Mouse Mammary Tumor Virus Infects Human Cells

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Abstract

Mouse mammary tumor virus (MMTV) has long been speculated to be involved in human breast cancer and more recently in human primary biliary cirrhosis. Despite complete proviral sequences markedly homologous to MMTV being identified in human breast cancer tissue, no convincing evidence has been presented to date that MMTV can infect human cells. Using both wild-type and a genetically marked virus (MMTV-EGFP), we show here the successful infection of a number of different human cells by MMTV. Furthermore, infection of human cells is shown to be almost as efficient as the infection of murine mammary epithelial cells. Sequencing of PCR products from integrated proviruses reveals that reverse transcription and integration of the viral genome has occurred as expected. Furthermore, sequencing of two independent MMTV proviral integration sites reveal them to be present only in the human and not in the mouse genome. Infection requires an intact MMTV envelope protein and is blocked either by heat inactivation of the virus or by specific neutralizing anti-MMTV serum, ruling out a nonspecific mechanism of viral transfer. Thus, MMTV can infect human cells and this finding provides a possible explanation for the detection by others of MMTV sequences in human breast cancer patients. (Cancer Res 2005; 65(15): 6651-9)

Introduction

Almost 70 years ago, mouse mammary tumor virus (MMTV) was shown to cause mammary carcinoma in mice following milk transmission from mother to suckling offspring (1). However, the cellular receptor with which MMTV interacts to initiate infection was only recently identified as the murine transferrin receptor 1 (Trf1) and it was reported that the human orthologue is not able to mediate infection of human cells (2). The involvement of MMTV in human breast cancer was proposed long ago based on the presence of MMTV-like retroviral particles in human breast cancer biopsies and milk and supported by the detection of proteins in breast tumors recognized by anti-MMTV antisera as well as by the presence of antibodies reactive with MMTV (3–10). Molecular hybridization studies identified significant homology at the nucleic acid level between MMTV and RNA isolated from human breast tumors (3). These data, however, were challenged by the finding that the human genome carries a number of distinct human endogenous retrovirus (HERV) sequences which exhibit sequence similarity to MMTV, potentially suggesting an explanation for the earlier observations of MMTV-like agents in humans (reviewed by ref. 11).

Recently however, interest in an exogenous viral etiology of human breast cancer was rekindled after the presence of MMTV-specific (distinctive from HERV) sequences were detected by at least three independent research groups in humans (12–15). In one study, MMTV env nucleotide sequences (95-99% homologous to MMTV) were detected in 38.5% of unselected human breast cancer samples but not in normal breast or other tissues (12). Independently, MMTV env-like sequences have also been found in 37% of human breast tumors (13) and more recently MMTV-env like sequences have been detected in 42% of tested breast cancer tissue but in only 1.8% of normal breast tissue samples from Australian women (14). Moreover, two complete proviral sequence were isolated from two human breast carcinomas and found to have 95% nucleotide homology with MMTV but only 57% homology to HERV-K (15). Using in situ hybridization, proviral sequences were only detected in chromosomal DNA from MMTV env-positive breast cancer tissue but not from normal breast cells. These findings, together with the absence of MMTV env-like sequences in normal breast tissues obtained from the same breast carcinoma patient, suggest an exogenous origin of the MMTV-like sequences in at least a certain percentage of human breast cancer cases (16).

In mice, MMTV causes mammary carcinoma by insertional mutagenesis and clonal expansion. Large amounts of virus are produced during pregnancy, dramatically increasing the chance of insertional mutagenesis but also allowing the virus to be transmitted to offspring in the milk. Milk-borne virus infects B cells in Peyer’s patches associated with the gastric tract and here, unique among retroviruses, MMTV depends upon a viral super-antigen (Sag)–induced immune response for efficient infection; without the Sag-mediated amplification of the few B cells initially infected by the virus, MMTV seems poorly infectious (17). In this light, the recent finding that MMTV-like long terminal repeat (LTR) sequences containing the sag open reading frame (ORF) were detected in 41.5% of human breast cancers is particularly interesting, as is the finding that these ORFs encode functional Sags which elicit the same amplification response in human cells as that required for efficient infection in mice (18).

Here we report direct evidence for the successful, and relatively efficient, infection of human cells, including human breast cancer derived cells, with nonpseudotyped MMTV virions. Because infection was dependent on the presence of a functional, wild-type, viral envelope and was blocked by specific anti-MMTV neutralizing serum or by heat inactivation, our results show an active and specific transfer of MMTV to the human cell and thus provide supportive evidence for the enzootic transmission of MMTV to humans.

