dropped about 40% in HepG2 cells. This result suggests that the presence of the LTR in the apoC-I locus contributes to the overall activity of the native promoter region, perhaps by providing position-dependent cis-acting elements, which work in combination with the native regulatory sequences. The genes encoding the three human apolipoproteins E, CI, and CII are located in a 45-kb cluster on chromosome 19 (34) and encode proteins with the ability to associate with lipids (35). The different apolipoproteins have distinct roles in lipid metabolism, where apoC-I is implicated to interact with apolipoprotein E in regulating the plasma lipid levels and in prolonging the residence time of lipoprotein particles in the circulation (36). Our analysis shows that all hominoids have the LTR integrated in the apoC-I locus, but it is absent in baboon. By introducing the LTR in the baboon apoC-I locus, we observed an increased expression relative to that seen for the natural baboon locus. At the time of integration, it is possible that the LTR was tolerated by either its neutral or beneficial effect on individuals. It is obvious that the presence of the LTR would have been selected against if it had a strong impact on apoC-I expression, resulting in hyperlipemic individuals (35), which has been suggested as a possible explanation for silencing of a second apoC-I (the apoC-I′) gene in humans (37). Although both the *in vivo* and transfection results suggest that the LTR has a moderate positive effect on the expression levels of apoC-I, one possibility is that the LTR replaced an existing function, for example the silenced second apoC-I gene. Another possibility is that the LTR had a selective advantage when it was first acquired, for example in ensuring the export of lipoprotein to peripheral tissues, thereby maintaining important cellular functions during periods of limited food supply.

In contrast to the LTR at the apoC-I locus, a significant portion of the EBR transcripts is derived from the LTR promoter in placenta. The LTR also increases the activity of the native EBR promoter region in transient transfection experiments, suggesting that this LTR has a dual role in acting both as promoter and enhancer for the expression of EBR in placenta. In human placenta, endothelins (ETs) are implicated in the fetoplacental circulation via ETB and ETA receptors, and as growth factors of placental cells (38, 39). The role for ETs and ET receptors in placental development is supported by studies in rats, where an increase in ET and ETB receptor density coincides with a rapid increase in placental growth (40), whereas elevated ET concentrations are observed in cases of placental growth retardation (41). Although the exact biological consequences of the interactions of ETs and the ET receptors in different parts of the placenta are complex and not well understood, our studies show that the LTR contributes significantly to expression of EBR. While the LTR-induced increase of EBR density in placenta might be an evolutionary event without physiological significance, another possibility is that an increased receptor density would serve as a clearance for the high levels of ETs that are present in the placenta, which in turn have implications in placental development and uteroplacental functions.

As is exemplified in this study, the capacity of LTRs and other retroelements to promote or, in other cases, polyadenylate genes is easily detectable because retroelement sequences are present within the transcript. Their enhancing potential is not readily detectable, because the element will not be part of the transcript. Effects due to retroelement enhancement on gene expression are likely to be more common due to less constraint on the distance and orientation of the element with respect to genes. It is probable that such elements have been used as evolutionary tools in the genomes of many organisms,

in that they may enable switches in the regulation of tissue specificity and levels of gene expression. Such genomic "retroelement experiments" resulting in sudden biochemical changes may have played an important role in adaptation. In humans, LTRs and other retroelements are implicated in the evolution of tissue-specific gene functions; for example, leptin is under control of a MER11 repeat element that acts as an enhancer for this gene in placenta (42). However, leptin is not expressed in mouse placenta because the MER11 element is absent in mice. Other examples where gene control elements have evolved during primate evolution involve replacement of an existing enhancer element (in the case of amylase) or creation of a novel regulatory UTR region (in the case of pleiotrophin) by HERV-E insertions (11, 12). It is intriguing that HERV-E elements are repeatedly found involved in gene regulatory functions although these elements are not as numerous as some other HERV families in the human genome (7). Although a selective advantage for the LTR insertions is not apparent, it is possible that the chromosomal location of HERV sequences or conserved LTR functions may influence gene expression.

In summary, we have identified two HERV-E elements that mediate increased transcription of the EBR and apoC-I genes in humans by donation of promoter and enhancer functions from their LTRs and add to the list where LTRs have been co-opted to serve gene regulatory functions.

Acknowledgments—We thank Doug Freeman, Paul Kowalski, and Holly Stamm for technical assistance.

