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Proviral Structure, Chromosomal Location, and Expression of HERV-K-T47D, a Novel Human Endogenous Retrovirus Derived from T47D Particles

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We previously described that type B retrovirus-like particles released from the human mammary carcinoma cell line T47D are pseudotypes and package retroviral RNA of different origins (W. Seifarth, H. Skladny, F. Krieg-Schneider, A. Reichert, R. Hehlmann, and C. Leib-Mösch, J. Virol. 69:6408–6416, 1995). One preferentially packaged retroviral sequence, ERV-MLN, has now been used to isolate the corresponding full-length provirus from a human genomic library. The 9,315-bp proviral genome comprises a complete retroviral structure except for a 3′ long terminal repeat (LTR) truncation. A lysine tRNA primer-binding site and phylogenetic analyses assign this human endogenous retroviral element, now called HERV-K-T47D, to the HML-4 subgroup of the HERV-K superfamily. The *gag*, *prt*, *pol*, and *env* genes exhibit 40 to 60% amino acid identity to HERV-K10. HERV-K-T47D is located on human chromosome 10, with five closely related elements on chromosomes 8, 9, 15, 16, and 19 and several hundred HERV-K-T47D-related solitary LTRs dispersed over the human genome. HERV-K-T47D-related sequences are detected in the genomes of higher primates and Old World monkeys but not in those of New World monkeys. High HERV-K-T47D transcription levels were observed in human placenta tissue, whereas transcription in T47D cells was strictly steroid dependent.

Human endogenous retroviral sequences (HERVs) are inherited genomic elements with structural features of integrated retroviruses. To date, HERVs are estimated to comprise at least 1% of the human genome (for reviews, see references 14, 16, and 44). The biologically most active HERVs are members of the HERV-K superfamily (for a review, see reference 20). Members of this family are characterized by the presence of primer binding sites (PBSs) for lysine tRNA, hence the designation K. They represent about 70 to 100 elements and a large number of solitary long terminal repeats (LTRs) in the human genome. These elements are related to type A, B, and D retroviruses and have been classified by alignments of short stretches of the reverse transcriptase (RT) domain into six different groups (HML-1 to -6) (23). The members within a subgroup are more than 85% identical, whereas the intersubgroup similarity does not exceed 75%. To date, full-length proviral elements from only subgroups HML-2 and -6 have been isolated and completely sequenced.

The group HERV-K(HML-6), which is the least closely related to mouse mammary tumor virus, comprises about 30 to 40 members with 40 to 68% nucleotide sequence similarity to mouse mammary tumor virus and intracisternal type A particles of the mouse and hamster (24). In addition to proviral sequences, about 50 solitary HML-6-related LTRs are found per haploid genome.

The HERV-K(HML-2) group consists of approximately 30 members with full-length genomes, a few elements with large deletions (23, 25, 31), and an estimated 10,000 to 25,000 solitary LTRs distributed throughout the human genome (15). The prototype HERV-K(HML-2) provirus is HERV-K10, which to date is the only completely sequenced full-length provirus of this group (31). HERV-K10 and most HERV-K10-related proviruses harbor a characteristic deletion of 292 nucleotides (nt) leading to a defective genome with a polymerase gene fused to the envelope gene. However, transcripts of other HERV-K-related proviruses with uninterrupted *pol* and *env* open reading frames have been detected in human teratocarcinoma cell lines (18, 19, 42), and *gag* and *pol* gene products of HERV-K(HML-2) family members have been demonstrated to be enzymatically active (12, 28, 37). The *env* gene of HERV-K-IDDM, which was isolated from patients with acute-onset type I diabetes, was found to encode an endogenous superantigen (4). These studies suggest that some proviruses of the HERV-K superfamily have the potential to encode functional retroviral enzymes, possibly even with sufficient genetic information for the formation of retrovirus-like particles, which have been observed in normal human placentas, oocytes, and fetuses (9, 13, 21, 26), in both malignant and nonmalignant breast tissue samples (1, 10, 11, 27), and in germ cell tumors or cell lines derived from these tissues (17). However, there remain many questions with regard to the biological significance or function of these particles, particularly since they appear to be generated by complementation between several expressed HERVs, resulting in pseudotype particles with retroviral RNA of different types (2, 32, 38). Such packaging mechanisms could lead to unforeseen consequences in the use of retroviral vectors in gene therapy or following interspecies organ transplants.

Previously, using degenerate primers from a conserved region of retroviral *pol* genes (39), we repeatedly amplified three different retroviral sequences from particles released by the

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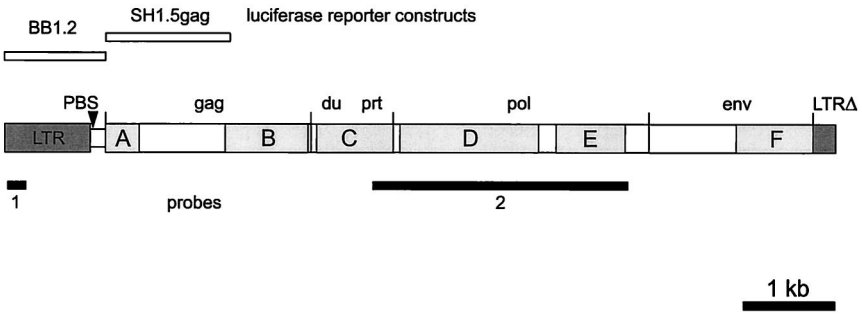


