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Retrovirus-Like Particles Released from the Human Breast Cancer Cell Line T47-D Display Type B- and C-Related Endogenous Retroviral Sequences

WOLFGANG SEIFARTH,[1]* HEYKO SKLADNY,[1] FRANK KRIEG-SCHNEIDER,[1] ANJA REICHERT,[1] RÜDIGER HEHLMANN,[1] AND CHRISTINE LEIB-MÖSCH[1,2]

III Medizinische Klinik, Klinikum Mannheim, Universität Heidelberg, D-68305 Mannheim,[1] and Institut für Molekulare Virologie, GSF-Forschungszentrum für Umwelt und Gesundheit, D-85758 Oberschleissheim,[2] Germany

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The human mammary carcinoma cell line T47-D releases retrovirus-like particles of type B morphology in a steroid-dependent manner (I. Keydar, T. Ohno, R. Nayak, R. Sweet, F. Simoni, F. Weiss, S. Karby, R. Mesa-Tejada, and S. Spiegelman, Proc. Natl. Acad. Sci. USA 81:4188–4192, 1984). Furthermore, reverse transcriptase (RT) activity is found to be associated with particle preparations. Using a set of degenerate primers derived from a conserved region of retroviral *pol* genes, we repeatedly amplified three different retroviral sequences (MLN, FRD, and FTD) from purified T47-D particles in several RT-PCR experiments. Screening of a human genomic library and Southern blot analysis revealed that these sequences are of endogenous origin. ERV-MLN represents a multicopy family of human endogenous retroviral elements (HERVs) with two closely related copies and up to 20 more distantly related members. In contrast, ERV-FRD and ERV-FTD comprise only one copy and five to seven related elements per haploid human genome. DNA sequence analysis of the proviral *pol* region of ERV-MLN revealed an uninterrupted stretch of 241 amino acids that shows 65% identity with the RT of the type B-related HERV designated HERV-K10. ERV-FRD and ERV-FTD are defective type C-related HERVs. The *pol* gene of ERV-FRD displays a nucleotide homology of 54% to the gibbon ape leukemia virus, and the *pol* gene of ERV-FTD is about 67% homologous to members of the RTVL-I family of HERVs. Our results thus indicate that the retroviral particles released by the breast cancer cell line T47-D are probably generated by complementation of several endogenous proviruses and can package retroviral transcripts of different origins.

The human genome contains many copies of inherited sequence elements with structural features of integrated retroviruses. Some of these human endogenous retroviral elements (HERVs) are single-copy elements, but most represent sequence families with up to 1,000 members, demonstrating the enormous reservoir of retroviral genes in the human genome. Most likely, these HERVs entered the primate genome at least 30 million to 40 million years ago and since then have spread by initiating their own retrotransposition. Today, at least 1% of the human genome consists of such endogenous retrovirus-related sequences (for a review, see references 4, 6, 21, 23, and 59).

The majority of these elements are inactivated by point mutations, frameshifts, or deletions, and to date, no replication-competent HERV has been detected. Nevertheless, a great number of HERVs are actively transcribed, and antigens immunologically related to retroviral structural proteins have been discovered in human sera and tissues (2, 3, 15, 20, 26, 56; for a review, see reference 21). Recently, transcripts of a family of HERVs (HERV-K) that are related to the mouse mammary tumor virus (MMTV) and that contain open reading frames possibly encoding functional Gag, Pol, and Env proteins have been characterized (27, 28, 37, 45).

Furthermore, particles with retrovirus-like morphology have been repeatedly detected in different types of human tissues and cell lines (for a review, see references 21 and 57). Retroviral particles have most commonly been observed in normal human placentas, oocytes, and fetuses (15, 22, 30, 34), both malignant and nonmalignant breast samples (1, 35), germ cell tumors, and cell lines derived from these tissues (25). In some cases, reverse transcriptase (RT) activity was found to be associated with particle fractions (9, 10, 17, 22, 25). In the case of the human teratocarcinoma cell line GH, immunological experiments demonstrated that the core proteins of the particles are probably coded for by members of the HERV-K family (3, 26). Taken together, these studies suggest that HERV proviruses can provide sufficient information to form retrovirus-like particles. However, there is still a lack of information on the biological function of these particles, their RNA genomes, and the origin of the RT activity. Furthermore, productive infectivity has not yet been demonstrated. Therefore, these particles may represent pseudotypes generated by complementation of several expressed HERVs and may contain defective retroviral genomes.

We have been studying this possibility by analyzing the retroviral RNA genomes packaged in particles released by the human mammary carcinoma cell line T47-D. This cell line was established from the pleural effusion of a patient with intraductal and invasive carcinoma of the breast and was found to release particles in a steroid-dependent manner (16). The particles produced by T47-D cells resemble type B virions, which has been demonstrated by electron microscopy, biochemical assays for RT activity, and immunological assays with antibodies raised against known infectious retroviruses (17). We used degenerate primers derived from a conserved sequence of the

* Corresponding author. Mailing address: III Medizinische Klinik, Klinikum Mannheim, Universität Heidelberg, Wiesbadener Str. 7-11, D-68305 Mannheim, Germany. Phone: (49) 621 383-4103. Fax: (49) 621 383-4201.

retroviral RT coding region to amplify RNA from purified T47-D particles. In this communication, we report the isolation, cloning, and sequence analysis of three different HERVs packaged as retroviral genomes in particles released from T47-D cells.

MATERIALS AND METHODS

Cells. T47-D cells (ATCC HTB 133) were obtained from the American Type Culture Collection and grown in RPMI-1640 medium (with Gln) containing 10% fetal calf serum, insulin (0.2 U/ml), and penicillin-streptomycin (100 U/ml). Nearly confluently grown cultures of T47-D cells were stimulated to produce retroviral particles by successive treatment with 10^{-9} M estradiol (days 1 and 2) and 10^{-8} M progesterone (days 3 and 4) according to the method of Keydar et al. (17).

RT assay. Retroviral T47-D particles were pelleted from supernatants (100 ml) of steroid-induced T47-D cell cultures by ultracentrifugation at 100,000 × *g* (Beckman SW-40/41). Particles were lysed on ice in 100 μl of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 0.2% Nonidet P-40, and 4 mM thioglycerol. After a brief centrifugation, 10 μl of the particle lysate was added to 40 μl of a standard RT cocktail containing 50 ng of DNase-treated control RNA supplied with the cDNA first-strand kit (Stratagene, Heidelberg, Germany). After reverse transcription, an aliquot of 5 μl was used for the subsequent PCR. The cDNA priming and PCR amplification were carried out with the control primers (sense primer, GCTGTTGGCTGTCTGCCTGG, and antisense primer, GGCATCCA CACAGGCCTGGA) supplied with the same kit. Reverse transcription and PCR were performed according to the instructions in the supplier's manual.