REFERENCES

1. Smit, A. F. A. (1999) *Curr. Opin. Genet. Dev.* **9,** 657–63
2. Boeke, J. D., and Stoye, J. P. (1997) in *Retroviruses* (Coffin, J., Hughes, S. H., and Varmus, H. E., eds) pp. 343–435, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Kidwell, M. G., and Lisch, D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7704–7711
4. Smit, A. F. A. (1996) *Curr. Opin. Genet. Dev.* **6,** 743–748
5. Larsson, E., Kato, N., and Cohen, M. (1989) *Curr. Top. Microbiol. Immunol.* **148,** 115–132
6. Tristem, M. (2000) *J. Virol.* **74,** 3715–3730
7. Wilkinson, D., Mager, D. L., and Leong J.-A. C. (1994) in *The Retroviridae* (Levy, J., ed) Vol. III, pp. 465–535, Plenum Press, New York
8. Britten, R. J. (1997) *Gene* (*Amst.*) **205,** 177–182
9. Sverdlov, E. D. (1998) *FEBS Lett.* **428,** 1–6
10. Brosius, J. (1999) *Gene* (*Amst.*) **238,** 115–134
11. Ting, C. N., Rosenberg, M. P., Snow, C. M., Samuelson, L. C., and Meisler, M. H. (1992) *Genes Dev.* **6,** 1457–1465
12. Schulte, A. M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A. T., and Wellstein, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14759–14764
13. Kapitonov, V. V., and Jurka, J. (1999) *J. Mol. Evol.* **48,** 248–251
14. Medstrand, P., Lindeskog, M., and Blomberg, J. (1992) *J. Gen. Virol.* **73,** 2463–2466
15. Wilkinson, D. A., Freeman, J. D., Goodchild, N. L., Kelleher, C. A., and Mager, D. L. (1990) *J. Virol.* **64,** 2157–2167
16. Medstrand, P., and Mager, D. L. (1998) *J. Virol.* **72,** 9782–9787
17. Goodchild, N. L., Wilkinson, D. A., and Mager, D. L. (1993) *Virology* **196,** 778–788
18. Repaske, R., Steele, P. E., O'Neill, R. R., Rabson, A. B., and Martin, M. A. (1985) *J. Virol.* **54,** 764–772
19. Rabson, A. B., Hamagishi, Y., Steele, P. E., Tykocinski, M., and Martin, M. A. (1985) *J. Virol.* **56,** 176–182
20. Lauer, S. J., Walker, D., Elshourbagy, N. A., Reardon, C. A., Levy-Wilson, B., and Taylor, J. M. (1988) *J. Biol. Chem.* **263,** 7277–7286
21. Nakamuta, M., Takayanagi, R., Sakai, Y., Sakamoto, S., Hagiwara, H., Mizuno, T., Saito, Y., Hirose, S., Yamamoto, M., and Nawata, H. (1991) *Biochem. Cell Biol. Commun.* **177,** 34–39
22. Ogawa, Y., Nakao, K., Arai, H., Nakagawa, O., Hosoda, K., Suga, S., Nakanishi, S., and Imura, H. (1991) *Biochem. Cell Biol. Commun.* **178,** 248–255
23. Tsutsumi, M., Liang, G., and Jones, P. A. (1999) *Gene* (*Amst.*) **228,** 43–49
24. Sibley, C. G., and Ahlquist, J. E. (1987) *J. Mol. Evol.* **26,** 99–121
25. Li, W. H., and Tanimura, M. (1987) *Nature* **326,** 93–96
26. Germain, A. M., MacDonald, P. C., and Casey, M. L. (1997) *Mol. Cell. Endocrinol.* **132,** 161–168
27. Dang, Q., Walker, D., Taylor, S., Allan, C., Chin, P., Fan, J., and Taylor, J. (1995) *J. Biol. Chem.* **270,** 22577–22585
28. Pastorcic, M., Birnbaum, S., and Hixson, J. E. (1992) *Genomics* **13,** 368–374
29. Arai, H., Nakao, K., Takaya, K., Hosoda, K., Ogawa, Y., Nakanishi, S., and Imura, H. (1993) *J. Biol. Chem.* **268,** 3463–3470
30. Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997) *Trends. Genet.* **13,** 335–340
31. Walsh, C. P., and Bestor, T. H. (1999) *Genes Dev.* **13,** 26–34
32. Shih, A., Coutavas, E. E., and Rush, M. G. (1991) *Virology* **182,** 495–502
33. Schulte, A. M., and Wellstein, A. (1998) *J. Virol.* **72,** 6065–6072
34. Smit, M., van der Kooij-Meijs, E., Frants, R. R., Havekes, L., and Klasen, E. C. (1988) *Hum. Genet.* **78,** 90–93
35. Li, W. H., Tanimura, M., Luo, C. C., Datta, S., and Chan, L. (1988) *J. Lipid Res.* **29,** 245–271
36. Jong, M. C., Hofker, M. H., and Havekes, L. M. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19,** 472–484
37. Freitas, E. M., Gaudieri, S., Zhang, W. J., Kulski, J. K., van Bockxmeer, F. M., Christiansen, F. T., and Dawkins, R. L. (2000) *J. Mol. Evol.* **50,** 391–396
38. Handwerger, S. (1995) *J. Lab. Clin. Med.* **125,** 679–681
39. Fant, M. E., Nanu, L., and Word, R. A. (1992) *J. Clin. Endocrinol. Metab.* **74,** 1158–1163
40. Shigematsu, K., Nakatani, A., Kawai, K., Moriuchi, R., Katamine, S., Miyamoto, T., and Niwa, M. (1996) *Endocrinology* **137,** 738–748
41. McMahon, L. P., Redman, C. W., and Firth, J. D. (1993) *Clin. Sci.* (*Lond.*) **85,** 417–424
42. Bi, S., Gavrilova, O., Gong, D. W., Mason, M. M., and Reitman, M. (1997) *J. Biol. Chem.* **272,** 30583–30588