

Multiple Integrated Copies and High-Level Production of the Human Retrovirus XMRV (Xenotropic Murine Leukemia Virus-Related Virus) from 22Rv1 Prostate Carcinoma Cells[∇]

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The human retrovirus XMRV (xenotropic murine leukemia virus-related virus) is associated with prostate cancer, most frequently in humans with a defect in the antiviral defense protein RNase L, suggesting a role for XMRV in prostate carcinogenesis. However, XMRV has not been found in prostate carcinoma cells. Here we show that 22Rv1 prostate carcinoma cells produce high-titer virus that is nearly identical in properties and sequence to XMRV isolated by others and consist primarily of a single clone of cells with at least 10 integrated copies of XMRV, warranting further study of a possible role for XMRV integration in carcinogenesis.

The association of human prostate cancer with mutations that impair the function of the antiviral defense protein RNase L has suggested a role for virus in prostate cancer. Indeed, analysis of cDNA from prostate tumors using a DNA microarray (Virochip) containing conserved DNA sequences from all known virus families indicated the presence of a novel gammaretrovirus in 40% of prostate cancer patients having homozygous R462Q mutations in RNase L (16). Cloning and sequencing of the virus revealed a close similarity to mouse xenotropic retroviruses; therefore, the new virus was named xenotropic murine leukemia virus-related virus (XMRV) (16). Importantly, XMRV has been found integrated into human genomic DNA from tumor-bearing prostatic tissue samples from 11 patients, showing that XMRV can indeed infect humans and is not a laboratory contaminant (2, 7). However, the possibility that XMRV plays a role in prostate cancer is weakened by the lack of an obvious oncogene in XMRV and by the finding that XMRV was not associated with prostate carcinoma cells but instead with tumor stromal cells, arguing against direct viral oncogenesis or insertional activation of oncogenes in the carcinoma cells by XMRV (16). Here we describe the detection of multiple integrated copies and high-level production of XMRV from 22Rv1 prostate carcinoma cells, which were derived from a primary prostatic carcinoma (14, 15). These cells secrete prostate-specific antigen, express an androgen receptor, and are responsive to dihydroxytestosterone (15), evidence that they are indeed of prostate epithelial cell origin.

Electron microscopic analysis of culture medium from 22Rv1 prostate carcinoma cells (ATCC CRL-2505) revealed the presence of gammaretrovirus-like particles (Fig. 1). To detect and characterize the biological activity of the presumptive virus, we used a marker rescue assay (10). In brief, HTX

cells (an approximately diploid subclone of human HT-1080 fibrosarcoma cells) transduced with the retroviral vector LAPSN (HTX/LAPSN cells) were exposed to culture medium conditioned by confluent layers of 22Rv1 cells for 24 h. The HTX/LAPSN cells were passaged for 2 weeks to allow virus spread. Next the cells were assayed for production of the LAPSN vector by measuring transfer of the alkaline phosphatase (AP) gene carried by the LAPSN vector to naïve HTX target cells and to *Mus dunni* tail fibroblast cells. The *Mus dunni* cells are wild mouse cells that are infectible by many gammaretroviruses, including xenotropic retroviruses (12). Medium samples from 22Rv1 cells originally obtained from the ATCC and maintained in the M. Tewari lab and from 22Rv1 cells freshly obtained from the ATCC tested highly positive for the presence of replication-competent virus in this marker rescue assay using either HTX or *Mus dunni* cells as targets for infection (data not shown).

To provide a quantitative measure of the virus released by the 22Rv1 cells, we used an S⁺L⁻ helper virus assay (11). This assay measures the ability of the test virus to rescue a transforming virus from PG-4 cat cells and induce foci of transformation. Assay of medium conditioned for 24 h by 22Rv1 cells maintained in the Tewari lab and by 22Rv1 cells freshly obtained from the ATCC gave titers from 2×10^6 to 10^7 focus-forming units (FFU) per ml, revealing high-titer virus production by 22Rv1 cells and showing that the 22Rv1 virus can infect and replicate in cat cells.