Purification of particle RNA. Retroviral particles from 240 ml of T47-D cell culture supernatant were pelleted at 100,000 × *g*, resuspended in 300 μl of 1× phosphate-buffered saline (PBS) and 10 mM $MgCl_2$, and treated with DNase (100 μg/ml) and RNase (50 μg/ml) for 30 min at 37°C. Proteinase K (100 μg/ml) was added, and incubation was prolonged for 30 min at 46°C. An equal volume of hot phenol–0.1% sodium dodecyl sulfate (SDS) (60°C) was added, and nucleic acids were extracted and purified by the standard phenol-chloroform method (43). Nucleic acids were precipitated with ethanol, air dried, and redissolved in 50 μl of diethyl pyrocarbonate (DEPC)–double-distilled H_2O.

Preparation of mRNA from T47-D cells. Cells were washed twice with ice-cold PBS. After the addition of a small volume of the same buffer, cells were scraped from tissue culture flasks with a rubber policeman and pelleted in a Heraeus Minifuge (at 1,500 rpm for 5 min at 4°C). Cells were resuspended in lysis buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, and 0.5% Nonidet P-40 and were lysed by swelling. After centrifugation of the suspension in a Heraeus Minifuge at 3,000 rpm for 5 min at 4°C, supernatant was extracted with an equal volume of hot phenol (65°C) and several times with an equal volume of phenol-chloroform-isoamyl alcohol until no proteinaceous interphase was detected. Total RNA was precipitated with ethanol overnight at −80°C and pelleted by centrifugation at 20,000 rpm at 4°C for 20 min (Beckman SW-40/41). Air-dried RNA was redissolved in binding buffer (10 mM Tris [pH 7.5], 0.5 M LiCl, 1 mM EDTA), and mRNA for RT-PCR was prepared with oligo$(dT)_{25}$ Dynabeads according to the supplier's protocol (Dynal, Hamburg, Germany).

RT-PCR and controls. Primers were synthesized with an Applied Biosystems oligonucleotide synthesizer. Mixed oligonucleotide primers (MOP) (50) were as follows: sense primer, CGCGGATCCTGGAAAGTG(C/T)T(A/G)CC(A/C)CA (A/G)GG, and antisense primer, CGCGGATCCGG(A/C)GGCCAGCAG (C/G)A(G/T)GTCATCCA(C/T)GTA. HERV-K10-specific, *pol*-specific primers (41) were as follows: sense primer, GCGCAGGATCCTAAACCCATACCAC TAACTTGG (nucleotides [nt] 3937 to 3961), and antisense primer, GCGCAG GATCCAGTCCAGCATTGGCAACCTCTGC (nt 4529 to 4553). ERV-MLN *pol*-specific primers were as follows: sense primer, CGACGGATTCATGTCG CT CAGGCTACATGC, and antisense primer, CGACGGATTCGTAGGGAT GCCCAAA GAAGG. ERV-FRD *pol*-specific primers were as follows: sense primer, GGCCGGATCCTT CTAGTTATCCTTGCAGAG, and antisense primer, GGCCGGATCCTTAATTAATT CCACCACATT. ERV-FTD *pol*-specific primers were as follows: sense primer, GCGAGGATCC TAGTGTCTGGT CCAAAGAAGGG, and antisense primer, GCGAGGATCCTTGAC TTGAT CAACTTCCTCAG. Synthetic *Bam*HI restriction sites are shown underlined. Human β-actin primers (38) were as follows: sense primer, GACCTTCAACAC CCCAGCCATGTAC (nt 1864 to 1889), and actin antisense primer, CTCCTT AATGTCAC GCACGATTTC (nt 2109 to 2133).

RNA (50 μl) extracted from T47-D particles was treated again with 10 U of DNase per 50 μl of RNA (RNase-free; Boehringer, Mannheim, Germany) for 30 min at 37°C in 100 mM sodium acetate (pH 5.0) and 5 mM $MgSO_4$. The extracted RNA (40 μl) was reverse transcribed with 10 pmol of the antisense MOP (Stratagene's first-strand cDNA synthesis kit). A portion (1/10) of the RT product was added to the PCR 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 deoxynucleoside triphosphates (each), 2.5 U of *Taq* DNA polymerase (Boehringer), and 50 pmol of each MOP. A Perkin-Elmer Cetus DNA thermal cycler was used for the PCR cycle, and the parameters were a hot start at 94°C for 5 min; 30 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C; and a final extension step of 7 min at 72°C.

A control procedure omitting the RT step was carried out to detect possible traces of genomic DNA in the T47-D particle preparations. To exclude contaminating cellular mRNA, a PCR with human β-actin primers was performed (38). Negative controls containing water instead of template were also included in each experiment. A portion (1/10) of the PCR mixture was separated by agarose gel electrophoresis (3% NuSieve [Bio-Rad] and 1% agarose), and amplification products were visualized with UV light after ethidium bromide staining.

Cloning and sequencing of RT-PCR products. After separation in agarose gels, the PCR products were extracted from the gels by the freeze and squeeze procedure (55) and purified by phenol extraction. Fragments were digested at the synthetic *Bam*HI restriction sites included in all the PCR primers and ligated into *Bam*HI-restricted pBluescript SK^+ (Stratagene) with T4 DNA ligase (Bethesda Research Laboratories). Plasmid DNA from positive clones was prepared with a Midiprep kit (Qiagen) and sequenced by the dideoxy chain termination method (44) with a T7 sequencing kit (Pharmacia). Sequence analysis and alignments were done with the software package Gene Works (IntelliGenetics, Inc.) on an Apple Quadra 840av.

Southern blot analysis. Genomic DNA was extracted from T47-D cells and human lymphocytes by the guanidinium isothiocyanate standard procedure (43). After restriction with the appropriate restriction endonuclease, DNA was separated in 1% agarose gels and transferred to Zeta-Probe membranes (Bio-Rad) by the vacu-blot procedure (Vacu-Gene XL; Pharmacia/LKB, Freiburg, Germany). Hybridization probes were excised from pBluescript SK^+ by digestion with *Bam*HI (Boehringer) and gel purified prior to being labelled with a nick translation or Megaprime kit (Amersham). The probes were cloned DNA fragments MLN (153 bp), FRD (151 bp), and FTD (151 bp) obtained by RT-PCR with MOP primers (MOP fragments) (see Fig. 2B); specific DNA fragments MLN (412 bp), FRD (770 bp), and FTD (945 bp) obtained by RT-PCR with specific primers (see Fig. 6A and B); and genomic restriction fragments MLN (2.9 kb [*Hin*dIII-*Hin*dIII]), FRD (3.0 kb [*Bam*HI-*Bam*HI]), and FTD (1.7 kb [*Eco*RI-*Hin*dIII]) spanning the *pol* region of the cloned proviruses ERV-MLN, ERV-FRD, and ERV-FTD (see Fig. 6A).

Prehybridization was carried out for 4 h at 60°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.05 M NaH_2PO_4 (pH 6.5), 0.1% SDS, 5× Denhardt's solution, 100 μg of denatured herring sperm DNA per ml, and 5.0% powdered milk. Hybridization was performed at high stringency in the same solution without powdered milk at 65°C (relaxed conditions, 55°C) for at least 16 h with 10^6 cpm of ^{32}P-labelled DNA per ml of hybridization solution. Washing was performed by raising stringency conditions in 0.1% SDS and 0.1× SSC from 60 to 68°C (relaxed conditions, 55 to 60°C).