Materials and Methods

Construction of mouse mammary tumor virus carrying enhanced green fluorescent protein. A recombinant MMTV-EGFP provirus was derived from a plasmid carrying a complete, productively infectious, MMTV provirus (pGR102; ref. 19). pGR102 was digested with ClaI and...
self-ligated (pGR102Ca). A CMV-EGFP expression cassette amplified by PCR was introduced into the unique SacI site carried by pGR102Ca-producing pEGFPs. Homologous recombination between pGR102 linearized by SacI digestion and the gel purified DraIII and PvuII restriction fragment of pEGFPs was done after heat shock transformation of competent JC5176 bacteria (20). The resulting plasmid was named pGR102ES.

Env gene mutation in mouse mammary tumor provirus carrying enhanced green fluorescent protein gene. A mutant MMTVmut-EGFP provirus carrying a premature stop codon upstream of the membrane spanning domain coding region of the env gene was also constructed. The 7.7 kb of the 3′-to-5′ env gene containing the premature termination codon (21) was amplified using primers 7516F (5′-CCGGCTTGTGGTATCAGTAGCTA-3′) and 8649R (5′-GTGATACGACCTCGGGGATTC-3′). PCR cycling conditions were one cycle at 94°C for 4 minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute followed by final extension at 72°C for 7 minutes. The amplicon was digested with ClaI and BgIII restriction enzymes and the resultant fragment ligated to the respective restriction site in pEGFPs (creating pEGFPMut). A 6.259-bp-long fragment resulting from ClaI and PvuII digestion of pEGFPMut was then used for homologous recombination with the DraIII/PvuII fragment of pGR102ES thereby generating pMMTVmut-EGFP carrying a premature termination codon in the env gene.

Transfection. A clon 293 cell line containing either the MMTV-EGFP or MMTVmut-EGFP provirus (293MMTV or 293MMTVmut) was prepared by cotransfection of the respective plasmid (pGR102ES or pMMTVmut-EGFP, respectively) with pcDNA3 carrying a neomycin resistance gene (15.1 molar ratio). Independent G418-resistant and enhanced green fluorescent protein-positive (EGFP+; 50% expressing single cell clones were isolated and 293MMTV clone was chosen because it expressed the highest levels of Env proteins and shed the highest levels of MMTV-EGFP virions (data not shown). 293MMTVmut clone expresses the highest levels of Env precursor (comparable with 293MMTV clone). Similarly, CGRES6 and NMTV13 clonal cell lines derived from feline kidney (CrFK) and normal mouse mammary gland cell line (NMuMG), respectively, were prepared.

Infection experiments. Filtered (Sarstedt, Nuembrecht, Germany; 0.45 μm) cell culture medium from dexamethasone stimulated producer cell lines 293MMTV, 293MMTVmut, CGRES6, and NMTV13 was used for infection of CrFK (feline kidney), NMuMG (normal mouse mammary gland), 293 (human kidney), and HeLa (human cervical adenocarcinoma) cells grown in 6-well plates (2x10^5 cells per well). The wild-type virus, MMTV(GR), produced by CR cells, a well-known and characterized cell line derived from an MMTV-induced mammary adenocarcinoma (22–26) was also used for infection. Virus supernatant containing polybrene (8 μg/mL) was incubated with recipient cells for 2 hours and fresh medium was added to the cells. EGFP expression was determined by fluorescence microscopy and transduction efficiency was measured by fluorescence-activated cell sorter (FACS) analysis (FACS Calibur cytometer, Becton Dickinson, Heidelberg, Germany). Genomic DNA was extracted from infected cells 1 week after transfection and the presence of proviral DNA was detected by PCR. A forward primer located in the EGFP gene (EGFP1: 5′-CCAAGCGACGAGGAGCAGTAC-3′) and a reverse primer located in the gag region (1637R: 5′-CCAGGCATGGACGAGCTGTA-3′) were used for detection of infection by MMTV-EGFP virus. Infection of cells by MMTV(GR) virus was detected using a forward primer 935F located in the U3 region (5′-AAAAGCGACATGAAACAA-3′) and the 1637R reverse primer. After 2 minutes at 94°C for 2 minutes, then 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds followed by a terminal extension at 72°C for 7 minutes. Specificity of the PCR reaction was confirmed by sequencing of products or Southern hybridization. The Southern blot hybridization was done using a DIG-labeled DNA probe prepared by using DIG PCR Probe Synthesis kit (Roche, Basel, Switzerland) with MMTV LTR–specific primers 9751R (5′-CCGGCTTGTGGTATCAGTAGCTA-3′) and 10187R (5′-CTCGCTGCTGCCGGTGACATG-3′), following the protocol supplied by the manufacturer. The prehybridization and hybridization was done in a buffer containing sodium phosphate (0.5 mol/L, pH 7.2), 7% SDS, 1 mmol/L EDTA, 1% bovine serum albumin. Prehybridization took place at 50°C for 2 hours followed by overnight hybridization at 50°C in the same solution with the addition of the denatured probe. The filter was washed with 2x SSC and 0.1% SDS twice for 5 minutes and with 0.5x SSC and 0.1% SDS twice at 30°C for 15 minutes. After blocking, the filter was probed with anti-DIG-AP Fab fragments (Roche, 750 units/mL) and the chemiluminescent signal was emitted after adding a CDP-Star substrate for alkaline phosphatase.