FIG. 1. Proviral organization of HERV-K-T47D, locations of hybridization probes, and regions of amino acid identity to HERV-K10. DNA fragments used as hybridization probes are shown as black bars (LTR, probe 1, 229 bp; *pol*, probe 2, 2.9-kb *Hin*dIII-*Hin*dIII fragment). Six regions with amino acid identity to HERV-K10 were identified and are depicted as shaded boxes A to F. Abbreviations: du, dUTPase; prt, protease; pol, polymerase; env, envelope. HERV-K-T47D fragments (BB1.2, nt 25 to 1243, 1,218 bp; SH1.5gag, nt 1062 to 2517, 1,455 bp) employed for the construction of recombinant pBL luciferase reporter plasmids used in transient transfection experiments are shown as open bars at the top of the figure. LTRΔ indicates the truncated 3′ LTR.

human mammary carcinoma-derived cell line T47D (38). One predominant sequence showed about 65% sequence identity to HERV-K10 within the RT region. By screening a human genomic library with the amplified product, we isolated a proviral *pol* sequence which we preliminarily termed ERV-MLN. The question was whether ERV-MLN is derived from an endogenous provirus with functional retroviral gene products, particularly with the packaging capabilities of Gag proteins. Therefore, we completely analyzed its proviral structure and genomic organization. Sequence comparisons assigned this novel HERV to the HML-4 subgroup of HERV-K elements. We also determined the chromosomal location and expression pattern of the provirus, now called HERV-K-T47D.

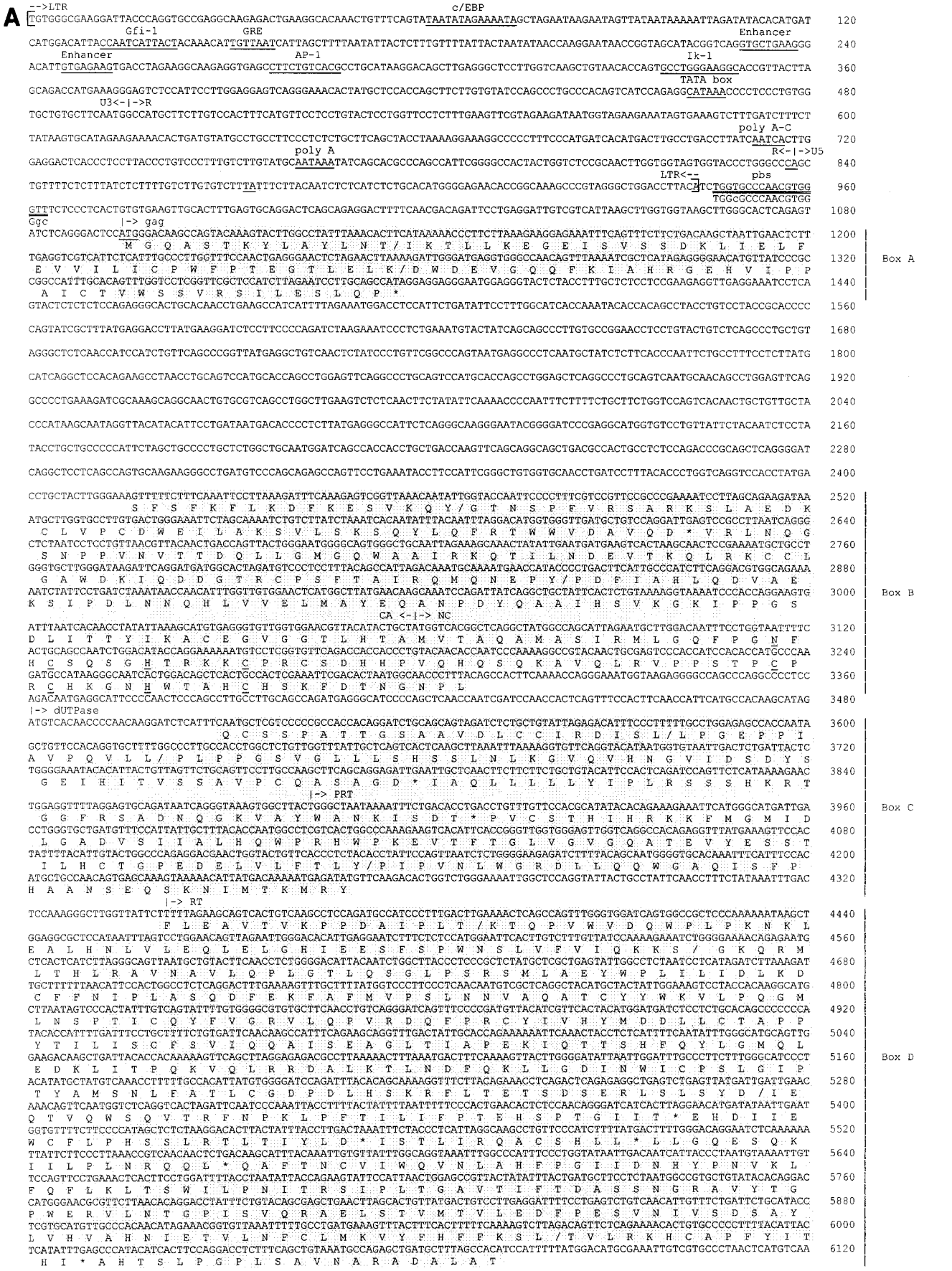
Classification of the HERV-K-T47D provirus. We previously isolated and cloned a human endogenous retroviral RT-related sequence from particles released by the human breast cancer cell line T47D. This *pol* fragment was used to isolate from a human genomic library, as described previously, a number of hybridizing λ clones (38) that were entirely sequenced by the dideoxy chain termination method (36). Two overlapping λ clones now revealed that this element, previously termed ERV-MLN, comprises an almost full-length proviral structure with an overall length of 9,315 bp (Fig. 1). Next to the 5′ LTR (nt 1 to 943) is a putative tRNA PBS (nt 946 to 963) which, despite a 3-bp mismatch, is most closely related to the complementary sequence of the 3′ end of human lysine tRNA (CUU anticodon) (Fig. 2A). The putative PBS is identical to that found in HERV-KC4 (6) and is closely related to the PBSs of other HERV elements belonging to the HERV-K superfamily. Therefore, ERV-MLN, a human endogenous retrovirus with lysine tRNA as the most likely primer for reverse transcription originating from retrovirus-like particles released by the T47D cell line, is now referred to as HERV-K-T47D.

