Characterization of Viral Particles Isolated from Primary Cultures of Human Breast Cancer Cells

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Abstract

The association of human breast cancer with sequences similar to the mouse mammary tumor virus (MMTV) has been shown, but convincing evidence for the presence of viral particles in breast tumors has been lacking. We have described the complete proviral structure of a retrovirus in human breast cancer. This provirus, designated as human mammary tumor virus (HMTV), was 95% homologous to MMTV and revealed features of a replication-competent virus. We have therefore investigated the production of viral particles in primary cultures of human breast cancer (MSSM). Cells isolated from ascites or pleural effusions of patients with metastatic breast cancer contained viral sequences in their DNA, expressed Env protein, and showed retroviral particles by electron microscopy. Viral particles from culture media exhibited morphologic features of β-retroviruses sedimenting at buoyant densities of 1.12 to 1.18 g/mL in sucrose gradients and showed reverse transcriptase activity. cDNA sequences from virion RNA were synthesized, amplified, and sequenced and all the virion genes were detected and 70% of the virion RNA was sequenced. The sequence homologies were, respectively, 85% to 95% compared with the MMTV and HMTV proviruses we have previously described. These results clearly show that breast cancer cells in primary cultures produced HMTV viral particles that are similar to the mouse virus and which may play a role in human breast cancer pathogenesis. [Cancer Res 2007;67(18):8960–5]

Introduction

The association of a virus similar to mouse mammary tumor virus (MMTV) with human breast cancer has been shown by us and others (1–8). We reported that a 660-bp sequence of the MMTV *env* gene is present in 38% of American women's breast cancers (1) and is expressed in most of the *env*-positive tumors (2). The sequence is not detected in the normal breast tissue of patients with *env*-positive carcinomas, thus ruling out germ line transmission or polymorphisms of endogenous retroviral sequences (3). Subsequent amplification experiments showed other gene segments homologous to their MMTV counterparts, but not to any of the human endogenous retroviruses including K-10 (HERV-K10), which is the most similar to MMTV (4). Using a 2.7 kb *env*-LTR sequence as a fluorescence *in situ* hybridization probe, several integration sites are visualized in different chromosomes in breast cancer cells freshly isolated from patients (4). Finally, a complete human mammary tumor virus (HMTV) proviral structure has been detected in two tumors (4). The provirus, with 96% sequence homology to MMTV, contains several hormone-responsive elements and the open reading frame for the superantigen (5). The cloned superantigen sequence has been expressed in human B cells demonstrating that it is functional and that it may play a role in pathogenesis. Pervasive differences between the LTRs of the human and murine viruses were recorded that may be related to disease determinants (5).

Because the complete proviral structure indicates that this is a replication-competent virus, we sought the presence of viral particles in primary cultures of human breast cancer cells. In this article, we report the characterization of such viral particles isolated from these cultures.

Materials and Methods

Cells. Ten primary cultures (MSSM) were obtained from discarded ascitic fluid or pleural effusions of patients with metastatic breast cancer (1). Cells were grown in MEGM media (Cambrex Bio Science Walkersville, Inc.). The medium contained 10 ng/mL of human epidermal growth factor, 5 μg/mL of insulin, 0.5 μg/mL of hydrocortisone, and bovine pituitary extract. Culture media and fluids were tested by PCR for *Mycoplasma* using the mycoplasma detection kit (American Type Culture Collection). MCF10F cells, a normal human breast–derived cell line (9) from American Type Culture Collection were grown in RPMI 1640 with 5% horse serum and antibiotics.

Isolation of viral particles. Four liters of medium from confluent monolayers of individual MSSM cell cultures were centrifuged at 500 rpm for 15 min at 4°C. The supernatant was centrifuged at 10,000 rpm for 30 min at 4°C in a Sorvall Rc-5B centrifuge and the resulting supernatant at 100,000 × *g* for 150 min at 4°C in an SW 27.1 rotor in an ultracentrifuge (Beckman L7-55). The high-speed pellet particulate fraction (PF) was resuspended in 10 mmol/L of Tris-HCl buffer (pH 7.4) and kept at −70°C for further analysis.

Electron microscopy. Cells and PF were fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydrated with ethanol, and embedded in Epoxil resin. Thin sections were double-stained with uranyl acetate and lead citrate, and examined in a Siemens 1A electron microscope (10).