In the infection neutralization study, recombinant MMTV-EGFP or wild-type MMTV(GR) virus was incubated before infection for 30 minutes with ice polyclonal goat anti-MMTV antisera (1:1,000; kindly supplied by Dr. Susan Ross), unrelated goat serum (1:1,000), or PBS. In heat inactivation experiments, cell culture medium from producer cells was first incubated at 60°C for 10 minutes before infection.

Detection of integration sites. To detect and characterize the integration of the MMTV-EGFP provirus in infected human cells, ligation-mediated nested PCR (LM-PCR) was done (27). Briefly, genomic DNA was extracted from 293 and HeLa cells 2 weeks after transduction with recombinant MMTV-EGFP virus and digested with EcoRI restriction enzyme. Blunt double-stranded DNA fragments were generated by primer extension with a biotinylated EGFP1 primer (5′-biontin-CCAAAGG- GAAGCCGCGATC-3′). DNA was purified with streptavidin paramagnetic beads (Dynal Biotech, Oslo, Norway) and ligated to the GenomeWalker adaptor (Clontech Laboratories, Inc., Palo Alto, CA). Two rounds of PCR amplification of the ligated fragments were done using EGFP primers (EGFP1 and EGFP2: 5′-CTCGGATTGAGCGGCGCTGA-3′) and Clontech primers, AP1 and AP2. The product was sequenced and a National Center for Biotechnology Information blast search4 done to identify DNA homologous to MMTV-EGFP flanking sequences.

Results

Infection of target cells with mouse mammary tumor virus produced by murine mammary tumor cells. Wild-type virus [MMTV(GR)] produced by an established cell line (GR) derived from an MMTV-induced murine adenocarcinoma (22) was used in initial infection experiments. Cell culture medium harvested from GR cells stimulated by the synthetic glucocorticoid dexamethasone was filtered (0.45 μm) and used to infect various target cells. Infection of recipient cells was assessed by PCR (Fig. 1B). An indicative 717-bp PCR product was detected in MMTV(GR)-infected (Fig. 1A, lanes 5 and 13) but not MMTV(GR)-uninfected (Fig. 1A, lanes 4 and 12) feline kidney (CrFK) cells, which are known to be permissive for infection with MMTV (28). Surprisingly, the same PCR product was also generated from infected human 293 (Fig. 1A, lane 7) and HeLa (Fig. 1A, lane 9) cells. No PCR product could be amplified from their uninfected counterparts (Fig. 1A, lanes 6 and 8), respectively, showing that PCR amplification was specific for acquired MMTV(GR) proviral sequences and was not the result of amplification of endogenous human sequences. The specificity of the amplification was confirmed by sequencing of the PCR products which were identical to the original MMTV(GR) sequence (data not shown). In contrast, when MMTV-permissive murine NMuMG mammary gland cells were subjected to a similar analysis, the 717-bp PCR product was obtained from both infected (Fig. 1A, lane 3) as well as noninfected (Fig. 1A, lane 2) cells. The PCR product obtained from noninfected cells reflects the presence of endogenous MMTV proviral sequences in this mouse cell line.