To further classify HERV-K-T47D within subgroups HML-1 to -6, so far characterized by a 242-bp stretch of the RT domain (23), we aligned the subgroup sequences with the corresponding HERV-K-T47D region by using the software package Gene Works (IntelliGenetics, Inc.). Sequence comparison revealed that HML-4.1 is the most closely related sequence, showing about 80% nucleotide homology. Therefore, HERV-K-T47D is the first identified full-length prototypic element of the HERV-K(HML-4) subgroup.

Genomic organization and coding regions. Since a full-length HML-4 group provirus has not yet been identified, we used the well-characterized HERV-K10 (31), a member of the HML-2 subgroup, for alignments with a computer-assisted translation of the complete nucleotide sequence of HERV-K-T47D in order to analyze its genomic organization and identify putative reading frames. At the amino acid level, this revealed an HERV-K-T47D retroviral structure, comprising *gag*, *prt*, *pol*, and *env* genes (Fig. 2A), with six regions exhibiting significant protein similarity to HERV-K10, ranging from 40 to 60% homology (boxes A to F in Fig. 1 and 2A). Within the HERV-K-T47D *gag* gene, which tentatively extends from nt 1095, based on comparison with HERV-K10 and the location of a suitable methionine, to nt 3480, two regions with significant amino acid similarity to HERV-K10 were found (box A, nt 1058 to 1385, 40% homology; and box B, nt 2416 to 3308, 49% homology). As shown in Fig. 2A, box A corresponds to the amino-terminal part of the matrix protein, whereas box B comprises the carboxy-terminal half of the capsid (CA) protein and almost the entire nucleocapsid (NC) protein. These regions are separated by 1,235 nt displaying no significant nucleotide (or, hence, amino acid) identity to the corresponding region of HERV-K10, which is shorter and comprises only 627 nt. FASTA database searches based on the differing 608 nt of this sequence revealed an 89% nucleotide homology in a 203-bp overlap with a human CpG island (5). Within the NC protein of HERV-K-T47D, two Zn finger domains of the $CX_2CX_4HX_4C$ type were identified (nt 3114 to 3155 and nt 3237 to 3275). The first motif is defective, lacking the initial Cys, whereas the second Zn finger is intact. A third conserved HERV-K-T47D region (box C in Fig. 1 and 2A) extends from nt 3510 to 4250. It exhibits 59% amino acid identity to the corresponding region of HERV-K10 and comprises the complete retroviral dUTPase (nt 3510 to 3883) and part of the retroviral protease (nt 3884 to 4340).

Retroviral *pol* genes are generally the most conserved sequences among retroviruses (22). This concurs with our observation that a 2.5-kb stretch of HERV-K-T47D *pol* (nt 4320 to 6998) shows a 60% overall amino acid identity to HERV-K10 (Fig. 2A, boxes D and E). The RT (nt 4341 to 5184) exhibits 65% identity, including some short stretches with almost absolute identity, while the tether region (nt 5185 to 5672), which connects the RT and RNase H protein domains, is less conserved (47% amino acid identity). RNase H (nt 5673 to 6086) shows 50% identity to HERV-K10, including a common feature of retroviral RNase H proteins, the DEDD motif.