Sucrose gradient. The PF was layered on top of a 10% to 50% sucrose gradient and centrifuged at 100,000 × *g* for 16 h at 4°C in a SW41 rotor in an ultracentrifuge (Beckman L7-55). The density of each fraction was determined by refractometry (Abbe 3L; Bausch&Lomb). Reverse transcriptase (RT) activity was determined in 10-μL aliquots of each fraction.

RT activity. RT activity was determined in the PF and in aliquots of the sucrose gradient fractions following the conditions described by Silver et al. (11) with minor modifications. Briefly, the PF pellet was resuspended in 50 μL of 10 mmol/L Tris-HCl (pH 7.4). To an aliquot of the PF, 0.2 mmol/L of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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DTT (Sigma-Aldrich Co.), 2 mmol/L of phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 0.2% NP40 (Sigma-Aldrich) were added to disrupt viral particles. The first step was done with 21 ng of Brome Mosaic Virus (BMV) RNA (Promega, Inc.) as template for cDNA synthesis, 0.2 μmol/L of the BMV-RT primer (5′-GGTCTCTTTTAGAGATTTACAGTG-3′; Invitrogen), 1 μL of RNaseOUT (Invitrogen), 1 μg of calf thymus DNA in 16.7 mmol/L of Tris-HCl (pH 8.3), 83.3 mmol/L of KCl, 8.3 mmol/L of $MgCl_2$, 0.8 mmol/L of deoxynucleotide triphosphate, 3.3 mmol/L of DTT. The mixture was incubated at 42°C for 60 min, then at 94°C for 10 min. Ten microliters of the reaction were assayed for PCR amplification using PCR beads (GE Healthcare Bio-Sciences Corp.), 2 μmol/L of specific forward primer BMV1 (5′-CGTGGTTGACACGCAGACCTCTTAC-3′; Invitrogen) and reverse primers BMV2 (5′-TCAACACTGTACGGCACCCGCATTC-3′; Invitrogen) for 40 cycles of 94°C for 15 s, 56°C for 15 s, 72°C for 15 s, and 1 cycle of 72°C for 5 min. The product of the reaction was detected in 2% agarose gels. The hybridization was carried out using radiolabeled specific BMV-Probe (5′-GCCTTTGAGAGTTACTCTTTG-3′; Invitrogen). The densitometry profile of the radioautograph was obtained using a Bio-Rad Imaging Densitometer (Bio-Rad Lab) and Molecular Analyst software (Bio-Rad Lab).

To increase viral production, MSSM3 and MCF10F cells were treated with 10 nmol/L of progesterone (Sigma-Aldrich), 1 nmol/L of estradiol (Sigma-Aldrich), and/or 50 ng phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 16 h (12, 13). Cells were irradiated for 1.5 min with a 15 W germicidal lamp (emission, 2,000 erg/cm^2/s) at 10 cm of distance. The culture medium was collected 24 h later and the RT activity determined in the PF.

RT-PCR. RNA was extracted from PF using the RNA isolation kit (Ambion Inc.) and treated with DNase (Ambion) before cDNA synthesis. Briefly, cDNA primers were designed to cover the entire putative viral RNA. Each of the cDNAs obtained were amplified by PCR with the corresponding forward and reverse primers and detected by hybridization with the specific labeled probe. All the primers and probes were designed based on the published HMTV sequences (1–4) using the OligoPerfect Designer software (Invitrogen). cDNA syntheses were done with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's protocol with 500 ng of RNA and specific cDNA primer for each viral fragment at 55°C for 60 min. The exception was the *gag* gene fragment cDNAs that were synthesized with HIV reverse transcriptase (Ambion) following the manufacturer's protocol at 65°C for 60 min after a cycle of denaturalization at 94°C for 5 min. The RNA template was removed from the cDNA/RNA hybrid by digestion with RNase H (Invitrogen) at 37°C for 30 min. The amplification step was done using Platinum Taq DNA Polymerase High-Fidelity (Invitrogen). Briefly, 10 μL of the cDNA synthesis reaction and 2 μmol/L of the forward and reverse primers were used. The product of the PCR reactions was run in agarose gel, stained with ethidium bromide, transferred to a nylon transfer membrane (Schleicher&Schuell), and hybridized with a specific labeled probe following the conditions previously described (2). Nested PCR was done using 5 μL of the first PCR reaction and appropriate second forward (2F) and reverse (2R) primers. A second nested PCR was done when necessary, using corresponding 3F and 3R primers. Amplification of the *gag* gene fragments was done with the GC cDNA PCR kit (BD Biosciences Inc.) using appropriate primers and following the conditions recommended by the manufacturer. The primers used and the conditions for PCR are shown in Supplementary Table S1. PCR products were sequenced by automated DNA sequencing (Applied Biosystems Inc.). The sequences were compared with others present in the Genbank using the BLAST program from National Center for Biotechnology Information, NIH.