Genomic library screening. For isolation of the corresponding genomic proviral HERVs, 5×10^6 recombinant λ phages of a human genomic library derived from a Burkitt lymphoma (Ly66II; kindly provided by M. Lipp, University of Munich) were screened. The genomic library Ly66 was constructed from a partial digest of human genomic DNA (average insert size, 18 kb) with λ vector EMBL3A and was once amplified (24a). Hybridization was performed with the isolated MOP fragments MLN, FRD, and FTD (see Fig. 2B) being used as probes under high-stringency conditions. Copy numbers of HERV sequences were estimated on the basis of the frequency of hybridizing clones per 5×10^6 recombinant λ phages. Positive λ phages were characterized by restriction endonuclease analysis and Southern blotting according to standard protocols (43). Hybridizing restriction fragments were separated on 1× Tris-borate-EDTA–1% agarose gels, isolated by electroelution, and subcloned into pUC18/19 and pBluescript SK^+ (Stratagene) as described above.

Nucleotide sequence accession number. The nucleotide sequences of the RT coding regions of ERV-MLN, ERV-FRD, and ERV-FTD have been deposited with GenBank under the following accession numbers: ERV-MLN, U27242; ERV-FRD, U27240; and ERV-FTD, U27241.

RESULTS

Demonstration of RT activity associated with T47-D particles. The presence of RT activity is a defining characteristic of retroviral particles. Therefore, we developed a very sensitive test based on RT-PCR that allows detection of minimal amounts of RT activity. For this purpose, we used a commercially available cDNA first-strand synthesis kit. The supplied control RNA was tested for contaminating DNA by a PCR omitting the RT reaction step and by using the control primer set supplied with the kit. After treatment of the RNA template with RNase-free DNase, no amplification products could be detected. Therefore, any amplification product obtained with this test would demonstrate an RT activity in the reaction mixture. Using this assay, we examined our T47-D particle

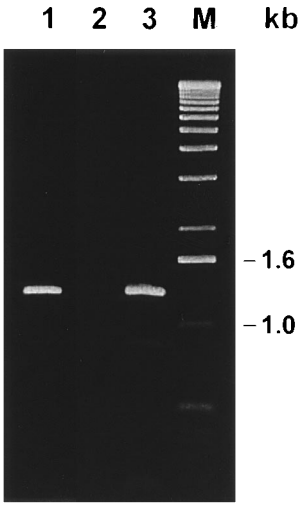
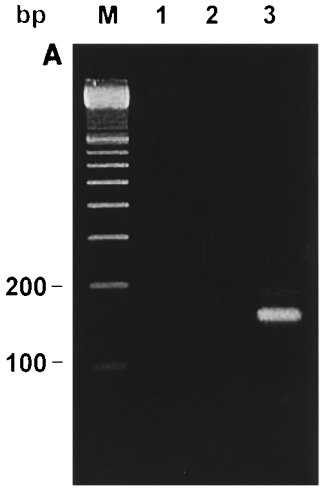


FIG. 1. PCR-based assay for RT activity associated with T47-D particles. A volume (1/10) of the RT-PCR products (expected size, 1.3 kb) was electrophoresed on a 1% Tris-borate-EDTA agarose gel and analyzed by ethidium bromide staining. Lanes: M, 1,000-bp marker (Bio-Rad); 1, RT reaction with 10 μl of particle lysate; 2, RT reaction with cleared cell culture supernatant; 3, positive control (RT reaction with Moloney murine leukemia virus RT).

preparations for RT activity after mild lysis. Amplification of the RNA template in a standardized PCR yielded products with the expected length of 1.3 kb (Fig. 1, lane 1). In a further experiment, cell culture supernatant obtained after the pelleting of T47-D particles was added to the RT reaction mixture. No specific amplification products could be detected (Fig. 1, lane 2), indicating that RT activity is bound to structures which can be pelleted by ultracentrifugation and that cDNA synthesis is not due to free enzyme in the cell culture supernatant. As a positive control, Moloney murine leukemia virus RT was added to the first-strand reaction mixture (Fig. 1, lane 3). These results demonstrate that retroviral particles associated with a functionally active RT are released from T47-D cells in our cell culture.

Purification, amplification, and sequence analysis of RNA extracted from T47-D particles. To avoid contamination by cellular nucleic acids originating from decaying T47-D cells, we placed much effort on the purification of RNA from T47-D particles. Since cellular genomic DNA and mRNA represent the major source of contamination, we established an experimental protocol similar to the method for λ phage preparation (43). This method was worked out with a type C virus-producing cell line as a test system and has been shown to be highly efficient for amplification and cloning of unknown retroviral genomes from virions (48). Supernatants of steroid-induced T47-D cultures (17) were harvested just before the cells were confluently grown. Particles were pelleted by ultracentrifugation, resuspended in PBS, and treated with a combination of DNase and RNase. This step was carried out to remove all nucleic acids accessible to the added enzymes, e.g., RNA and DNA not packaged in the virions and therefore not protected by the viral core proteins. Subsequently, proteinase K was added to inactivate the degradative enzymes, and the RNA genome of the particles was extracted with hot phenol-SDS. After several phenol-chloroform purification steps, the extracted particle RNA was treated again with RNase-free DNase. RT-PCR were performed to amplify the viral RNA genomes. A set of MOP (50) derived from highly conserved motifs (amino acids VLPQG and Y(M/I)DD(L/I)L) (14) from retroviral RT was used for RT-PCR.

Several control experiments to test particle RNA preparations for contamination with cellular DNA and RNA were carried out. To demonstrate that no genomic DNA is present in our RNA preparations, a PCR omitting the reverse transcription step was performed. To check for contaminating cellular RNA, we performed an RT-PCR with a human β-actin-specific primer set. Amplification of an abundantly expressed transcript, such as β-actin, should indicate any random contamination with cellular mRNA copurified with T47-D particles. For amplification of particle genomes, we used only prep-



B

ATGCTTAATAGTCCGACTATTTGTCAGTATTTTGTGGGGCGTGTGCTTCAACCTGTCAGGGATCAGTTTCCCCGATGTTACATCGTTTAC 90
M L N S P T I C Q Y F V G R V L Q P V R D Q F P R C Y I V Y

TTCAGAGATAGTCCCCATTTGTTTGGGCAAGCCTTGGCTAGATATTTGCAGGACCTAAGTCTTTATATGGAAGGGCATCTCCTACAG 87
F R D S P H L F G Q A L A R Y L Q D L S L Y M E G H L L Q

TTCACAGACTCCCCTAATATTTTTGGTGAAATTTTAGAACAAGCATTAGAAAAAGTTTTCATTCCAGAACAAATATGCCTTCTCCAG 87
F T D S P N I F G E I L E Q A L E K V F I P E Q I C L L Q

FIG. 2. (A) RT-PCR amplification products derived from T47-D particle RNA. PCR products (1/10 of reaction mixture volume) were separated by 3% NuSieve (Bio-Rad)–1% agarose–1× Tris-borate-EDTA gel electrophoresis and visualized with UV light after ethidium bromide staining. Lanes: M, 100-bp ladder (Bio-Rad); 1, template of double-distilled water; 2, template of 10 μl of particle RNA omitting the reverse transcription step; 3, template of 10 μl of reverse-transcribed (antisense MOP) particle RNA. (B) Nucleotide and deduced amino acid sequences of the three HERV amplification products predominantly isolated from T47-D particles. Clones were termed MLN, FRD, and FTD on the basis of the first three amino acids (shown underlined) of their putative open reading frames. The PCR primer (MOP) sequence is not included. The numbers at the right indicate the number of nucleotides.