An identical infection and PCR analysis was done on a number of human breast cancer cell lines (T-47D, MDA, MCF-7, BT-20, H3396, and HS 578T) and allowed the detection of the same specific 717-bp PCR product from T-47D (Fig. 1A, lane 14), MCF-7 (Fig. 1A, lane 18),

4 http://www.ncbi.nlm.nih.gov/BLAST
Establishment of recombinant mouse mammary tumor virus–enhanced green fluorescent protein producer clones.

Both the natural low infectivity of MMTV and the presence of closely related endogenous retroviral sequences in the target cells, hamper the facile detection of MMTV infection events in cell culture. We have previously shown expression of MMTV Gag and Env proteins as well as production of infectious MMTV virions upon transfection of CrFK cells with the MMT provirus encoded by the plasmid pGR102 (19). To confirm the ability of MMTV to infect human cells as well as to facilitate detection and quantification of infected cells, we modified the infectious MMTV provirus carried by pGR102 to express an EGFP gene under control of the cytomegalovirus (CMV) promoter. The resulting plasmid, pGR102EGFP, carrying the CMV-EGFP cassette in the 3′ LTR of a complete MMTV provirus was used to generate stable virus producing 293, CrFK, or NMuMG cell clones (293MMTV, CGRES6, and NMTV13, respectively).

To validate the infection assay, filtered (0.45 µm) virus containing supernatant derived from 293MMTV cells was used to infect permissive CrFK cells (Fig. 2). Cytoplasmic expression of EGFP was detected in these cells by UV microscopy. Two days after infection small foci, usually consisting of a few EGFP-expressing cells, were observed (Fig. 2, CrFK, left, 2 dpi). After three further days in culture a larger number of EGFP-positive cells were observed (Fig. 2, CrFK, right, 5 dpi), suggesting that proviral DNA was integrated in the genome of target cells and transmitted to daughter cells. EGFP expression was observed in transduced CrFK cells over the complete 12-week observation period, confirming that the provirus was integrated in the infected cells. Similar results were obtained using virus produced from the CGRES6 cell clone (data not shown).

Infection of human cells with a mouse mammary tumor virus carrying an enhanced green fluorescent protein gene produced from human, feline, and mouse cells. Next, filtered supernatant from the 293MMTV clone was used to successfully infect human HeLa and 293, as well as murine mammary gland (NMuMG) cells as judged by fluorescence microscopy (Fig. 2) and FACS analysis (Fig. 4; Table 1). Again, an increase in the number of cells in clusters expressing EGFP 5 days after infection (Fig. 2, right, 5 dpi) compared with 2 days after infection (Fig. 2, left, 2 dpi) was observed. Similar results were obtained using virus produced from the CGRES6 cell clone (data not shown). One month after infection, expression of EGFP was still observed by fluorescence microscopy in all infected cell lines and long-term cultures of infected 293 cells showed EGFP expression at least 10 months after infection (data not shown).

The titer of the MMTV-EGFP virus on 293 and HeLa cells, as determined by FACS analyses, was the same to 10-fold lower than that determined on NMuMG cells (Table 1), irrespective of whether the virus plated on target cells was produced in human 293 (293MMTV), feline CrFK (CGRES6), or murine NMuMG (NMTV13) cells. The titers obtained on CrFK were consistently higher than on the murine NMuMG cells, confirming that these cells are particularly susceptible to infection by MMTV (28).

PCR analysis with primers specific for EGFP and MMTV gag (Fig. 1D) revealed that a 451-bp product could be amplified using genomic DNA from infected HeLa (Fig. 1C, lane 5), 293 (Fig. 1C, lane 7), and NMuMG (Fig. 1C, lane 3) cells, indicative of reverse transcription resulting in the duplication and translocation of the CMV-EGFP containing U3 region from the 3′ to the 5′ LTR in infected cells. In contrast, no PCR product could be detected using these primers and genomic DNA from noninfected NMuMG cells, validating the exogenous origin of the PCR template.

Thus, the use of an EGFP carrying MMTV as shown here, in contrast to the use of a wild-type MMTV, not only allows easy visualization of infected cells and the possibility for FACS screening but also enables the facile differentiation between endogenous and exogenous MMTV by PCR (compare Fig. 1A, lane 2 and Fig. 1C, lane 2).
Analysis of sequences at the site of proviral integration.