The *env* gene of HERV-K-T47D shows the least homology to HERV-K10, with the exception of the transmembrane domain (TM). At the amino terminus of the HERV-K-T47D TM, a region with 59% amino acid identity to HERV-K10 is found (Fig. 1 and 2A, box F, nt 8347 to 8991). Specifically, two clusters of hydrophobic amino acids (nt 8401 to 8478 and 8931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c/EBP
Gfi-1
GRE
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Enhancer
AP-1
Ik-1
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poly A
R<-|->U5
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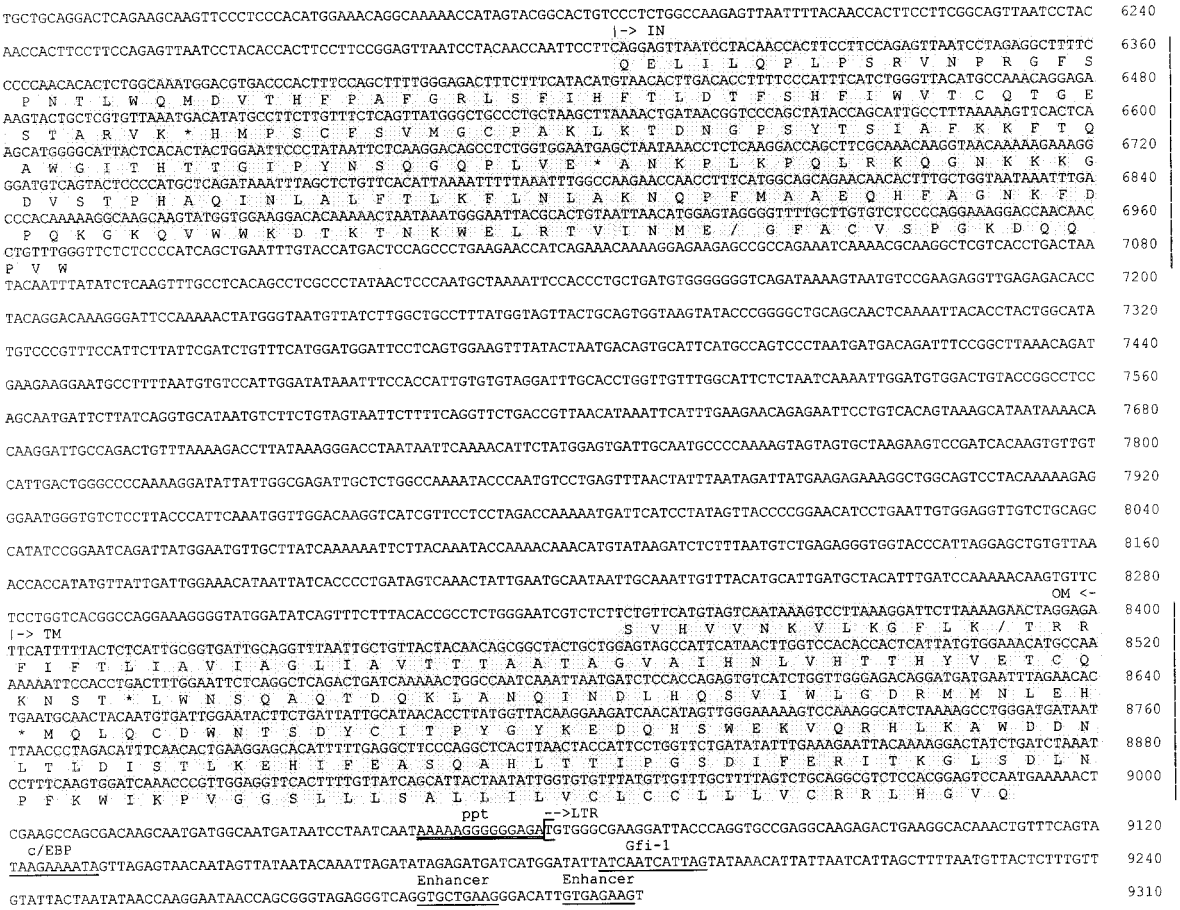

B

matrix	IR TGTGGG		c/EBP binding site V$CEBP_01		Gfi-1 binding site V$GFI_01	
HERV-K-T47D LTR	TGTGGG	53	TATAATATAGAAAATAGCTA	49	TTACCAATCATTACTAC	130
HumU2 Solo LTR	.....	53	..C.GC.A.....AT..*	53	...TT.....G.TT	131
Gorilla Solo LTR	.....	53	....G..A.....AT..	53	...TT.....G.TT	131
Pongo Solo LTR	.....	52	....A..A.....AT..	53	C..TT.....G.TT	130
Pan Solo LTR	.....	53	....G..A.....TAT..	53	...TT.....G.TT	131

matrix	AP-1 binding site V$AP1FJ_Q2		IK-1 binding site V$IK1_01		TATA box (putative)	
HERV-K-T47D LTR	GCCTTCTGTCACGC	45	AGTGCCTGGGAAGGCACC-	104	AGGCATAAAC	290
HumU2 Solo LTR	...C..AT.....*	45	.....C.TG	171	....TCC...	302
Gorilla Solo LTR	...C..AT.....*	43	.....-..C.TG	170	....T.C...	306
Pongo Solo LTR	...C..AT.....*	44	.....C.....C.TG	173	.C.-T.C...	298
Pan Solo LTR	...C..AT.....*	43	.....C.TG	170	....TCC...	298

	poly A signal		poly A tailing start		IR
HERV-K-T47D LTR	TGCAATAAATATCAG	71	TTCTCTTTATCTCTTTTGTC	67	CTTACA
HumU2 Solo LTR	.A.....G...	76	....TCC.....-...		
Gorilla Solo LTR	.A.....G...	76	....TCC.....-...		
Pongo Solo LTR	.A.....G...	72	....TCC.....-...		
Pan Solo LTR	.A.....G...	76	....TCC.....-...		

FIG. 2. (A) Nucleotide sequence of HERV-K-T47D proviral DNA. LTRs are enclosed in brackets, and the inverted termini TGT and ACA are indicated by arrows. Transcriptional regulatory sequences, i.e., c/EBP, Gfi-1, AP-1, Ik-1, a glucocorticoid-responsive element (GRE), enhancer-like elements, a putative TATA box, a polyadenylation signal, and polyadenylation sites (CA and TA), are underlined once and labeled above. PBS and the polypurine tract (ppt) are double underlined. Sequence complementary to the 3′ end of human lysine tRNA is depicted below the PBS sequence, with lowercase letters being used for mismatches. Translated amino acid sequences with significant homology to HERV-K10 (31), shown under the nucleotide sequence in the six shaded boxes A to F, are those of *gag* (box A, 40% identity; box B, 49% identity), dUTPase-protease (box C, 59% identity), RT-RNase H (box D, 59% identity), integrase (box E, 59% identity), and *env* (box F, 58% identity). Frameshifts in the amino acid sequence are indicated with slashes; asterisks correspond to stop codons. Conserved zinc finger motifs (type $CX_2CX_4HX_4C$) in the NC (box B) region are marked by underlining of the corresponding amino acids. (B) Alignment of putative regulatory elements of the HERV-K-T47D 5′ LTR with corresponding elements from solitary HERV-K-T47D-related LTRs of higher primates (33). Asterisks indicate binding sites which would not have been found with the default parameters of MathInspector (34). However, they were found when a lower threshold was used. Dots and dashes show identical and missing nucleotides, respectively. Under the binding site designations are search string variables used by the program MathInspector. IR, inverted repeat.

to 8970) are highly conserved (86% identity). The 3′ end of HERV-K-T47D *env* is followed by a polypurine tract (nt 9046 to 9059), which is a conserved motif of the retroviral *env*-LTR border. Despite their well-defined structure, the coding regions of HERV-K-T47D are interrupted by nonsense and frameshift mutations.