XMRV is unable to infect Chinese hamster ovary (CHO) cells, but expression of the xenotropic and polytropic retrovirus receptor Xpr1 from human cells (1) in CHO cells renders them susceptible to XMRV infection (2). Xenotropic viruses from mice were named on the basis of their ability to infect “foreign” cells but not those of laboratory mice; however, the ability of XMRV to infect laboratory mouse cells has not been determined. To test whether the virus released from 22Rv1 cells has these properties of mouse xenotropic viruses, we used virus generated in the marker rescue assay to infect hamster and mouse cells that expressed or did not express human Xpr1 (Table 1). For a control, we measured infection by the LAPSN

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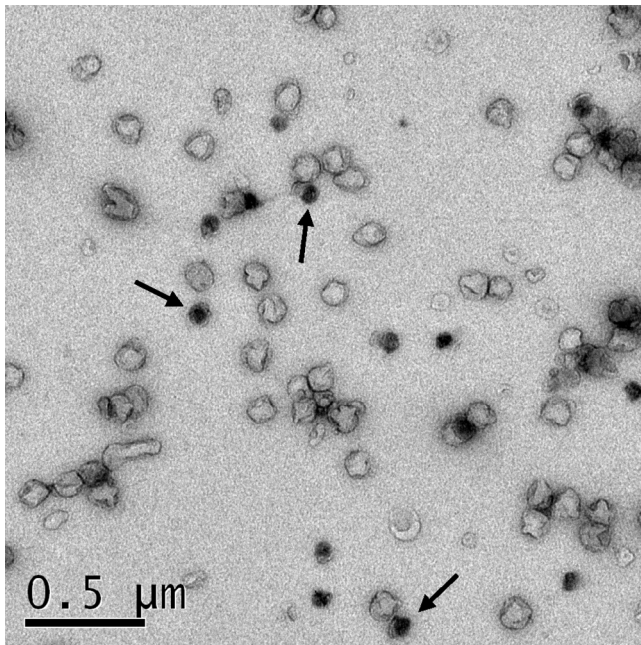


FIG. 1. Presence of retrovirus-like particles in culture medium from 22Rv1 cells. Medium was harvested from 22Rv1 cells, clarified by centrifugation at $16,500 \times g$ for 20 min, filtered through 0.22- μm -pore-size filters, and centrifuged at $120,000 \times g$ for 70 min, and the pelleted material was analyzed by transmission electron microscopy. Arrows indicate retrovirus-like particles with dark cores surrounded by a membrane.

vector pseudotyped with the NZB mouse xenotropic retrovirus [LAPSN(NZB)] produced from *Mus dunni*/LAPSN+NZB cells (12). We found that infection of the mouse and hamster cells by the 22Rv1 virus-pseudotyped LAPSN vector required the presence of Xpr1. Infection of mouse cells by LAPSN(NZB) virus also required the presence of Xpr1, but infection of hamster cells by LAPSN(NZB) was facilitated by

TABLE 1. 22Rv1 retrovirus host range and receptor use^a

Helper virus	LAPSN vector titer (AP ⁺ FFU/ml) on target cells ^b :			
	NIH 3T3	NIH 3T3/Xpr1	CHO	CHO/Xpr1
22Rv1 (MT lab) ^c	<1	100	<1	3×10^5
22Rv1 (ATCC) ^c	<1	100	2	3×10^5
NZB xenotropic	<5	100	3×10^3	6×10^5
None	<1	<1	<1	<1

^a Conditioned medium was harvested from confluent layers of 22Rv1 cells. Medium samples were filtered through 0.45- μm -pore-size filters and added to HTX/LAPSN cells, and the cells were passaged for 2 weeks to allow virus spread. Medium was harvested and filtered 12 to 24 h after feeding confluent layers of HTX/LAPSN cells exposed to medium from the 22Rv1 cells, from naïve HTX/LAPSN cells (no helper virus), and from *Mus dunni*/LAPSN cells infected with the NZB mouse xenotropic retrovirus.