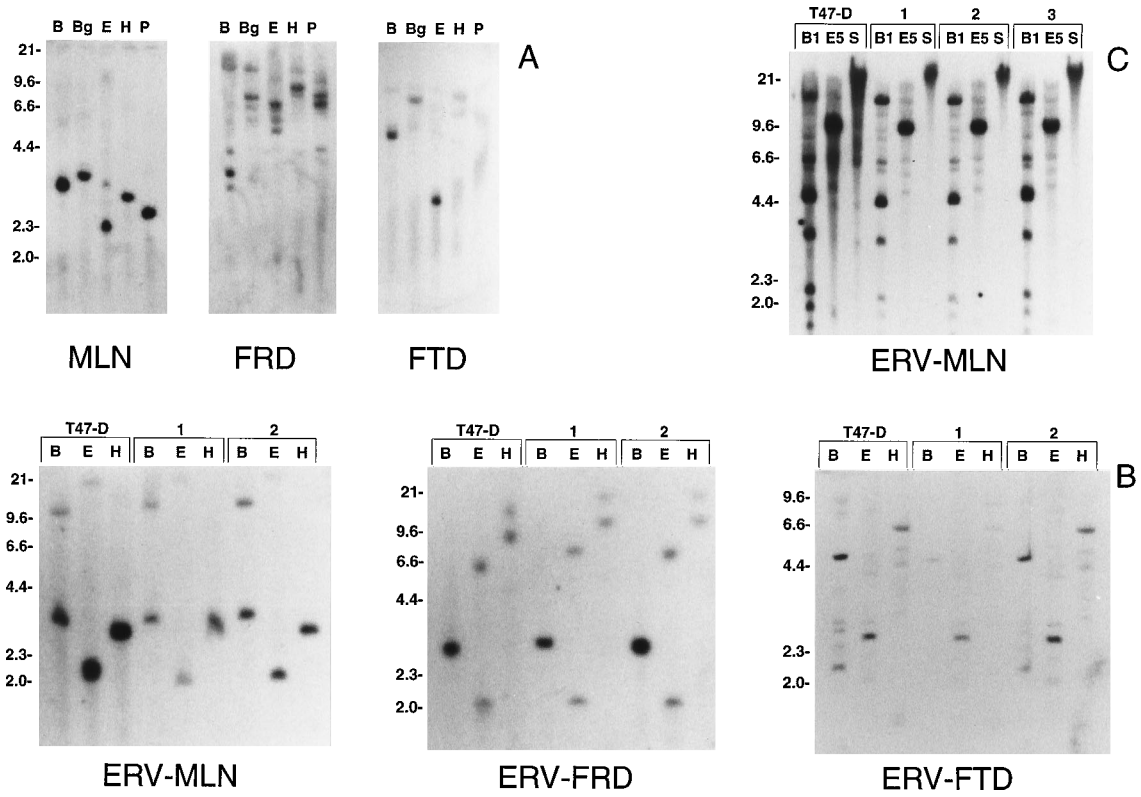


FIG. 3. Southern blot analysis of human genomic DNA with probes specific for the three T47-D HERVs. (A) T47-D DNA was restricted to completion and hybridized under relaxed stringency conditions. For hybridization, cloned RT-PCR fragments (MOP amplification products MLN, FRD, and FTD [Fig. 2B]) derived from T47-D particles were used. (B and C) Genomic DNAs isolated from T47-D cells and from mononuclear cells of healthy persons (lane groups 1 to 3) were hybridized under high-stringency conditions with cloned DNA fragments derived from proviral clones (ERV-MLN, 2.9-kb *Hin*dIII-*Hin*dIII; ERV-FRD, 3.0-kb *Bam*HI-*Bam*HI; and ERV-FTD, 1.7-kb *Eco*RI-*Hin*dIII [Fig. 6A]). Restriction enzymes: B, *Bam*HI; B1, *Bgl*I; Bg, *Bgl*II; E, *Eco*RI; E5, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sma*I. Marker sizes are indicated in kilobases to the left of the gels.

arations which showed negative results for both types of contamination.

Amplification products that were about 150 bp in length (Fig. 2A, lane 3) were extracted from the agarose gels and cloned, and a representative number of clones were analyzed by nucleotide sequencing. To make sure that the amplified and cloned retroviral sequences were reproducibly derived from particles, the PCR was repeated three times, starting from different supernatant pools. Sequences that had been amplified in all three experiments were further characterized.

We reproducibly isolated three types of retroviral sequences in several copies. Their nucleotide and deduced amino acid sequences are shown in Fig. 2B. Preliminarily, the three retroviral clones were termed MLN, FRD, and FTD on the basis of the first three positions of their deduced amino acid sequences, respectively. Together with the primer (MOP) sequences, the MLN fragment is 153 bp in length and FRD and FTD each comprise 151 bp. Additionally, some closely related retroviral sequences with 56 to 65% homology to clone MLN, 89 to 99% homology to clone FRD, and 69 to 89% homology to clone FTD were obtained; however, they were obtained in only one of the three experiments (sequence data not shown). Our findings indicate that MLN, FRD, and FTD are preferentially packaged members of HERV families comprising several closely related proviruses.

HERV-K elements are to date the only known HERV genomes that possess open reading frames possibly encoding functional proteins. We therefore used HERV-K-specific primers derived from the *pol* region of clone HERV-K10 (42). However, we could not detect HERV-K-specific amplification products either in mRNA preparations of T47-D cells or in particle RNA (data not shown). Our results indicate that the T47-D cells grown in our laboratory do not express HERV-K *pol* sequences on a level sufficient to be detected by a one-round PCR.

Copy number and endogenous origin of retroviral genomes from T47-D particles. Southern analysis to identify the copy number of the three HERV sequences isolated from T47-D particles was performed. Two sets of Southern experiments differing in stringency conditions and the origins of the DNA probes used for hybridization were carried out. For the Southern experiment shown in Fig. 3A, T47-D DNA was digested with different restriction endonucleases (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I) and hybridized under relaxed stringency conditions to the amplification products synthesized from T47-D particle RNA (MOP fragments). In the second set of Southern blots (Fig. 3B), high-stringency hybridization of human genomic DNA from T47-D cells and two unrelated individuals (lane groups T47-D, 1, and 2, respectively) was performed with proviral DNA fragments of ERV-MLN, ERV-FRD, and ERV-FTD isolated from a once-amplified human genomic library by screening with the respective MOP fragments being used as probes. Therefore, hybridization probes 2.9 HH (ERV-MLN, 2.9 kb), 3.0 BB (ERV-FRD, 3.0 kb), and 1.7 EH (ERV-FTD, 1.7 kb) are of human genomic origin (see Fig. 6A) but are not derived from T47-D cells or particles.