MMTV integration sites in the human host genome were mapped by LM-PCR to unequivocally show that cells had acquired MMTV DNA by infection, as well as to confirm the human origin of the infected cells. The LM-PCR method (Fig. 3B) was established using DNA from the 293MMTV producer cell clone that had been stably transfected with the plasmid pGR102ES. As expected, a 517-bp LM-PCR product was amplified from these cells (Fig. 3A, lane 5), whereas analysis of DNA from parental 293 cells yielded no LM-PCR product (Fig. 3A, lane 1). The specificity of amplification was confirmed by sequencing of the LM-PCR product (data not shown).

No LM-PCR product was obtained using genomic DNA of uninfected 293 or HeLa cells (Fig. 3A, lanes 1 and 2, respectively). In contrast, analysis of genomic DNA from 293 and HeLa cells transduced with MMTV-EGFP virus yielded products of around 400 bp (Fig. 4A, lane 3) and 600 bp (Fig. 3A, lane 4), respectively. The LM-PCR products were sequenced and the chromosomal localization of the two proviral integration sites determined (Fig. 3C). Each integrated provirus has lost the terminal 2 bp of MMTV but has retained the neighboring CA residues, hallmarks of retroviral integration (Fig. 3C). Host cell sequences of 288 and 112 bp juxtaposed the 3' end of each provirus and BLAST searches allowed both to be unambiguously mapped to a specific human chromosome (Fig. 3C). One MMTV-EGFP provirus is integrated in the intron sequence of a syntaxin binding protein 5-like gene on human chromosome 3. The other MMTV-EGFP provirus is inserted in the intron sequence of a glutamate receptor, ionotropic, AMPA 1 gene on human chromosome 5. Interestingly, probably because both integrations occurred in introns, no significant homology to either site was detected in the mouse genome, confirming the human origin of the infected cells.

Neutralization of infection and heat inactivation.

Stable virus producers were used in all the previously described experiments to rule out the possibility of carryover of plasmid DNA that often occurs in transient transfections. Similarly, filtered supernatant was used to prevent the transfer of cells. Nevertheless, to verify that the infection of human cells with MMTV was the result of the specific interaction between MMTV and specific receptor molecules rather than nonspecific virus uptake or infection with virions...
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Table 1. Infection and neutralization of infection of various cell lines with MMTV-EGFP viruses produced in 293MMTV, CGRES6, and NMTV13 cells and infection with MMTVmut-EGFP recombinant virus

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Titer (GFU/mL)*</th>
<th>293MMTV</th>
<th>293MMTV + Anti-MMTV ¹</th>
<th>293 MMTVmut-EGFP</th>
<th>CGRES6</th>
<th>CGRES6 + Anti-MMTV ¹</th>
<th>NMTV13</th>
</tr>
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<td>293</td>
<td>150</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>300</td>
<td>&lt;1</td>
<td>220</td>
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</tr>
<tr>
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<td>500</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>400</td>
<td>&lt;1</td>
<td>120</td>
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<tr>
<td>NMuMG</td>
<td>1,500</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1,000</td>
<td>&lt;1</td>
<td>220</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>1,800</td>
<td>&lt;1</td>
<td>1,100</td>
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</tr>
</tbody>
</table>

NOTE: Representative titers of at least two independent experiments for all infections are shown.

*Green fluorescent units/mL.

¹Neutralization with goat anti-MMTV polyclonal serum.

Discussion

Although MMTV, the prototype β-retrovirus, was discovered by Bittner more than half a century ago (1), the biology of this virus is still not completely understood mainly due to difficulties in obtaining high titers of the virus in cell culture, its poor infectivity compared with γ-retroviruses, as well as the lack of a simple and sensitive in vitro assay to evaluate the infectivity and transforming potential of the virus.

To date, there is also much controversy about the possible infection of human cells by MMTV and the involvement of this virus in human breast tumorigenesis (11). Recently, studies with MLV pseudotypes bearing MMTV envelope proteins suggested that mouse but not human Trf1 is the receptor for MMTV and the human homologue of the murine MMTV receptor cannot (2) mediate efficient infection of human cells.

These apparently contradictory findings prompted us to critically reinvestigate whether MMTV can infect human cells using sensitive quantitative techniques. Two approaches were taken. In the first, wild-type MMTV obtained from a well-characterized mouse mammary tumor cell line was used and infection events detected by PCR. In the second approach, a recombinant MMTV carrying a CMV-EGFP expression cassette inserted into the U3 region of the 3′LTR of a complete provirus was used. Virus was generated by stable transfection of human (293), murine (NMuMG), and feline (CrFK) cells and infection events detected by fluorescence microscopy, FACS, and PCR. Regardless of the type or source of the virus, human cells of different origin were consistently and reproducibly infected at efficiencies that were at best similar to and at worst only 10-fold lower than efficiencies obtained on murine target cells.