^b Target cells were exposed to various amounts of the harvested medium and were stained for alkaline phosphatase-positive (AP⁺) cell foci 2 days later. Target cells included NIH 3T3 mouse cells, NIH 3T3 cells transduced with a retrovirus vector expressing the human xenotropic retrovirus receptor Xpr1 (LhXprSN) (1), CHO cells transduced with a control retrovirus vector LXS, and CHO cells transduced with the retrovirus vector expressing Xpr1. LAPSN vector titer is expressed as AP⁺ FFU per ml of medium.

^c 22Rv1 cells obtained from the ATCC and maintained in the M. Tewari lab (MT lab) or from 22Rv1 cells freshly obtained from the ATCC (ATCC).

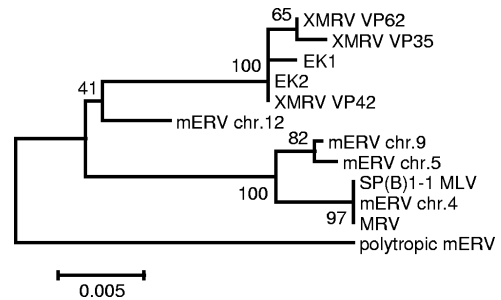


FIG. 2. Phylogenetic tree of 22Rv1 *gag* sequences (clones EK1 and EK2), most closely related GenBank sequences, and one more distant sequence (polytropic mouse endogenous retrovirus [GenBank accession number FJ544576.1]). Sequences were aligned using ClustalX, and an unrooted neighbor-joining tree was generated using this alignment and MEGA version 4 software. Bootstrap values (500 replicates) are shown as percentages, and the scale bar indicates evolutionary distance in base substitutions per site. mERV, mouse endogenous retrovirus; MRV, mouse AIDS-related virus; chr.12, chromosome 12.

but did not require Xpr1. These results show that the phenotype of the 22Rv1 virus is identical to that previously reported for XMRV (2) but is somewhat different from the typical mouse xenotropic retrovirus NZB. In addition, our results show that XMRV can infect human, cat, and wild mouse cells, but it does not infect laboratory mouse or Chinese hamster cells.

The 22Rv1 cells were derived from cells that had been grown by xenotransplantation in nude mice (13, 14, 17); therefore, it was possible that the 22Rv1 virus was acquired from mice. To further establish the identity of the 22Rv1 virus, we cloned and sequenced a 600-bp region of the 22Rv1 virus *gag* gene that is different in XMRV and related mouse retroviruses by using previously described GAG-OF and GAG-OR primers (16) to PCR amplify the region from reverse-transcribed viral RNA. A BLAST search of the nonredundant GenBank sequences for sequences similar to two cloned sequences (EK1 and EK2 [GenBank accession numbers FJ907198 and FJ907199, respectively]) revealed nearly exact matches to XMRV clone VP42, and phylogenetic analysis revealed that the 22Rv1 sequences clearly cluster with all previously cloned XMRV sequences (Fig. 2). From these data, we conclude that the 22Rv1 virus is XMRV and is not a mouse xenotropic virus acquired during passage of the cells in culture or in mice (see additional supporting data in the Addendum in Proof).

We were interested to see whether other commonly used prostate carcinoma cell lines produce XMRV. Previous work indicates that LNCaP and DU145 prostate carcinoma cells do not produce XMRV (2). For our analysis, medium exposed to test cells for 24 h was assayed for the presence of replication-competent retrovirus using the S⁺L⁻ assay described above. LNCaP (5) and PC-3 (6) cells tested negative for virus production (<1 FFU/ml), while VCaP cells (8) tested weakly positive for virus production (20 FFU/ml), and the transformed foci formed slowly, indicating poor replication of this virus in feline S⁺L⁻ cells. In contrast, XMRV virus from 22Rv1 cells gave titers of 2×10^6 to 10^7 FFU/ml, and the foci formed rapidly, indicating efficient replication of XMRV in the feline cells.