A comparison of blot MLN (Fig. 3A) and blot ERV-MLN (Fig. 3B) reveals that for all investigated DNA samples, the

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ERV-MLN     SKGLVILFRSSHCQASRCHPFDLKT-QPVWVDQWPLPKNKLEALHNLVLE      49(frame1)
HERV-K10    RRNR.SFLGAVTVEPPKPI.LTW..EK....N......Q......L.AN.      50
MMTV        ---FTGFMIGAIESNLFADQISW.SD....LN....KQE..Q..QQ..T.      47
MuIAP       W.PRQTGSGF.LAAIGAAR.IPW..GD....P..H.SSE....VIQ..E.      50

                                        GKQRMLTHLRAVNAVLQPLGTLQSG    25(frame3)
ERV-MLN     QLELGHIEESFSPWNSLVFVIQKKS*                              74(frame1)
HERV-K10    ...K....P.......P..........WHT..D.......I..M.P..P.      100
MMTV        ..Q...L...N....TP....K.....W.L.QD......TMHDM.A..P.      97
MuIAP       ..K....DP.T...TPI...K.....W.L.HD..PI.EQMNLF.PV.R.      100

ERV-MLN     LPSRSMLAEYWPLILIDLKDCFFNIPLASQDFEKFAFMVPSLNNVAQATC      75(frame3)
HERV-K10    ...PA.IPKD....I........T....E..C.....TI.AI..KEP..R     150
MMTV        ...PVAVPLG.EI.I...Q.....K.HPE.CKR...S...P.FKRPYQR      147
MuIAP       ..VL.A.PRG.N..I..I.....S...CPR.RPR...TI..I.SDEPDNR     150

ERV-MLN     YYWKVLPQGMLNSPTICQYPVGRVLQPVRDQFPRCYIVHYMDDLLCTAPP     125(frame3)
HERV-K10    FQ................T....A.....EK.SD...I..I...I..A.ET     200
MMTV        FQ........K....L..K..DKAILT...KYQDS........I.LAH.S     197
MuIAP       .Q........S....M..LY.QEA.L...E...SLILLL....I.LCHKE     200

ERV-MLN     YTILISCFSVIQQAISEAGLTIAPEKIQTTSHFQYLGMQLEDKLITPQKV     175(frame3)
HERV-K10    KDK..D.YTFL.AEVAN...A..SD....STP.H.....I.NRK.K...I     250
MMTV        RS.VDEILTSMI..LNKH..VVST....KYDNLK...THIQGDSVSY..L     247
MuIAP       L.M.QKAYPFLLKTL.QW..Q..T..V.ISDTG.F..SVVSPDK.V....     250

ERV-MLN     QLRRDALKTLNDFQKLLGDINWICPSLGIPTYAMSNLFATLCGDPDLHSK     225(frame3)
HERV-K10    EI.K.T.................R.T............SI.R..S..N.Q     300
MMTV        .I.T.K.R..........N....R.F.KLT.GELKP..EI.N..SNPI.T     297
MuIAP       EI...H.H...NF.........LR.F.K..SAELRP..WY.E...HIS.P     300

                                LIEQTVQWSQVTRFNPKLPFTILIFPTEHSPTGI    34 (frame2)
ERV-MLN     RFLTETSDSERLSLSY*                                       241(frame3)
HERV-K10    .I..PEATK.IK----.V.EKI.SA.IN.ID.LA.LQL...A.A......      346
MMTV        .R..PEACKALQ----.MNERLSTAR.K.LDLSQ.WSLC.LK..YT..AC     343
MuIAP       .T..LAANQALQ----KV.KAL.NA.LQAIEDSQ..SLCV.K.AQL..AV     346
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FIG. 4. Homology of ERV-MLN with type B retroviruses. Amino acid sequence alignment of the RT coding region of ERV-MLN and the corresponding regions of type B-related retroviruses (HERV-K10 [41], MMTV [36], and murine intracisternal type A particle gene [40]). Amino acid domains corresponding to the loci of MOP (50) used for RT-PCR are indicated with underlines. Identities are depicted as dots. Asterisks are used to denote frameshifts.

same pattern of prominent bands can be detected with both the particle-derived MOP fragments and the proviral DNA probes isolated from the genomic library. In most cases, only one single band in each lane of restricted DNA is observed, suggesting that only one copy of ERV-MLN per haploid genome exists. However, the hybridizing bands are small in size and may also represent internal restriction fragments. Therefore, additional Southern analyses were performed under the same conditions with rare cutting enzymes (Fig. 3C). We used sequence data obtained from the proviral ERV-MLN clone to select enzymes cutting only once in the proviral ERV-MLN sequence and not in the hybridization probe (see Fig. 6A). Two strongly hybridizing bands are observed in each lane of *Bgl*I- and *Eco*RV-restricted DNA, suggesting that two closely related copies of ERV-MLN in the human genome exist. Beside these strong signals, about 10 weaker hybridizing bands can be detected, indicating that ERV-MLN represents a novel family of HERVs that includes at least 10 more distantly related members. To determine the copy number of these more distantly related proviral ERV-MLN sequences in the human genome, we screened a human genomic library under high-stringency hybridization conditions. Comparing the number of hybridizing phages with the number of λ phages representing one complete human genome, we found that ERV-MLN represents a HERV family with up to 20 related members.

Southern analyses with FRD MOP fragments and genomic ERV-FRD probes also revealed identical patterns of strong signals (*Bam*HI, 3.0 kb; *Eco*RI, 6.6 and 2.1 kb; and *Hin*dIII, 9.0 kb) for all DNA samples. Additionally, three weak bands appear in the Southern blot (FRD) hybridized under relaxed stringency conditions (Fig. 3A). DNA restricted with *Eco*RI and *Hin*dIII shows two signals with lower intensities than that of the signal observed in *Bam*HI-digested DNA (Fig. 3B), which is presumably due to internal *Eco*RI and *Hin*dIII restriction sites in the proviral ERV-FRD probe used for hybridization (see Fig. 6A). Therefore, we conclude that ERV-FRD is a single-copy HERV in the human genome and that there are at least three related HERVs which cross-hybridize with the FRD probe under relaxed stringency conditions. The corresponding FTD blots also reveal similar signal patterns (*Bam*HI, 5.0 kb; *Eco*RI, 2.6 kb; and *Hin*dIII, 7.0 kb). The high-stringency ERV-FTD blot shows about three additional weakly hybridizing bands compared with the low-stringency FTD blot. This may be due to the use of the 1.7-kb *Eco*RI-*Hin*dIII probe (see Fig. 6A) which contains conserved regions of the *prt-pol* genes of ERV-FTD and which may detect further related HERVs. Taken together, these results show that identical patterns of strong signals are observed in all investigated DNA samples with hybridization probes originating from T47-D particles and from human genomic DNA. Therefore, we conclude that ERV-MLN, ERV-FRD, and ERV-FTD are endogenous elements of the human genome.

Copy number calculations for ERV-FRD and ERV-FTD based on genomic library screening revealed that they are probably present in the human genome as single-copy HERVs with approximately five to seven related elements, confirming the data obtained on the basis of the banding patterns observed on Southern blots.