Env expression were detected in dexamethasone-stimulated 293MMTVmutEGFP and nonmutated 293MMTV clones (data not shown), no EGFP expression was detectable by FACS analysis in CrFK, NMuMG, 293, and HeLa cells exposed to the filtered culture supernatant from 293MMTVmutEGFP (Table 1). Thus, infectivity was completely abolished when a premature stop codon was introduced into the env coding region showing (i) the requirement for authentic processed MMTV Env for infection of human and mouse cells and (ii) that 293 producer cells do not express heterologous endogenous envelope proteins that can complement for the defective MMTV Env.

Infection is dependent on the presence of functional envelope proteins. To confirm that functional envelope protein is a prerequisite for MMTV infection of human cells, we constructed an EGFP carrying MMTV provirus with a premature termination codon just upstream of the membrane spanning domain-coding region (MMTVmut-EGFP). After transfection of 293 cells with this plasmid, independent cell clones were tested for the expression of MMTV Env proteins. In agreement with previously published data (19), lack of the complete membrane spanning COOH terminus in the truncated Env precursor (gp73) seems responsible for the lack of processing into SU and TM subunits, gp52 and gp36 (data not shown). Although comparable levels of pseudotyped with envelopes from other viruses, several experiments were conducted.

First, virus neutralization experiments were done using a neutralizing goat anti-MMTV serum (kindly donated by Susan Ross). Infection of human, feline, and mouse target cells was completely blocked by preincubating recombinant MMTV-EGFP virus produced from either CGRES6 or 293MMTV with neutralizing goat anti-MMTV antibodies (Fig. 4A; Table 1). The infection of human and feline cells by wild-type MMTV(GR) virus was also neutralized, because no MMTV sequences could be PCR amplified from human (Fig. 5, lanes 9 and 12) and feline cells (Fig. 5, lane 5) when this virus was preincubated with anti-MMTV antiserum. No signal could be detected even after hybridization with an MMTV-specific LTR probe (Fig. 5, lanes 17, 21, and 24). In contrast, pretreatment of MMTV-EGFP or MMTV(GR) virus with a normal goat serum had no effect on the infectivity of the recombinant and wild-type virus, confirming the specificity of the interaction between the virus and its cellular receptor (Fig. 4A). Nonspecific inhibition of the goat anti-MMTV serum could be ruled out, because this serum did not neutralize infectivity of recombinant murine leukemia virus (MLV-EGFP) carrying an amphotrophic Env (4070A) thus showing the specificity of the anti-MMTV serum used (data not shown).

Retroviruses are known to be heat labile (29). Thus, next we incubated virus-containing supernatants from CGRES6 and 293MMTV cells for 10 minutes at 60°C. No EGFP expression could be detected in cells transduced with heat inactivated MMTV-EGFP virus, whereas those handled in a similar fashion without the heat inactivation retained their ability to infect target cells (Fig. 4B), showing that transmission is temperature sensitive as expected for retroviral infection but not for DNA transfer.

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EGFP is a more sensitive marker, particularly when driven by a strong CMV promoter, than the lacZ marker gene (driven by the MLV promoter) used in the experiments leading to the identification of MMTV receptor (2). This might account for the discrepancy between the results presented here and the results of the aforementioned study. Furthermore, in the receptor study, MLV pseudotypes carrying the MMTV envelope, generated transiently by triple transfection of 293T cells, were used, whereas the present study employed stably produced wild-type MMTV or authentic recombinant MMTV-EGFP virus produced from well-characterized, stably transfected 293, CrFK, and NMuMG cells after only limited passages.

A number of experiments were done to show that the infection of human cells was the result of bona fide MMTV infection. First, a passive transfer of EGFP protein (30) could be ruled out (i) because detection of EGFP by fluorescence microscopy and FACS analysis was possible up to 10 months after infection. (ii) Stable cytoplasmic EGFP expression observed in these cells was of relatively high mean fluorescence intensity (MFI in average 1,000). (iii) No EGFP expression was detected in target cells when the MMTV Env protein was truncated, although equivalent amounts of the wild-type and truncated unprocessed Env protein were detected in producer cells.