Antibodies

P2 Monoclonal antibody. Amino acid sequences from the 660 bp *env* gene sequence (AF239172) were examined by the antigenicity index (PeptideStructure). The sequence designated P2 (amine-LLGLPHLI-DIEKRGSTFHIS-amide) was predicted to be the most immunogenic and was synthesized at the Microchemistry Laboratory of the New York Blood Center. A murine monoclonal antibody, designated as mAbP2, was prepared by Viro/Dynamics.

SU Polyclonal antibody. The sequence for the SU component of the *env* gene from a virus associated with human primary biliary cirrhosis (14, 15) was inserted into a modified pET bacterial expression vector. In addition to the usual his6tag, this vector had been modified to include an Avitag (Avidity Inc.). Expression was done in a modified BL21 (DE3) bacterial strain containing the plasmid pBIRAcm, which expresses the biotinylating

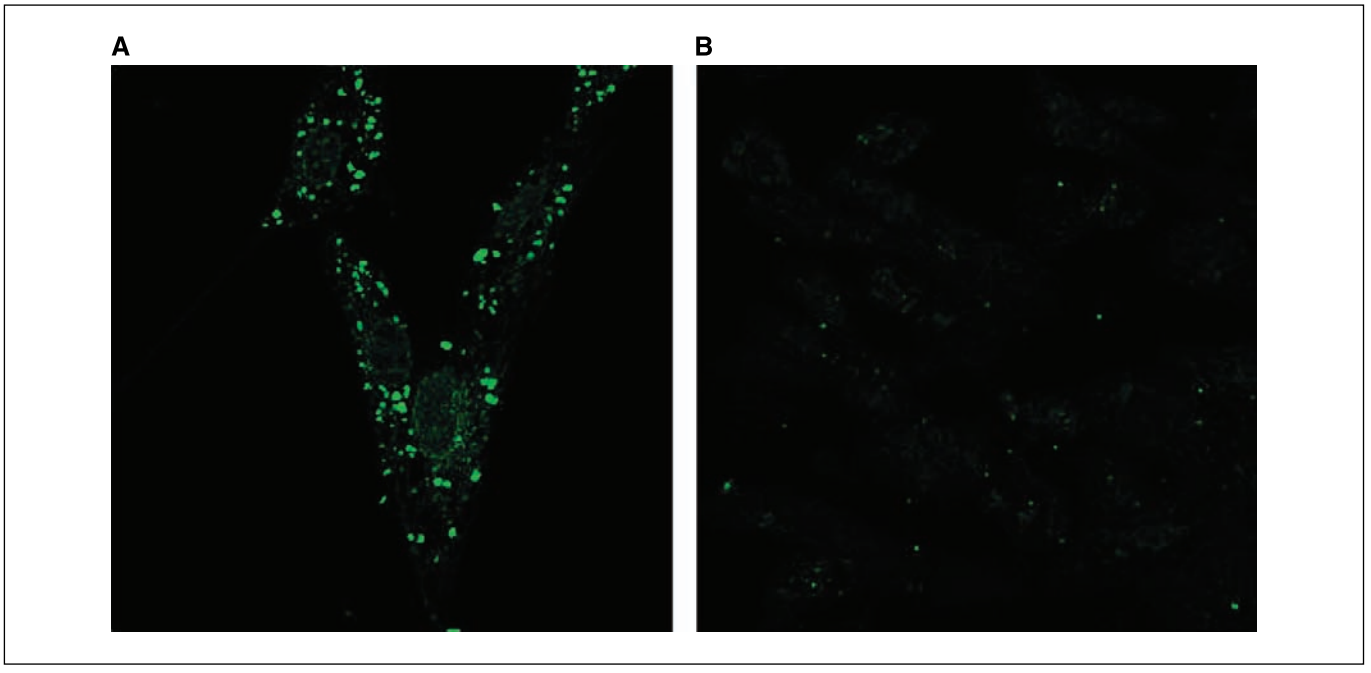

Figure 1. Detection of Env in MSSM7 cells by immunofluorescence. The experimental conditions used are described in Materials and Methods. *A,* MSSM7 cells; *B,* MCF10F cells.