Isolation of genomic clones and sequence analysis of RT coding regions. To identify the proviral genomes of HERVs packaged in T47-D particles, we screened a human genomic library (Ly66II) under high-stringency conditions with the MLN, FRD, and FTD MOP sequences being used as hybridization probes. Ten clones hybridizing strongly with MLN and six clones each hybridizing with FRD and FTD were isolated and further characterized by restriction mapping and Southern hybridization. All λ clones which had been isolated with the same MOP fragment as the probe showed identical hybridization patterns for the internal restriction fragments (data not shown). Furthermore, partial sequencing of the *pol* genes revealed nucleotide identities, at least within the analyzed re-

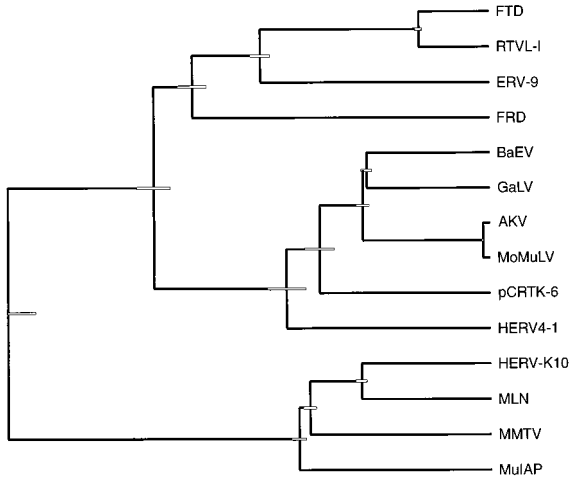

FIG. 5. Phylogenetic relationship of ERV-MLN, ERV-FRD, and ERV-FTD. For multiple alignments, a conserved stretch of 400 bp (starting with the RT motif of amino acids VLPQG [14]) from the RT coding region was compared with the corresponding RT sequences of other known HERVs, including the S71-related clone pCRTK-6 (11), HERV-K10 (41), HERV4-1 (42), ERV-9 (19), and RTVL-I (31), as well as those of several mammalian endogenous and exogenous retroviruses, including baboon endogenous virus (BaEV) (54), Gibbon ape leukemia virus (GaLV) (7), endogenous murine leukemia virus (AKV) (8), Moloney murine leukemia virus (MoMuLV) (51), MMTV (36), and the murine intracisternal type A particle gene (MulAP) (40).

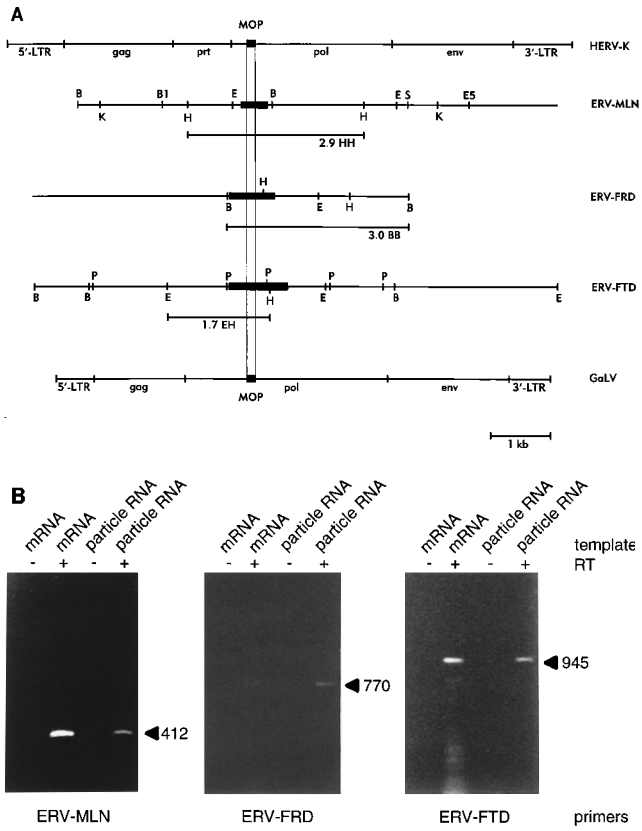

FIG. 6. (A) Partial restriction map of the proviral λ clones ERV-MLN, ERV-FRD, and ERV-FTD and localization of specific HERV probes used for Southern hybridization. Proviral genomic probes (ERV-MLN, 2.9 HH; ERV-FRD, 3.0 BB; and ERV-FTD, 1.7 EH) are depicted as lines (Southern hybridization [Fig. 3B]). Probes amplified from T47-D particle RNA (MLN, 412 bp; FRD, 770 bp; and FTD, 947 bp) are represented as black bars (Southern hybridization [Fig. 7]). For purposes of comparison, the proviral organizations of typical type B (HERV-K) (41) and type C (Gibbon ape leukemia virus [GaLV]) (7) retroviruses are shown. LTR, long terminal repeat; MOP, MOP-derived amplification product; B, *Bam*HI; B1, *Bgl*I; E, *Eco*RI; E5, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I. (B) Reamplification of ERV-MLN-, ERV-FRD-, and ERV-FTD-specific fragments from T47-D particles by RT-PCR. PCR primers (see Materials and Methods for the sequences) were deduced from the sequences of proviral λ clones. +, PCR with RT step. −, PCR without RT step. A 1/10 portion of PCR reaction mixture was analyzed on 1.5% agarose gels. The amplification products corresponded to the expected sizes (MLN, 412 bp; FRD, 770 bp; and FTD, 947 bp).

gions. Therefore, a representative member of each HERV type was selected and subcloned into plasmid vectors for further analysis. Subsequently, the putative *pol* coding regions of HERVs MLN, FRD, and FTD were sequenced. In each case, the nucleotide sequence of the genomic clone was found to be identical in the region of the MOP fragment to that of the originally amplified particle RNA genome. A search of sequence databases revealed an overall homology of 65% for the ERV-MLN *pol* gene and that of the type B-related HERV-K10 (41). In contrast, the *pol* gene of ERV-FRD displays a nucleotide homology of 54% with that of the type C gibbon ape leukemia virus (7). The *pol* gene of ERV-FTD was found to be 67% homologous with those of members of the RTVL-I family of HERVs (31).

Figure 4 shows an amino acid sequence comparison of the RT coding region of ERV-MLN and those of type A- and B-related retroviruses. The deduced amino acid sequences share a 65% amino acid identity with that of the RT of HERV-K10. The RT coding sequence of ERV-MLN is disrupted by two frameshifts (amino acid 74, frame 1→3, and amino acid 241, frame 3→2) and is split over all three frames. The putative RT coding regions of the type C-related ERV-FRD and ERV-FTD are interrupted by several stop codons and frameshifts that prevent expression of a functional protein.

To compare HERVs MLN, FRD, and FTD with other known HERVs, a well-conserved stretch of 400 bp from the RT coding region was used for a multiple alignment. The resulting phylogenetic tree reveals that ERV-MLN is closely related to the HERV-K family of HERVs (Fig. 5) and forms one group together with the MMTVs (41) and murine intracisternal type A particles (40). The single-copy element FRD represents a new type of HERV. Its closest relative is the HERV ERV-9 (19). ERV-FTD shares a common ancestor with ERV-FRD and is most closely related to RTVL-I, an endogenous retroviral element first identified in the human haptoglobin cluster (31). ERV-FRD, ERV-FTD, RTVL-I, and ERV-9 are clustered into a separate group of type C-related HERVs.