The putative LTRs of HERV-K-T47D were defined by aligning the sequences flanking the proviral coding regions at the 5′ and 3′ ends. Sequence repeats of 254 bp which differ from one another in 23 positions (91% homology) were identified. However, database searches revealed that the 943-bp region from the 5′ end of HERV-K-T47D exhibits 70% homology to a solitary retroviral LTR sequence at the human RNU2 locus on chromosome 17q21 (33). This LTR can be traced back to a complete retroviral element of 6 kb which still exists in the corresponding chromosomal locus of the baboon. During primate evolution, excision of the provirus by homologous recombination created the solitary LTR now found in the genomes of the chimpanzee, gorilla, orangutan, and human (33). This LTR is considered to be associated with the concerted evolution of the tandem array encoding U2 snRNA. Direct sequence alignment of this solitary RNU2 LTR with the ends of HERV-K-T47D revealed that its 5′ LTR is intact whereas the 3′ LTR is truncated after nt 254. The 5′ LTR is bordered by short inverted repeats (TGT. . .ACA) and is followed by an untranslated leader sequence of 150 bp (nt 944 to 1094) containing the PBS (nt 946 to 963). Several potential regulatory elements were identified by using the program ModelInspector (8). Putative binding sites for transcription factors C/EBP, Gfi-1, AP1, and Ik-1 were detected within the U3 region (Fig. 2A and B) by using the program MathInspector (34). These sequences were found to be conserved in solitary human and various solitary primate RNU2 LTRs (Fig. 2B). However, a putative TATA box at position 462 of HERV-K-T47D is not present in those solitary LTRs. A glucocorticoid-responsive element and two enhancer-like structures were also tentatively assigned (29). A polyadenylation signal [poly(A)] was detected in the 5′ LTR but not in the 3′ LTR, which is truncated in this region. Therefore, a poly(A) signal located either within the coding region of the provirus or within 3′ cellular flanking sequences may be used to generate HERV-K-T47D mRNA. To examine these possibilities, we screened a cDNA library from steroid-induced T47D cells with an HERV-K-T47D LTR probe (Fig. 1, probe 1) generated by PCR with forward primer CCGAGGCAAGAGACTGAAGG CAC (nt 25 to 47) and reverse primer ACTTCTCACAATGT CCCTTCAGC (nt 232 to 254). We were not able to isolate an HERV-K-T47D cDNA by this method, but we obtained several clones containing cellular sequences which are polyadenylated by solitary HERV-K-T47D-related LTRs (data not shown). Based on these clones, we identified two possible poly(A) addition sites (CA and TA) within the HERV-K-T47D LTR. The poly(A) addition site observed in the majority of cDNA clones was used to assign the R-U5 border (Fig. 2A).

Chromosomal location and evolution of HERV-K-T47D and related elements. Southern blot analysis was performed under high-stringency conditions as described previously (38) to determine the copy number and chromosomal location of HERV-K-T47D and closely related sequences in the human genome. *Hin*dIII-digested DNA from a panel of 24 human-rodent monochromosomal hybrid cell lines (mapping panel no. 2; NIGMS Human Genetic Cell Repository, Camden, N.J.) was hybridized to an HERV-K-T47D *pol* DNA fragment (Fig. 1, probe 2). The observed banding pattern suggests that HERV-K-T47D is located on human chromosome 10. Furthermore, five related elements, probably representing other members of the HERV-K(HML-4) family, could be assigned to chromosomes 8, 9, 15, 16, and 19. Southern blot analysis of human DNA samples digested with a set of restriction enzymes revealed that in addition to those proviral sequences, several hundred solitary HERV-K-T47D LTRs may exist in the human genome (Fig. 3A). As is known from studies of HERV-K(HML-2) (15) and HERV-H elements (7), multiple solitary LTRs are a common feature of HERV families. As evolutionary relics, they reflect high-level retrotransposon activity and subsequent homologous recombination during evolution.

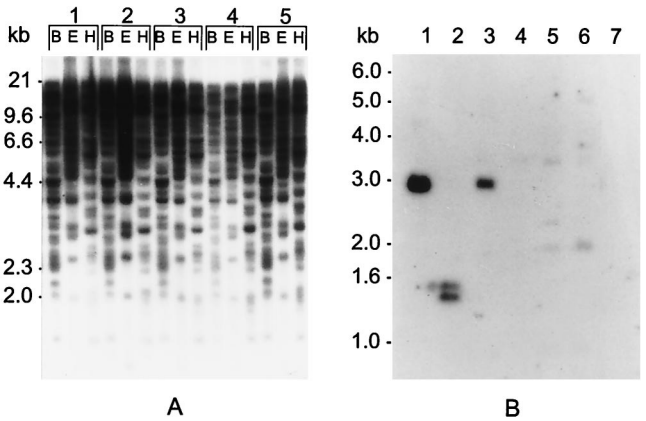

FIG. 3. (A) Southern blot analysis of human genomic DNA with a probe specific for the HERV-K-T47D LTR (Fig. 1, probe 1). DNA samples (10 μg/lane) from healthy individuals (1 to 5) were restricted to completion, blotted, and hybridized under relaxed stringency conditions (5× SSC, 60°C). Restriction enzymes are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; and H, *Hin*dIII. Marker sizes are indicated on the left. (B) Southern blot analysis of DNA derived from Old World and New World monkeys and higher primates, using a probe specific for the HERV-K-T47D *pol* gene (Fig. 1, probe 2). High-molecular-weight DNA (10 μg/lane) was restricted to completion with *Hin*dIII, blotted, and hybridized under relaxed stringency conditions. The DNAs analyzed were as follows: lane 1, human; lane 2, chimpanzee; lane 3, orangutan; lane 4, *Presbytis*; lane 5, baboon; lane 6, rhesus monkey; and lane 7, *Aotes*.