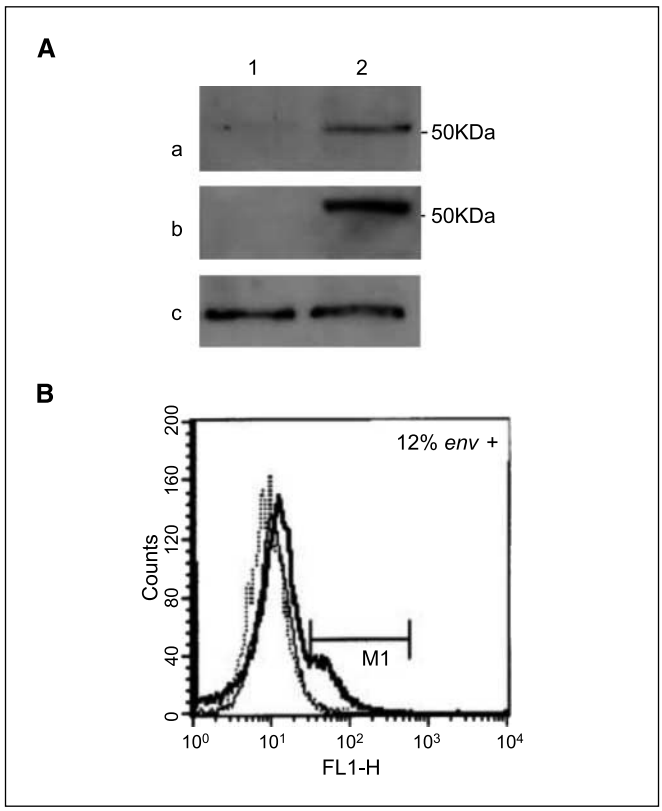

Figure 2. Detection of Env in MSSM7 cells by immunoblotting and flow cytometry. The experimental conditions used are described in Materials and Methods. *A,* immunoblotting of MCF10F cells (*lane 1*) and MSSM7 cells (*lane 2*) done with (*a*) mAbP2, (*b*) pAbSu cells, and (*c*) antitubulin. *B,* surface expression of HMTV Env proteins in MSSM7 cells. MSSM7 and MCF10F cells were stained with the mAbP2 specific for HMTV envelope proteins followed by FITC-labeled anti-mouse IgG antibody and analyzed by FACS. *M1,* the FITC-positive cells. The percentage of Env-positive is shown. Histograms depict representative results from five independent experiments. *Solid line,* MSSM7 cells; *dotted line,* MCF10 cells; *dashed line,* isotype control.

enzyme BirA (16) under control of Ptac promoter. The resulting SU protein was specifically biotinylated *in vivo* by induction with isopropyl-L-thio-β-D-galactopyranoside in the presence of 50 mmol/L of biotin. After purification under denaturing conditions on a nickel chelation column (Nickel NTA; Qiagen) the protein was linked to streptavidin agarose (Pierce) and sent for production of rabbit polyclonal antiserum, designated pAb-Su (Lampire Biological Laboratories).

α-Tubulin monoclonal antibody. A mouse monoclonal antibody against α-tubulin, designed pAbTu was employed (Sigma-Aldrich).

Western blot. Protein lysates were prepared from $\sim 1 \times 10^7$ cells. Confluent cell monolayers were washed with PBS, treated with radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology Inc.) for 15 min at 4°C, and then scraped from the plates. Lysates were clarified by centrifugation at 12,000 rpm for 10 min. Protein concentration was determined by the Bradford method (Bio-Rad Lab). Equal amounts of protein from each sample were loaded onto an SDS-PAGE/10% polyacrylamide gel, followed by transfer to nitrocellulose membranes. Western blot analysis was done using mAbP2, pAb-Su, and mAbTu as primary antibodies. Proteins were visualized using horseradish peroxidase–labeled sheep anti-mouse IgG for mAbp2 and mAbTu and anti-rabbit IgG for pAb-Su (GE Healthcare Bio-Sciences), as a secondary antibody followed by enhanced chemiluminescence (GE Healthcare Bio-Sciences).