Reamplification of particle genomes with specific primers derived from proviral T47-D HERV sequences. To confirm our results that T47-D particle genomes are identical with ERV-MLN, ERV-FRD, and ERV-FTD isolated from human genomic DNA, RT-PCR were performed with specific primers derived from the *pol* sequences of the corresponding proviral genomic phage clones. The lengths of the expected amplification products indicated in Fig. 6A are 412 bp (ERV-MLN), 770 bp (ERV-FRD), and 947 bp (ERV-FRD). Figure 6B shows fragments amplified with those primers by using mRNA isolated from T47-D cells and particle RNA as templates for RT-PCR. In all cases, the expected amplification products could be observed. These data suggest that transcripts of the proviral HERV sequences MLN, FRD, and FTD are present in T47-D cells and are packaged in particles.

PCR amplification products were further analyzed by cloning, nucleotide sequencing, and Southern blot hybridization. Four different samples of human genomic DNA (T47-D and three unrelated healthy individuals) were hybridized under high-stringency conditions with the HERV sequences reamplified from the particle genome (Fig. 7). All DNA samples display identical banding patterns after hybridization with the HERV probes MLN (412 bp), FRD (770 bp), and FTD (945 bp). Compared with the Southern blot shown in Fig. 3B (ERV-MLN panel), that for MLN shows the same prominent bands of 3.2 kb (*Bam*HI), 2.2 kb (*Eco*RI), and 2.9 kb (*Hin*dIII). In contrast, the 770-bp FRD probe does not detect the 2.1-kb hybridization signal in the *Eco*RI-digested DNA which is observed in the corresponding FRD blot shown in Fig. 3B. This is because of the location of the reamplified 770-bp FRD sequence, which lacks the internal *Eco*RI and *Hin*dIII restriction sites present in the genomic 3.0 kb BB ERV-FRD probe (Fig. 6A). Therefore, the 770-bp FRD probe recognizes only one unique restriction fragment in *Eco*RI- and *Hin*dIII-digested DNA. Hybridization with the FTD probe (945 bp) revealed a number of signals with different intensities, indicating that ERV-FTD belongs to a family with at least five to seven members that are not identical but are closely related. The sizes of the prominent bands (*Bam*HI, 5.0 kb; *Eco*RI, 2.6 kb; and *Hin*dIII, 7.0 kb) in Fig. 7 are consistent with the sizes of fragments detected with the genomic hybridization probe in the corresponding lanes in Fig. 3B. These results indicate that transcripts packaged in T47-D particles are derived from ERV-MLN, ERV-FRD, and ERV-FTD proviruses. Furthermore, partial sequence analysis and comparison of the regions of the transcripts with the corresponding RT coding regions of

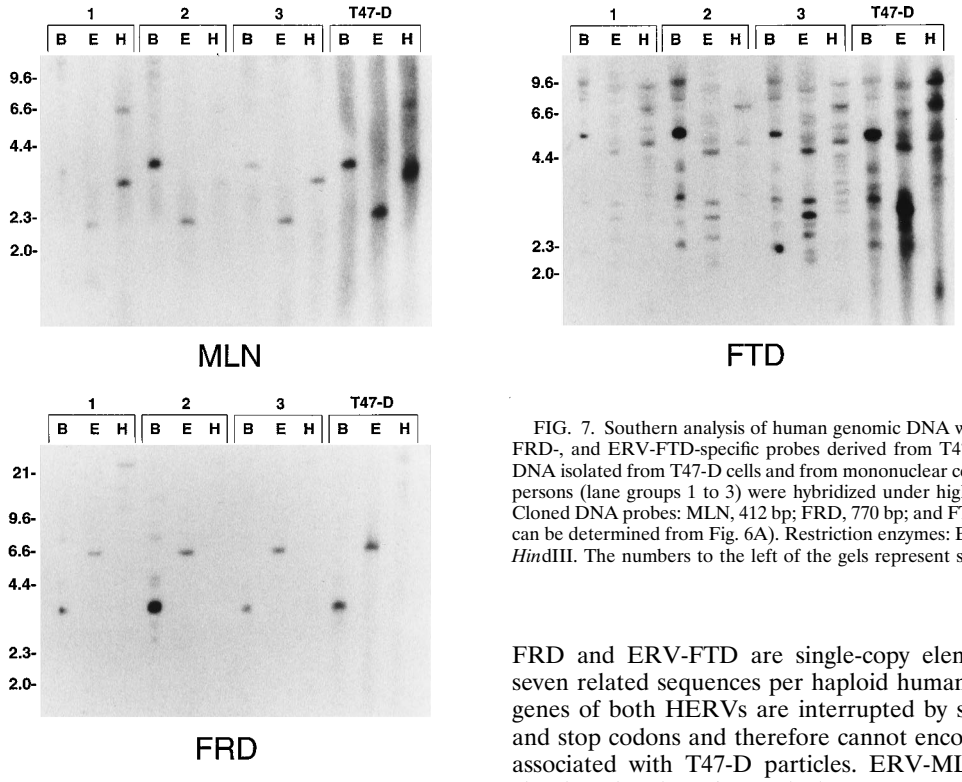

FIG. 7. Southern analysis of human genomic DNA with ERV-MLN-, ERV-FRD-, and ERV-FTD-specific probes derived from T47-D particles. Genomic DNA isolated from T47-D cells and from mononuclear cells of unrelated healthy persons (lane groups 1 to 3) were hybridized under high-stringency conditions. Cloned DNA probes: MLN, 412 bp; FRD, 770 bp; and FTD, 947 bp (localization can be determined from Fig. 6A). Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The numbers to the left of the gels represent sizes (in kilobases).

the genomic clones (data not shown) confirm that the particle sequences are identical to the proviral sequences isolated from the genomic library.

DISCUSSION

In the past, particles resembling retroviruses have been observed in many human tissues and cell lines and have been associated with various human tumors (for reviews, see references 13, 21, and 57). In most cases, these retrovirus-like particles were produced in very small amounts and therefore were characterized only by electron microscopy, measurement of RT activity, or immunological methods. Since efficient and sensitive molecular techniques were not available to further investigate these particles and clarify their origin, these observations were not confirmed in subsequent studies, and therefore these particles were often considered to be laboratory contaminants (for a review, see reference 57).

In the last few years, however, very sensitive methods that allow the molecular analysis of only traces of DNA and RNA have been developed. We have used the sensitive RT-PCR method to study particles released after steroid hormone stimulation of the human mammary carcinoma cell line T47-D in two different ways. In order to identify low levels of particle-bound RT activity, we developed an RT-PCR-based test using a commercially available cDNA synthesis kit. With the help of degenerate primers derived from a well-conserved sequence of the RT coding region of retroviruses (50), we were able to amplify three different retroviral sequences from purified T47-D particles. These three sequences were predominantly isolated from three independently prepared T47-D particle fractions. Sequence analysis revealed homology with either type B (MLN) or type C retroviruses (FRD and FTD).