Second, a nonspecific transfer of DNA or virus can be ruled out. (i) Infection is dependent on a specific interaction between MMTV proteins and cognate receptors on the target cell. An MMTV-neutralizing antibody specifically inhibited infection with both wild-type MMTV (GR) as well as with recombinant MMTV-EGFP virus. The same antibody did not neutralize an MLV-based vector carrying an amphotropic envelope (MLV-EGFP); thus, the serum used is not generally able to block retroviral infection. (ii) Similarly, truncation of the MMTV envelope by insertion of a Figure 3.

**Figure 3.** LM-PCR detection of MMTV integration sites. A, LM-PCR protocol was optimized with genomic DNA from the 293MMTV clone (lane 5). The genomic DNA from this clone was mixed with human genomic DNA (2 μg of human genomic DNA were added to 0.5 μg of 293MMTV DNA), digested with the restriction enzyme EcoRI, and subsequently subjected to LM-PCR using a combination of EGFP and adaptor-specific primers. The EcoRI restriction site on the plasmid is present 222 bp downstream of the 3′ end of MMTV LTR, allowing us to predict the size of the LM-PCR product. Because the EcoRI restriction site is relatively close to the MMTV LTR, the probability that the plasmid would be linearized between the EcoRI site and the end of the LTR before incorporation in the genome was low. Genomic DNA from 293 and HeLa cells (lanes 3 and 4) infected with supernatant from 293MMTV cells, as well as their respective uninfected counterparts (lanes 1 and 2) was digested with EcoRI and subjected to an optimized LM-PCR protocol. Nested LM-PCR products were analyzed by agarose gel electrophoresis: products excised and sequenced (arrows); lane M, 1-kb molecular weight marker. B, schematic outline of LM-PCR. Genomic DNA was digested with EcoRI and fragments containing MMTV-EGFP and host-flanking sequence were hybridized with a biotinylated EGFP-specific primer and primer extension performed. After enrichment of the products using streptavidin-coated paramagnetic beads, an adaptor (black box) was ligated to the flanking DNA and solid-phase nested PCR done with adaptor-specific (AP1 and AP2) and EGFP-specific (EGFP1 and EGFP2) primers. C, MMTV-EGFP integration sites in transduced 293 and HeLa cells determined by LM-PCR. A Genbank BlastN search was done and integration sites were mapped to specific human chromosomes. The identified flanking sequence in HeLa cells matched intron sequence of sytaxin binding protein 5-like gene on the human chromosome 3. In infected 293 cells, the MMTV-EGFP virus landed in the intron sequence of glutamate receptor, ionotropic, AMPA1 gene on the chromosome 5. No significant homology to the identified flanking sequences was determined in mouse genome. The identified flanking sequences are in capital letters. The MMTV LTR sequence (shaded) and terminal CA dinucleotide (bold letters).
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Figure 4. FACS analysis of MMTV-EGFP-infected cells. A, neutralization of MMTV-EGFP infectivity. Infection of CrFK and HeLa cells was detected by FACS analysis 2 days after infection. Infection of MMTV-EGFP recombinant virus produced from the 293MMTV clone was blocked using goat anti-MMTV antibodies (1:1,000). In contrast, preincubation of the virus with normal goat serum (1:1,000) did not neutralize infection. B, heat inactivation of MMTV-EGFP virus. Cell culture supernatant from 293MMTV cells was incubated at 60°C for 10 minutes and used for infection. Control infection was done with nonheat-inactivated virus. A-B, FACS histograms as uncompensated RAW data measured in the GFP (530 nm) and Autofluorescence (585 nm) channels. Region R3 corresponds to the EGFP-positive cells and R2 to EGFP-negative cells. Percentage of infected cells is shown.

Finally, MMTV recombinant virions generated in human (293), feline (CrFK), and murine (NMuMG) were used for infection of human cells and all yielded similarly good infection efficiencies on human, feline, and murine target cells. It thus seems highly unlikely that three cell lines from three different species (i) produce an endogenous envelope with a similar infection spectrum that can pseudotype the recombinant MMTV and (ii) produce the same host range variant allowing infection of human cells, especially because the stably transfected producer cells were only in culture for a relatively short time.