Immunofluorescence. Cells were plated on coverslips to subconfluency, washed with PBS, fixed with 2% formaldehyde (Sigma-Aldrich) for 10 min and washed thrice with PBS. Blotting was done with 1% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature, then cells were exposed to the mAbP2 at different dilutions in bovine serum albumin-PBS, washed thrice with PBS and incubated with fluorochrome-conjugated secondary antibody at 1:100 (AFTC green-rodhamine; Sigma-Aldrich) for 1 h, washed thrice with PBS and mounted in diglyceride (Sigma-Aldrich).

Flow cytometry. Cells were detached from the flasks using a nonenzymatic cell dissociation buffer (cell dissociation buffer; Sigma-Aldrich). After blocking, 5×10^5 cells were stained in a two-step protocol with mAbP2 followed by anti-mouse IgG FITC coupled antibody (Roche Diagnostics) and subjected to fluorescence-activated cell sorting (FACS) analysis. Cell counts were acquired on a FACScan cytometer (Becton Dickinson). Approximately 20,000 cells were analyzed per sample with dead cells excluded by FSC/SCC profiles. Surface marker analysis was done using Cell Quest Software.

Results

Evidence for the presence of viral particles in breast cancer cells. The primary cultures of human breast cancer cells used in these experiments were obtained from discarded ascitic fluids or discarded pleural effusions from 10 patients with HMTV *env*-positive breast cancer. The full characterization of these cultures

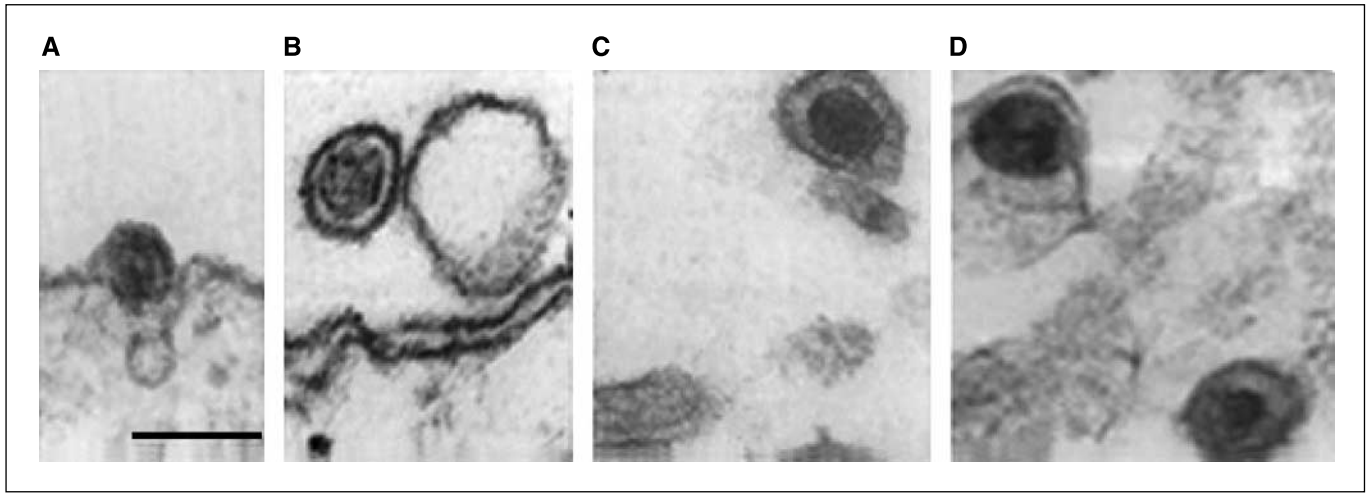

Figure 3. Electron microscopy of MSSM3 cell associates and PF viral particles. *A* and *B,* selected examples of cell-associated viral particles. *C* and *D,* selected examples of viral particles in PFs. Note that the nucleoids are condensed to varying degrees. Original magnification, ×130,000 (*bar,* 0.2 μm).

will be published elsewhere.[4] They were found to have the normal 46 XX karyotype with minor chromosomal changes and to express the epithelial and some of the cancer-related genes (17). The presence of HMTV *env* gene sequences was confirmed in all of them before using these cells for further experiments.

The expression of *env* sequences was investigated in MSSM cells by immunofluorescence, Western blot, and FACS analyses using mAbP2 and by Western blot using pAbSU. The results of immunofluorescence showed that mAbP2 reacted with 15% of the MSSM7 cells, but not with MCF-10F cells (Fig. 1*A–B*). Similar results were obtained by Western blotting, which revealed a protein of molecular weight approximately 52 kDa reacting with mAbP2 only in the tumor cells (Fig. 2*A*). A protein of the same molecular weight was detected with pAbSu (Fig. 2*A*). As a control for equal loading, tubulin expression was examined in the same protein extracts (Fig. 2*A*).