We further characterized the virus from VCaP cells by marker rescue assay as follows. HTX/LAPSN and *Mus dunni*/

LAPSN cells were exposed to filtered medium from VCaP cells and passaged for 2 weeks to allow virus spread, and medium from these cells was tested for the presence of the LAPSN vector by assay on naïve HTX and *Mus dunni* cells. High-level LAPSN vector production was detected from the VCaP medium-exposed *Mus dunni*/LAPSN cells when measured on *Mus dunni* target cells (5×10^6 AP⁺ FFU/ml), but when measured on HTX cells, the titer was 1,000-fold lower (6×10^3 AP⁺ FFU/ml). No LAPSN vector production was detected from the VCaP medium-exposed HTX/LAPSN cells when measured on *Mus dunni* or on HTX cells (<1 AP⁺ FFU/ml). These results show that the VCaP virus replicates well in *Mus dunni* cells but only poorly infects and replicates in HTX cells, unlike XMRV, which efficiently infects and replicates in HTX cells (Table 1 and data not shown). Thus, of the five prostate carcinoma cells tested, only the 22Rv1 cells produce XMRV.

XMRV is most frequently detected in humans with homozygous R462Q mutations in RNase L (16). To determine whether this mutation was present in 22Rv1 cells, we used PCR to amplify this region of the *RNASEL* gene from 22Rv1 cell DNA, HTX cell DNA, and from a 1:1 mixture of the two DNAs. Sequencing of the resulting products revealed only the wild-type amino acid codon (CGA) in HTX cell DNA, only the R462Q mutant codon (CAA) in 22Rv1 cell DNA, and a mixture of the two codons in the mixed DNA sample, which shows that simultaneous detection of both codons was possible. These results show that 22Rv1 cells are homozygous for the R462Q mutant *RNASEL* allele.

Oncogenesis by retroviruses can result from insertional activation of oncogenes followed by outgrowth of clonal tumor cell lines that are often marked by multiple retrovirus integrations. To examine XMRV integration sites in the 22Rv1 cells, we performed Southern analysis of 22Rv1 cell DNA by using our cloned XMRV *gag* sequences as a probe and by using restriction enzymes that cut on both sides of the XMRV *gag* region (KpnI) or that cut on one side of the *gag* region and at variable sites in surrounding human genomic DNA (BamHI) (Fig. 3). KpnI digestion produced the expected 2.81-kb band (Fig. 3), and phosphorimager analysis indicated a band intensity corresponding to at least 10 integrated virus copies per cell. BamHI digestion revealed at least 10 bands at approximately single-copy-per-cell levels, indicating that the 22Rv1 cell population consists primarily of a clone of cells with at least 10 XMRV integration sites. Note that BamHI also creates an internal 2.13-kb viral fragment that hybridizes with the *gag* probe (Fig. 3). This fragment contains only 10% of the *gag* probe sequence, and therefore, the observed band is not as intense as the KpnI band but is clearly more intense than the other bands in this lane. We found no hybridization of the *gag* probe to DNA from human HTX cells, consistent with the fact that no endogenous retroviruses closely related to XMRV are present in the human genome. In summary, these results are consistent with the restriction enzyme patterns predicted by previous sequencing of XMRV and are consistent with but do not prove a model of prostate carcinogenesis involving insertional activation of cellular oncogenes by XMRV.

In summary, we report here the first identification of XMRV in prostate carcinoma cells. Earlier studies found the virus in prostate tumor stromal cells by fluorescence in situ hybridization and by immunohistochemistry using Gag-specific antibod-