To investigate HERV-K-T47D evolution, we analyzed DNAs of Old and New World monkeys and higher primates by performing Southern blot hybridization under relaxed hybridization conditions as described previously (38). High-molecular-weight DNA was digested with *Hin*dIII and probed with the 2.9-kb HERV-K-T47D *pol* fragment (Fig. 1, probe 2). A strong signal of the same size (2.9 kb) was detected in restricted DNA from both the human and the orangutan (Fig. 3B, lanes 1 and 3). DNA derived from the chimpanzee resulted in two smaller bands (1.3 and 1.4 kb) of similar intensity (lane 2), suggesting the presence of an additional *Hin*dIII restriction site in this element. In Old World monkeys (lanes 4 to 6), a series of weak signals differing in size (approximately 2.0, 2.4, and 3.5 kb) were detected, while DNA from the New World monkey genus *Aotes* (lane 7) gave no detectable hybridization signal. These data concur with previous findings indicating that most HERV elements arose early in primate evolution (for a review, see reference 16).

Transcription of HERV-K-T47D in human tissues. Regardless of whether active, functional proteins are encoded, a crucial role of HERVs may be their ability to act as promoters of either immunologically related retroviral antigens or cellular genes or, conversely, to act as premature transcription terminators. Since the production and release of T47D particles is steroid dependent (11, 32, 38), T47D cells were treated with 10^{-9} M estrogen for 48 h, at which time was added 10^{-8} M progesterone, with subsequent incubation for 24 h (11, 30).

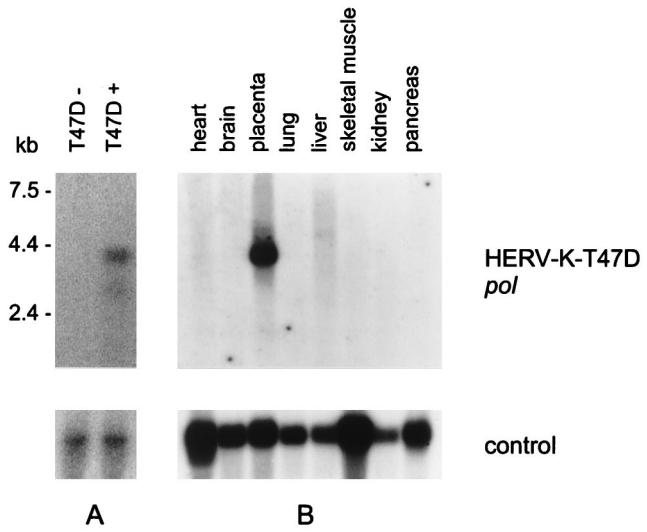

FIG. 4. HERV-K-T47D transcription in human tissues. (A) Total RNA derived from estradiol- and progesterone-induced (T47D+) or noninduced (T47D−) cells was blotted onto Hybond membranes. All filters were probed with an HERV-K-T47D *pol* fragment (Fig. 1, probe 2) and washed under conditions of high stringency (0.1× SSC, 65°C). (B) Multiple-tissue Northern blot with 2 μg of mRNA per lane from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas tissues. To assess RNA quality, the blots (A and B) were rehybridized with a human APC probe and a ubiquitin probe, respectively. Marker sizes are indicated on the left.

Total RNA was prepared from steroid-treated and untreated cells in accordance with a CsCl ultracentrifugation protocol (35), separated by denaturing 1% formamide–agarose gel electrophoresis, transferred to Zeta-Probe membranes (Bio-Rad, Munich, Germany) by the vacublot procedure (Vacu-Gene XL; Pharmacia/LKB, Freiburg, Germany), and hybridized with a ^{32}P-labeled HERV-K-T47D *pol* fragment (Fig. 1, probe 2) under high-stringency conditions (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% sodium dodecyl sulfate, 5× Denhardt's solution, and 100 μg of denatured sheared herring sperm DNA per ml for 16 h at 65°C). Expression of HERV-K-T47D was found exclusively in steroid-stimulated T47D cells (Fig. 4A), therefore correlating well with the production and release of HERV-K-T47D particles. The HERV-K-T47D transcript was about 4.5 kb, which does not match the size of a full-length or a regular spliced retroviral transcript.