FACS analysis indicated that 12% of the MSSM7 cells expressed the protein on the cell surface (Fig. 2*B*). Electron microscopy examination of MSSM3 cells showed virus particles budding from the cells as well as outside the cells. The morphologic features of these particles: 100 to 110 nm in size, spherical shape with an eccentric dense core, and with either an immature or matured capsid structure are consistent with a β-retroviruses like MMTV (Fig. 3*A–C*).

Isolation and characterization of viral particles. PF from MSSM cells were examined for RT activity (Mg^{2+}-dependent), density gradient centrifugation, electron microscopy, and viral sequences. RT activity was detected in the PF of all 10 *env*-positive MSSM primary cultures. As shown in Fig. 4*A*, no increase in the level of RT activity was found in the PF of MSSM3 cells treated with agents known to increase retrovirus production. To rule out that RT activity could be due to a contaminant in the culture media, uncultured tissue culture medium was processed as the conditioned medium, and RT activity was determined (Fig. 4*A, lane 1*). No activity was found in the uncultured medium. By contrast, PF from MCF-10F cells showed RT activity only after PMA treatment, which is known to activate endogenous retroviruses (13).

To assess the particles' buoyant density, the PF was centrifuged in a 10% to 50% sucrose gradient. The presence of viral particles was monitored by RT activity in each gradient fraction. A representative experiment is shown in Fig. 4*B*, in which two main peaks of RT activity could be seen at 1.12 and 1.18 g/mL density, with the higher density peak being within the correct range of retrovirus densities. In other experiments, more than two peaks were detected, but always within the same density range. Fractions containing the peaks of RT activity were pooled, RNA was extracted and RT-PCR done, and the HMTV *env* gene was detected and sequenced. Viral particles with the characteristic features of β-retroviruses were observed by electron microscopic examination of the PFs, as shown in Fig. 3*D*.

Finally, virion RNA was examined by RT-PCR (Fig. 5). The entire viral cDNA was synthesized in 10 fragments called A to J. All the viral genes were detected by PCR using the specific primers and probes. The primers and probes were designed with the same variables and allowed for the possibility to overlap each other. Fragment A cDNA was obtained with a polyd(TTT) as cDNA primer. Fragment B cDNA was synthesized with LTR3 primer, fragment C cDNA with ENV3L primer from *env* gene, fragment D with ENV5LR primer from *env* gene, fragment E with POL-GR primer from *pol* gene, fragment F with POLFR from *pol* gene, fragment G with ENV8R from *env* gene, fragment H with G2724R from *pol* gene, fragment I with POL8R from *pol* gene, fragment J1 with GAG3R, and fragment J3 with G1931R from *gag* gene. These cDNAs were then amplified by PCR with specific primers as shown in Supplementary Table S1. The fragments were obtained from PF of all of MSSM cells. The results are summarized in Fig. 5. Fragments F and H (data not shown) were detected but not sequenced. Fragment A was sequenced in 6 out of 10 specimens, B in 7, C in 2, D in 2, and I, J1, and J2 in 1. Sequence homology between fragments from different specimens ranged from 90% to 98%. Two of the fragments (D and E) were smaller than the expected size. The sequences obtained were 88% to 98% homologous to the MMTV and to the proviral sequences published by us (ref. 4; Table 1) and to HMTV sequences reported by others (6, 7, 14). The LTR sequences were characteristic of HMTV (5). No significant homology was found in any of the human endogenous retrovirus sequences present in Genbank. A representative sequence of each of the fragments has been transmitted to the Genbank and the accession numbers are shown in Table 1.