Viral tropism is determined by the presence of cognate receptor on target cells. Contradictory findings have been reported about the nature of the cellular MMTV receptor. On one hand, restricted presence of the receptor on mouse and rat cells was shown (2, 31, 32); on the other hand, other studies have suggested a broad distribution of the receptor on mouse, mink, cat, and human cells (28, 33–37) as well as on different murine cell types (38). Furthermore, genetic studies mapped a gene associated with susceptibility to MMTV infection to mouse chromosome 16, but it is of interest that also in this study, chromosomes 7 and 17 were also postulated to be involved (39). Additionally, by analogy to γ-retroviruses, ectropic and xenotropic MMTV variants have been proposed (32, 40). Recently, proteoglycans like heparin have also been shown to play a role in MMTV-cell interactions, perhaps by facilitating attachment of the virions to the surface of target cells (41). Tfr1 has been identified as a molecule mediating internalization of the virus (2) and infectivity was correlated with the level of expression of the Tfr1 receptor on the cell surface (41).

MMTV is transmitted via the milk to suckling mice. The virus transverses the gut via M cells and then infects B cells in the Peyer’s patches. These infected B cells express Sag that is then presented to cognate T cells resulting in the activation of both B and T cells. This produces a reservoir of infected cells from a few initially infected cells, which then eventually transmit the virus to epithelial cells in the mammary gland (42, 43). Intriguingly, Tfr1 is, however, undetectable on the surface of resting B cells, which are the primary target of MMTV (44), whereas there is currently no evidence that crypt enterocytes, which express Tfr1 for iron uptake (45), are infected by MMTV. Taken together, this data, and our results suggest that MMTV may use other receptors for cell entry, which are also present on human cells. Alternatively, as already suggested in the 1970s (32, 40), different variants of MMTV may exist, with different infection spectra. However, at least in the region proposed to be involved in receptor binding to either Tfr1 or heparin, there are no differences in the amino acid residues between GR virus and other sequenced MMTV isolates (41).

Irrespective of receptor usage and potential MMTV variants, our data shows for the first time the efficient infection of human cells and provides a missing link between observations made by others of MMTV-like sequences, proteins, and antibodies associated with human breast cancer. In this light, it is of interest that epidemiologic studies of migrant populations have revealed that environmental factor(s) play an important role in the risk of developing human breast cancer. The incidence of breast cancer in women from low-risk regions (e.g., China, Vietnam, Far East) moving to high-risk regions (United States and Western Europe) increased rapidly within one or two generations (46). It has been noted that the prevalence of Mus domesticus, an important reservoir for MMTV, correlates with these same regions (47), suggesting enzootic transmission of MMTV from mice to humans. Potential enzootic transmission of MMTV is also supported by phylogenetic analysis of MMTV-like sequences.
obtained from human breast cancer (HMLV) and MMTV sequences that revealed HMLVs are indistinct from MMTVs (48).

Transfer of retroviruses from one species to another is a highly contentious issue. On one hand, it seems clear that simian immunodeficiency virus has been transmitted from monkeys to humans on more than one occasion thus generating various isolates of HIV (49) associated with AIDS. Human cells can also be infected by murine leukemia retroviruses and such viruses are used in human gene therapy protocols as vectors. Sensitive techniques have been developed to monitor unwanted spread of such viruses in treated patients (50). That such infection events may be pathogenic, albeit under specific conditions (51), has recently been clearly documented in the unfortunate outcome of the otherwise highly successful gene therapy trials for X-linked severe combined immunodeficiency (X-SCID) in which to date three patients, whereas “cured” of their X-SCID disorder, later succumbed to lymphoma as a result of retroviral insertion mutagenesis (52). Clearly, there is also some concern that endogenous porcine retroviruses may be pathogenic in recipients of porcine xenotransplants because at least some of these viruses can productively infect human cells (53–55), a fear that has overshadowed other potential risks from infectious agents in xenotransplantation (reviewed by ref. 56). On the other hand, feline retroviruses are also able to infect human cells (57, 58), and yet there is no documented evidence of transfer of these common viruses of cats to cat owners. Ability to infect human cells is thus not necessarily automatically correlated with disease; however, it will increase the likelihood of promoter/ enhancer insertions associated with retrovirus mediated tumorigenesis occurring. Whereas breast cancer clearly has a multifactorial etiology, it remains to be seen whether in some cases an infectious agent like MMTV may play some role in this etiology. Obviously, however, a prerequisite for such a role is the infection of human cells as shown here.

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