To confirm our Northern blotting results and further analyze the observed truncated HERV-K-T47D transcript, PCR experiments were performed on T47D cDNA, using primers derived from HERV-K-T47D *gag* (reverse primer, CGCGGATCCTA TGGCTGCAAGGATTCTAAG, nt 1358 to 1381), *pol* (forward primer, CGCGGATCCCTCAACAATGTCGCTCAGG CTAC, nt 4741 to 4766; reverse primer, CGCGGATCCCCA AGTAACTTTTGAAAGTC, nt 5104 to 5126), *env* (forward primer, CGCGGATCCGTTTAATTGCTGTTACTACAACA GC, nt 8432 to 8456), and LTR (forward primer, CGCGGAT CCGCAACTTGGTGGTAGTGGTACC, nt 805 to 829) in various combinations and in combination with an oligo(dT)$_{22}$ primer. T47D mRNA was reverse transcribed, using a cDNA first-strand kit (Stratagene, La Jolla, Calif.) and the target-specific reverse primers. Amplification of HERV fragments was carried out in a reaction mixture (total volume, 100 μl) containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.25 mM each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany), and 50 pmol of each mixed oligonucleotide primer pair. Using a Perkin-Elmer Cetus DNA thermal cycler, PCR was performed with the following cycle parameters: a hot start at 94°C for 5 min; 30 cycles of 1 min at 94°C, 2 min at 55 to 65°C, and 2 min at 72°C; and a final extension step of 7 min at 72°C. A control reaction in which the DNA template was omitted was carried out to detect any traces of contaminating genomic DNA in the solutions used.

Only amplification with *gag* and *pol* primers yielded fragments, comprising 575 and 387 bp, respectively. These PCR products were sequenced, revealing 100% sequence identity to the corresponding *gag* and *pol* sequences of HERV-K-T47D (data not shown). However, despite several trials, amplification of HERV-K-T47D *env* sequences by using either *env*-derived primers or *pol* and *env* primers in combination with oligo(dT) primers failed. Therefore, we conclude that the 4.5-kb truncated HERV-K-T47D transcript contains only *gag* and *pol* sequences and lacks a complete HERV-K-T47D *env* gene. The lack of a functional poly(A) signal within the 3′ LTR of HERV-K-T47D correlates with the DNA sequencing data indicating that the polyadenylation signal detected in the 5′ LTR is located in the region that is truncated in the 3′ LTR. This suggests that the 4.5-kb transcript may be generated by premature termination, using a poly(A) signal located within the coding region of the provirus, or by splicing into 3′ cellular sequences which may provide the required poly(A) site.

To investigate the transcriptional activity of HERV-K-T47D in other human tissues, Northern blot analyses were carried out with a commercial human multiple-tissue Northern blot (HMT-blot; Clontech, Palo Alto, Calif.). In all Northern blot hybridization experiments, either the adenomatous polyposis coli (APC) gene, a human tumor suppressor and housekeeping gene (kindly provided by H.-J. Butterfass, German Cancer Research Center, Heidelberg, Germany), or the human ubiquitin gene (Clontech) was used to monitor mRNA integrity. Hybridization with the HERV-K-T47D *pol* fragment under high-stringency conditions revealed a high level of transcription exclusively in full-term placental tissue (Fig. 4B). The signal corresponds in size to the band obtained from T47D RNA after steroid treatment (Fig. 4A). With the exception of the breast carcinoma cell line T47D, a correlation between HERV-K-T47D expression and the occurrence or progression of malignancy was not found. No HERV-K-T47D transcripts could be detected in RNA from human tumor cell lines such as melanoma (S361), lung cancer (A549), colorectal adenocarcinoma (SW480), cervix carcinoma (HeLa), Burkitt's lymphoma (Raji), lymphoblastic leukemia (Molt-4), promyelocytic leukemia (HL-60), and chronic myeloid leukemia (K562) and in RNA from peripheral blood mononuclear cells of patients with chronic myeloid leukemia, acute lymphatic leukemia, or acute myeloid leukemia (data not shown). These results suggest that HERV-K-T47D transcription at levels detectable by Northern blot analysis is tissue specific and steroid hormone dependent.

Transcriptional activity of the 5′ LTR of HERV-K-T47D. To examine the promoter activity of the putative 5′ LTR of HERV-K-T47D in T47D cells, plasmids containing the luciferase reporter gene downstream of an LTR-containing DNA fragment of HERV-K-T47D were constructed (Fig. 1, BB1.2). The BB1.2 fragment was generated by PCR from the proviral sequence by using forward primer GCGGGATCCGAGGCA AGAGACTGAAGGCAC (nt 25 to 47) and reverse primer CGCGGATCCCTCAGTTGGAAACCAAGGGC (nt 1221 to 1243). Employing the newly introduced *Bam*HI restriction sites, BB1.2 was cloned in both the sense (pBL-BB1.2s) and the antisense (pBL-BB1.2as) directions into the multiple cloning site of the luciferase expression vector pBL (Fig. 5A) (kind-