The amplified sequences were used to isolate the corresponding proviruses from a human genomic library. ERV-FRD and ERV-FTD are single-copy elements with five to seven related sequences per haploid human genome. The *pol* genes of both HERVs are interrupted by several frameshifts and stop codons and therefore cannot encode the RT activity associated with T47-D particles. ERV-MLN comprises two closely related copies and about 20 more distantly related sequences in the human genome. The *pol* gene of one proviral ERV-MLN clone was analyzed by nucleotide sequencing. The ERV-MLN RT coding region contains only two frameshifts. Taking into account the large number of ERV-MLN-related sequences present in the human genome, it seems to be possible that some of these sequences could contain intact *pol* genes and might be expressed at least at low levels in T47-D cells. Previously, Shih et al. (50) reported the detection of RT coding sequences in human and mouse genomic DNAs. Amplification from human DNA revealed a subset of PCR clones which are related to the HERV-K family. One of these clones (A13) has an amino acid sequence identical to that of clone MLN amplified from T47-D particles. Additionally, 14 related PCR clones were characterized by Shih et al., confirming that MLN/A13 is a member of a multicopy family with at least 14 related sequences in the human genome.

The particle-associated RT and also the particle-forming proteins must not necessarily be coded for by retroviral genomes packaged into the virions. Both structural proteins as well as retroviral enzymes could be provided in *trans* by other HERVs. Candidates for such HERVs are members of the HERV-K family. To date, HERV-K type 1 (HERV-K10) and type 2 are the only known human endogenous retroviruses with open reading frames encoding genes required for retroviral replication (26, 28, 37, 45). Furthermore, enzymatic activity has been demonstrated at least for HERV-K protease (37, 45). This enzyme shows autocatalytic cleavage when expressed in *Escherichia coli* and can process the HERV-K Gag polyprotein. HERV-K sequences have been found to be transcribed in various normal human tissues (32) and leukocytes (5, 18, 33) and have been associated with particle formation in a human teratocarcinoma cell line (3, 25–27). Furthermore, steroid-dependent expression of HERV-K elements has been reported to occur in T47-D cells (39). Therefore, we used primers derived from the *pol* gene of clone HERV-K10 (41) to search for

transcripts encoding HERV-K RT. However, we were not able to amplify HERV-K *pol* transcripts either from isolated T47-D particles or from mRNA preparations, even though the same primers amplified HERV-K transcripts from various normal human tissues and tumors and human leukocytes (48). In accordance with our findings, spliced or unspliced HERV-K transcripts could not be detected in cytoplasmic T47-D mRNA preparations by Northern (RNA) blotting (25a). However, the involvement of HERV-K elements in the particle formation of T47-D cells cannot absolutely be excluded. The inability to detect even low levels of HERV-K *pol* transcripts detectable by PCR could also be explained by the use of HERV-K10-specific primers which might not fit properly to other members of the HERV-K family. Furthermore, HERV-K elements represent only one subgroup of type B-related HERVs. At least six different subgroups of human MMTV-like HERVs with similarity to MMTVs and intracisternal type A particles have been identified by sequence comparisons of conserved regions within the RT (32). HERV-K elements represent the human MMTV-like HERV subgroup HML-1, whereas ERV-MLN could be assigned to subgroup HML-4. Among these groups, further, yet unknown HERVs with open reading frames encoding retroviral proteins might exist.

The fact that we could not isolate sequences encoding an intact RT from T47-D particles may be due to a protective mechanism to prevent packaging of replication-competent endogenous retroviral genomes. Possibly, HERVs with open reading frames for the essential retroviral proteins are defective in their packaging signal, whereas retroviral mRNAs containing an intact packaging signal are inactivated by mutations in the protein coding regions. This would mean that a strategy similar to that used in the construction of packaging cell lines for retrovirus-mediated gene transfer is used by nature to inactivate possibly infectious HERVs and protect the organism from further infection.

The phylogenetic analysis revealed that MLN, FRD, and FTD are completely divergent HERVs that are related to animal type B or C retroviruses. This raises the question whether there might be different types of particles released from T47-D cells. Analysis of T47-D particles by Keydar et al. (17) revealed some clues to the existence of two heterogenous forms of T47-D particles. Analyzing clonal derivatives of the human breast carcinoma cell line T47-D, we found two types of particles which can be distinguished by biochemical characteristics. One type (T47-D clone 11) has a density of 1.18 g/ml and is released from cells in a steroid-dependent manner, whereas T47-D cells of clone 8 produce particles with a higher density (1.195 g/ml) that are independent of steroid hormone induction. These observations could be explained by the release of two types of particles which are assembled with distinct proteins derived from type B and C HERVs.

The formation of phenotypically mixed particles is a well-known process that occurs during virus assembly in cells coinfected with two or more retroviruses and has, for example, repeatedly been observed with avian and mammalian retroviruses (24, 58). Investigations by Spector et al. (52) revealed that dual infection of cells with the human immunodeficiency virus type 1 and a murine amphotropic retrovirus leads to the production of human immunodeficiency virus type 1 pseudotypes with expanded cellular and species tropism. Furthermore, endogenous murine retroviruses can provide structural proteins in *trans* to form murine leukemia virus-human immunodeficiency virus type 1 pseudotypes (29). Copackaging of defective endogenous retroviral elements by type C retroviruses has also been demonstrated in several murine cell lines (47, 49, 53), including a commonly used packaging cell line (12, 46). VL30 sequences, a family of retroelements that are found at 100 to 200 copies in the mouse and rat genome, do not encode any essential retroviral genes. However, these elements have been shown to form pseudotypes with various type C retroviruses (49). In transmission experiments, Scolnick et al. (47) demonstrated the infectivity of VL30 transcripts packaged by murine or primate retroviruses. Further studies indicating the biological activity of these pseudotypes have been reported on by Hatzoglou et al. (12). Rat hepatoma cells acquire mouse VL30 retroviral elements when infected with Moloney murine leukemia virus recombinant retrovirus produced from Ψ2, a packaging cell line currently used for gene therapy. Undesirable transmission of endogenous murine proviral sequences to human cells has been reported by Scadden et al. (46). We showed that a 5.2-kb mouse transcript originally derived from a defective mink cell focus-forming provirus is packaged in Ψ2 pseudotype virions and integrated into the genome of human target cells upon infection.

Taken together, the published data indicate that pseudotyping and copackaging of defective endogenous retroviral sequences is a common retroviral mechanism and therefore has to be considered as an explanation for our findings that different HERVs can be isolated from T47-D particles. Although these particles seem to be replication-defective pseudotypes and cannot infect cells productively, they might still be able to attach to cellular receptors and penetrate the cell. The weak RT activity detected in the particle preparations suggests that reverse transcription and subsequent integration of the defective retroviral genomes into the cellular DNA might occur and lead to insertion mutagenesis in the infected cells. The fact that a human cell line can act as a natural packaging cell line, albeit with low efficiency, may have consequences for the use of retroviral vectors in gene therapy. Therefore, it will be important to further investigate the nature of T47-D particles and to identify the proviral sequences encoding the RT and the structural proteins of these particles.

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