Discussion

The results reported here clearly show for the first time that primary cultures of human breast cancer cells produce viral

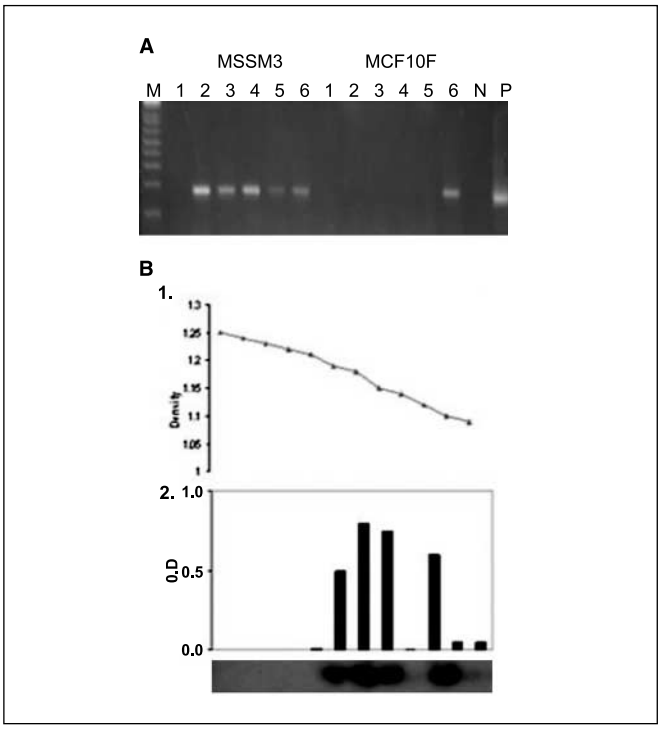

Figure 4. Detection of RT activity in PF of MSSM cells and in sucrose gradient density fractions. *A,* RT activity in PF. High-speed pellets from MSSM3 and MCF10 cells were prepared and RT activity assayed as described in Materials and Methods. Molecular weight marker (*M*), culture media (*1*), untreated cell pellets (*2*), and cells exposed to UV light (*3*). Cells exposed to 1 nmol/L of estrogen (*4*), 10 nm progesterone (*5*), and 50 μg of PMA (*6*). *N,* negative control; *P,* positive control for RT. *B,* sucrose density gradient centrifugation of high-speed pellets. PF from MSSM3 cells were centrifuged through a 10% to 50% sucrose gradient for 16 h at 100,000 × *g* in a SW41 rotor. The gradient was fractionated and the buoyant density of each fraction was determined. *1,* density of gradient fractions; *2,* RT activity determined in each fraction as described in Materials and Methods. *Graph,* quantitation of RT activity. Density of peak fractions (*bottom*).

[4] Melana et al., manuscript in preparation.

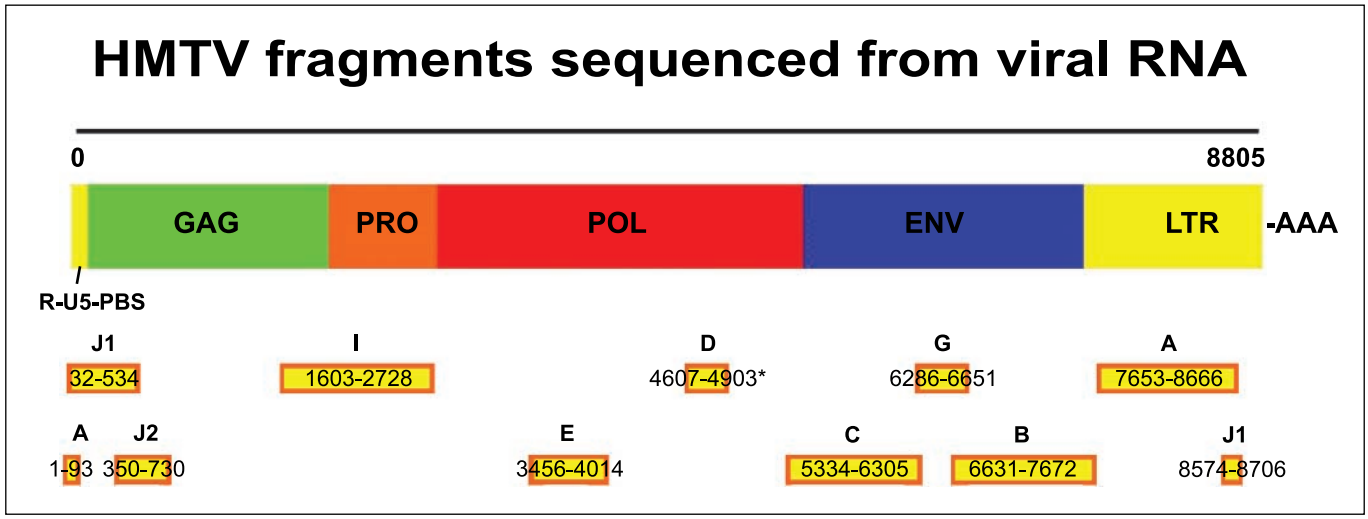

Figure 5. cDNA sequences from virion RNA. *Top,* the putative structure of the virion genome. *Bottom,* the sequenced fragments of the viral genome. *Numbers,* nucleotide location of fragments in the viral genome. Experimental conditions as described in Materials and Methods.

particles with morphologic and molecular characteristics of a β-retrovirus similar to MMTV.