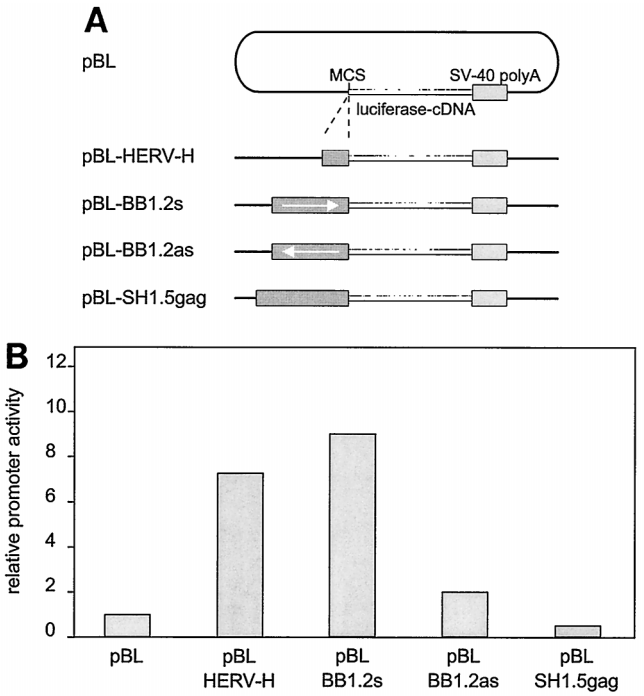

FIG. 5. Analysis of HERV-K-T47D putative LTR promoter activity in T47D cells. (A) pBL-HERV reporter constructs used for luciferase expression assays. The putative LTR 1.2-kb PCR fragment (Fig. 1, fragment BB1.2) was cloned in the sense (pBL-BB1.2s) and the antisense (pBL-BB1.2as) orientations into the luciferase expression vector pBL. As controls, plasmid pBL-SH1.5gag with the insert (SH1.5gag, Fig. 1) and pBL-HERV-H containing the HERV-H LTR promoter of H6 (7) were similarly constructed. MCS, multiple cloning site; SV-40, simian virus 40. (B) Transient expression in T47D cells of HERV-pBL luciferase reporter constructs. T47D cells were transiently transfected according to standard procedures. The luciferase expression driven by the retroviral promoter was measured by a standardized luciferase assay and is shown as bar graphs representing relative promoter activity. All results shown are derived from triplicate experiments.

ly provided by Karin Butz, German Cancer Research Center, Heidelberg, Germany). As a negative control, the SH1.5gag fragment was included in the T47D cell transfection experiments. Plasmid pBL-SH1.5gag (sense direction) was constructed by cloning a 1.5-kb *Spe*I-*Hin*dIII DNA fragment (Fig. 1) into pBL. Amplification of this fragment was performed with forward primer GCGACTAGTTGGGCACTCAGAGTA TCTCAG (nt 1062 to 1087) and reverse primer GCGAAGC TTCTGCTAAGGATTTTCGGGCGG (nt 2488 to 2517). As a positive control, a transcriptionally active HERV-H LTR was used (7). A 393-bp fragment containing the HERV-H promoter was amplified from clone H6 (kindly provided by D. Mager, Terry Fox Laboratory, Vancouver, British Columbia, Canada), using forward primer CGCGGATCCTGTCAGGCC TCTGAGCCCAA and reverse primer CGCAAGCTTATGTG AGCAACATGGCTGTT, and cloned via *Bam*HI and *Hin*dIII restriction sites into pBL. The identity and correct insertion orientation of each construct were verified by nucleotide sequencing.

Transfection of plasmids into T47D cells was performed by the calcium phosphate precipitation method (3). The day before transfection, 3×10^5 cells were seeded per 6-cm-diameter petri dish and cultivated for 24 h at 37°C and 5% CO_2. For transfection, triplicate dishes were incubated in parallel with a calcium phosphate-DNA mixture containing 0.8 pmol of reporter plasmid and 1 μg of pZ (a β-actin–luciferase construct used for internal standardization). The total amount of DNA per dish was adjusted to 6.5 μg with pBluescript SK(+). Incubation was carried out for 16 to 18 h at 37°C and 5% CO_2. T47D cells were then further incubated in fresh RPMI 1640 medium for 48 h. T47D cells were treated with estradiol and then progesterone (in dimethyl sulfoxide solvent), each for 24 h, as described by Keydar et al. (11), while the control dishes received medium with dimethyl sulfoxide alone. At 48 h postincubation (37°C, 5% CO_2), cells were harvested and lysates were prepared according to the recommendations of the Enhanced Luciferase Assay Kit (Berthold Detection Systems, Pforzheim, Germany). Relative HERV promoter activity was calculated as the ratio between the levels of luciferase expression of the constructs and the pBL vector. Transient expression of the constructs in T47D cells revealed that pBL-BB1.2s displayed about the same relative transcriptional activity as the active promoter of the HERV-H LTR (pBL-HERV-H). Upon steroid induction of T47D cells, an about twofold enhancement of luciferase activity was observed (data not shown). This suggests that the 5′ LTR of HERV-K-T47D contains regulatory elements that are steroid dependent and can mediate efficient transcriptional activity of HERV-K-T47D or other sequences in T47D cells.

In conclusion, our results show that HERV-K-T47D is actively transcribed in T47D cells in a steroid-dependent manner, and this active transcription is easily accounted for by the promoter activity and the presence of a number of putative transcription factor binding sequences found in the 5′ LTR. Such activity may also apply to a number of related solitary LTRs which were also detected, perhaps resulting in transcriptional activation of disease-associated antigens. However, the HERV-K-T47D-specific transcript, containing only *gag* and *pol* sequences, does not comprise a full-length proviral sequence but is presumably irregularly spliced or terminated. Since HERV-K-T47D does not have the coding capacity for full-length structural proteins, the origin of the retroviral proteins responsible for particle formation and the RT activity found associated with T47D particles (11, 32, 38) is still unclear. Particularly, the *gag* gene, which is essential for virus packaging and particle formation, is inactivated by stop codons and frameshifts in HERV-K-T47D. As rescue experiments with defective retroviruses lacking the *gag*, *pol*, and *env* open reading frames suggest (41), these activities must be provided in *trans* by other coding-competent HERV elements. Examples of such coding-competent HERVs are members of the HERV-K(HML-2) subgroup, transcripts of which have been detected in some human teratocarcinoma cell lines (20, 42). Since we were not able to isolate intact protein-coding HERVs from particle preparations, the packaging signals of these genomes may be defective in order to prevent the generation of replication-competent and possibly infective retroviral particles. Particularly in light of the use of retroviral vectors in gene therapy or the prospect of xenotransplantation (40, 43), identification of such HERV sequences and understanding the mechanisms and risks of generating new, infectious retroviral particles will be of major importance.

Nucleotide sequence accession number. The complete nucleotide sequence of HERV-K-T47D has been deposited in GenBank under accession no. AF020092.

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