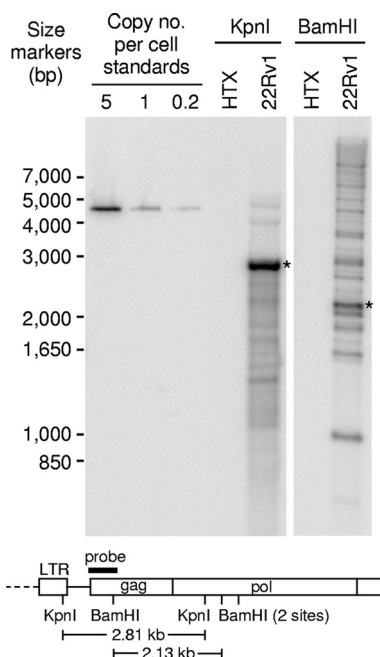


FIG. 3. Southern analysis of genomic DNA from 22Rv1 and HTX cells using an XMRV *gag* probe. Both panels shown are from the same exposure of the same blot with some internal lanes removed. At the bottom of the figure is a scale diagram of XMRV based on the sequence of the VP42 XMRV clone (GenBank accession number DQ241302) with sizes indicated for the internal restriction fragments that hybridize with the *gag* probe (2.81 and 2.13 kb). Bands in the blot corresponding to these fragments are marked with asterisks. Copy number standards were made using a plasmid containing the *gag* probe sequences cut with a restriction enzyme that cuts once in the bacterial plasmid sequences and using an estimate of 6×10^9 bp for the genome size of 22Rv1 cells. LTR, long terminal repeat.

ies (2) or in bulk tumor material by PCR without determination of the infected cell type (4). Unfortunately, we are unable to determine the extent of carcinoma cell infection in the original tumor, and it is possible that the 22Rv1 carcinoma cells were infected by virus produced by tumor stromal cells during passage as a xenograft in mice. Interestingly, we found that the 22Rv1 carcinoma cell line is primarily composed of a single clonal cell line marked by ≥ 10 XMRV integration sites, consistent with a model of carcinogenesis involving insertional activation of oncogenes by XMRV. However, this result is also consistent with the presence of multiple integrations in a particular cell followed by outgrowth of the cell because of oncogenic events unrelated to the virus integrations, or more trivially, to clonal outgrowth during passage of the cells in mice or in culture, although we never cloned the 22Rv1 cells and the literature indicates these and the parental cells have always been grown as a bulk population (13–15, 17). Initial studies by others have found no common XMRV integration sites within or near proto-oncogenes or tumor suppressor genes in tumor tissue from nine prostate cancer patients (7), arguing against a role for virus insertion in prostate cancer. Accordingly, it is possible that XMRV has other roles in prostate cancer, such as the alteration of stromal cells to facilitate cancer development or the generation of aneuploid genetically unstable cells by virus-mediated cell fusion (3).

Finally, production of XMRV by 22Rv1 and potentially other prostate cancer cell lines should be carefully considered from the standpoint of possible virus transmission to laboratory personnel, to other cells cultured in parallel, and as a confounding factor in the interpretation of experimental results. For example, 22Rv1 cells have been reported to produce 80- to 150-nm “exosomes” (9) which look very much like the presumed ~100-nm retrovirus particles shown in Fig. 1, and the possible role of XMRV in the phenomena ascribed to exosomes will need clarification.

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ADDENDUM IN PROOF

Recently, Sardana et al. (G. Sardana, K. Jung, C. Stephan, and E. P. Diamandis, *J. Proteome Res.* 7:3329–3338, 2008) performed a proteomic analysis of conditioned medium from 22Rv1 cells in search of prostate cancer biomarkers, but the presence of XMRV was not reported. For this analysis, proteins in medium exposed to the cells were digested with trypsin, and the resulting peptide fragments were separated by chromatography, identified by mass spectroscopy, and mapped to known human proteins to determine the identities and amounts of human proteins secreted by 22Rv1 cells. To determine whether XMRV was present in medium from 22Rv1 cells, the XMRV Gag, Gag-Pro-Pol, and Env protein sequences were added to the human protein database, and the peptide data were reanalyzed, revealing that the XMRV proteins were actually more abundant than any of the human proteins secreted into the culture medium by 22Rv1 cells (E. P. Diamandis and C. R. Smith, personal communication). Exact peptide matches to XMRV proteins spanned 54% of the Gag, 47% of the Gag-Pro-Pol, and 33% of the Env protein sequence. A BLAST search of the GenBank database using the compiled peptide sequences revealed perfect or near-perfect matches to the existing XMRV isolates, with lower similarity to any other retroviruses in the database. These results provide independent confirmation of retrovirus production by 22Rv1 cells and show that the virus produced by 22Rv1 cells is similar to XMRV over its entire length and not just over the *gag* region

that we sequenced, further supporting our conclusion that the 22Rv1 virus is indeed XMRV.

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