The presence of undefined viral particles in 37% of the human breast cancer biopsies was first reported by Feller and Chopra (18), whereas Moore et al. described viral structures morphologically similar to MMTV in milk from humans and mice (19). Convincing evidence for the presence of viral particles in human breast tumors molecularly similar to MMTV has been lacking, however (20).

The particles described here resemble the β-retrovirus reported by Xu et al. (14) in human primary biliary cirrhosis, but differ from the endogenous β-retrovirus described in the breast cancer cell line T47D, which was released upon treatment with steroids (21). In addition to β-retrovirus–like particles, type C particles were also observed in T47D cultures (21). In contrast, we have observed few C type–like particles (data not shown). Results from sucrose gradient centrifugation and RT activity showed peaks of activity in the range of 1.16 to 1.20 g/mL density, which is consistent with the retroviruses' buoyant density. Analysis of the cDNA in the peaks revealed the presence of MMTV-like *env* sequences, suggesting that the density peaks contain particles

Table 1. Homology of the viral RNA HMTV with MSSM proviral and MMTV sequences

Fragment	Location	Accession no.	Homology % with		
			HMTV proviral		MMTV (gi9626965)
			MSSM1*	MSSM2*	
A	7653–8665 1–92	DQ910868	91	92	92
B	6631–7672	DQ910867	92	92	94
C	5334–6305	DQ910869	94	93	93
D	4607–4903	DQ910870	99	98	88
E	3456–4014	DQ917493	96	95	91
G	6286–6651	DQ925473	98	98	91
I	1603–2718	DQ917496	96	96	94
J1	32–534 8574–8706	DQ917494	97	96	92
J2	350–730	DQ917495	97	97	91

*The HMTV proviral sequences of MSSM1 and MSSM2 tumors were described by Liu et al. (4).

belonging to the same virus, probably at different stages of maturation.

Sequences from virion RNA were 88% to 98% homologous with MMTV and with the human provirus HMTV previously reported by us (4). Approximately 70% of the genome was sequenced. Some regions of the *gag* and *pol* genes could not be sequenced due to lack of sufficient amplified material. The inability to reverse transcribe some parts of the *gag* and *pol* sequences may have been due to pseudoknots, which have been described to occur in MMTV mRNA (22) and which are responsible for the expression of *pro* and *pol* frameshifting (23). Furthermore, two of the fragments (D and E) were smaller than the expected size, although different strategies were used to obtain them.

Detection of human retroviruses has always been difficult, as pointed out by Poiesz et al. (24). We have succeeded in detecting all the genes by RT-PCR and by hybridization, and were able to sequence most of the putative virion genes and gene junctions. This has facilitated, for the first time, the identification of a human retrovirus that is closely related to MMTV and to the previously described human provirus (4, 15).

Primary cultures of human breast cancer cells have the advantage of being closer to the original tumor than established cell lines, as has recently been shown by expression profiling (17). Their shortcomings are variable rate of growth, limited number of passages and low virus production. Results from immunofluorescence and FACS analysis revealed that only 10% to 15% and 8% to 12% of the cells expressed the viral proteins, respectively. In this context, they resemble HUT102 cells from which 20 L of culture media was used to isolate HTLV-1, the first oncogenic human retrovirus described (24). By comparison, our largest culture yielded only 4 L, from which we successfully identified HMTV. Attempts to increase virus production using hormone or chemical treatments as reported by others (12, 13) did not improve the level of RT activity as shown in Fig. 4*A*.

The origin of the HMTV is still unknown. The possibility that it originates from mice by zoonosis has been proposed by Stewart et al. (25) and by Szabo et al. (26). Epidemiologic studies show a correlation between breast cancer incidence and the habitat of the feral mouse *Mus domesticus*, which carries more copies of MMTV than other mouse species (25). Successful infection of human cells with MMTV (14, 27–29) and studies of viral sequences in human breast cancer in different geographic locations (30) support this hypothesis. To establish causality, the potential of the viral particles described here for infectivity and transformation is under investigation in our laboratory.

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