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Acutely Transforming Retrovirus Expressing Nras Generated from HT-1080 Fibrosarcoma Cells Infected with the Human Retrovirus XMRV[▽]

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Virus from HT-1080 fibrosarcoma cells infected with the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) can induce rare foci of transformation in rat 208F fibroblasts. Characterization of three such foci revealed that one produced an acutely transforming virus at a high titer. The virus consists of a mutant Nras cDNA from the HT-1080 cells inserted into a retroviral vector (added to the HT-1080 cells as a marker for infection) in place of internal vector sequences. These results show that XMRV can generate acutely transforming viruses at a low rate, as is typical of other replication-competent retroviruses, and reveal the potential for transforming virus contamination of retroviral vectors made from transformed cell lines.

XMRV (xenotropic murine leukemia virus-related virus) has been associated with prostate cancer (19, 20) and chronic fatigue syndrome (12), although some researchers fail to detect XMRV in other populations with these diseases (4, 8). XMRV is found integrated into human genomic DNA from prostate cancer samples, indicating that it is indeed a human retrovirus and not a laboratory contaminant (3, 9). Because of the potential role of XMRV in prostate cancer, we previously tested XMRV for transforming activity in fibroblast and epithelial cell lines. Although XMRV is a simple retrovirus that does not carry a host-derived oncogene, there is precedence for transformation by retroviral Env genes (21, 22). However, transfection of XMRV proviral DNA or viral envelope expression vectors into 208F rat fibroblasts did not result in transformation, and infection of most cell types tested with XMRV did not induce transformation (13). In contrast, infection of 208F cells with XMRV did result in rare transformed foci suggestive of oncogene activation by XMRV. Characterization of cells from three transformed foci produced by infection of 208F cells with virus from HTX cells (a pseudodiploid subclone of HT-1080 fibrosarcoma cells [18]) infected with XMRV and the LAPSN retroviral vector (included as a marker for infection) revealed that all produced XMRV and that one produced a highly active transforming virus (13).

Transforming virus structure. To investigate the genesis of this transforming virus, we amplified genomic DNA from the 208F focus 3 cells, which produce the transforming virus (13), by using primers that matched both murine leukemia virus (MLV) and XMRV long terminal repeats (LTRs) (small arrows near LTRs in Fig. 1). In addition to ~8- and ~5-kb products corresponding to full-length XMRV and LAPSN, 2.8-kb and 1.3-kb PCR products were obtained. The 1.3-kb product was sequenced and determined to be XMRV with an internal deletion from the 5′ LTR-tRNA primer binding site (PBS) junction through bp 784 of the Env gene. The 2.8-kb product was a recombinant composed of an Nras cDNA flanked by LTRs from the LAPSN vector (Fig. 1) (GenBank accession no. GU934326). The recombinant virus included LAPSN LTRs and ~250 bp of the packaging signal (ψ), which would allow efficient packaging of the virus, as well as the entire simian virus 40 (SV40) early promoter and *neo* gene. The full-length Nras coding region found in the transforming virus was 98.6% identical to the human Nras sequence (NM_002524.3) and only 89.8% identical to the rat sequence (NM_080766.2), showing that the Nras sequence was acquired from the HTX cells in which the virus was generated, rather than from the rat 208F cells. In particular, the Nras insert has the glutamine-to-lysine activating mutation at residue 61 (Nras*), which has been shown to be the mutation responsible for the transformed phenotype of HT-1080 cells (1, 7, 14). PCR using primers specific for human Nras confirmed the presence of this sequence in focus 3 cells and the absence of this sequence in the parental rat 208F cell line. Both recombination events occurred within short regions of sequence identity (Fig. 1).

To confirm that the Nras* recombinant virus that we sequenced was indeed capable of transforming cells, we inserted the cloned and sequenced 2.8-kb PCR product between the LTRs of the pLAPSN plasmid to make pLNras*SN (Fig. 1). 208F cells transfected with pLNras*SN exhibited transformed foci between 1 and 2 weeks after transfection while no such foci were observed following transfection with pLAPSN.

We considered the possibility that 208F foci 1 and 2, which do not produce transforming virus (13), were transformed by transfer of Nras* cDNA through a retrofection mechanism (11) that did not generate a recombinant virus. However, PCR using human Nras-specific primers (small arrows near Nras* in Fig. 1) did not detect human Nras in DNA extracted from these two foci or from an additional three transformed foci generated in 208F cells by virus from XMRV-infected HTX cells. This shows that these foci were not transformed by trans-

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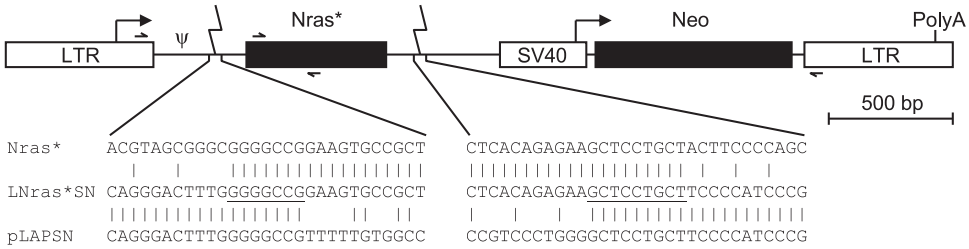

FIG. 1. Proviral structure of the acutely transforming retrovirus LNras*SN. Retroviral LTR and SV40 transcription start sites are indicated by large arrows, protein coding regions are shown in black, PolyA indicates the polyadenylation signal in the downstream LTR, and PCR primers are shown with small arrows. Nras* indicates the mutant human Nras sequence (Gln-to-Lys activating mutation at protein residue 61). Sequences at the recombination sites are shown at bottom with vertical lines indicating identical bases and underlined bases indicating regions of microhomology.

fer of Nras* from HTX cells and suggests that they were transformed by retroviral insertional mutagenesis.

Analysis of HT-1080-based retrovirus packaging cells for production of transforming virus. Retrovirus packaging cells have been made from HT-1080 and other transformed cell lines. Such packaging cells are used to make replication-incompetent retroviral vectors for laboratory and clinical applications, and inadvertent production of a transforming virus such as the one reported here would be problematic, especially for clinical applications. To test this possibility, we transduced FLYA amphotropic HT-1080-based packaging cells (2) with the LAPSN vector, grew the cells in G418 to select for cells expressing the vector, and assayed virus produced by the cells for transformation of 208F cells. For the transformation assay, 208F cells seeded the day before at 6×10^5 per 10-cm-diameter dish were exposed to filtered (0.45-μm-pore-size) virus in the presence of 4 μg Polybrene per ml and then were fed every 3 to 4 days for a month with culture medium containing 5% fetal bovine serum. This assay is identical to the one used previously to detect transformation by virus from XMRV-infected cells (13). A total of 8 ml of virus from FLYA/LAPSN cells induced no foci of transformation in four 10-cm dishes. Staining the cells for alkaline phosphatase (AP) at the end of the experiment revealed that 30 to 50% of the 208F cells were AP^+, showing that they had been transduced by the LAPSN vector produced by the FLYA/LAPSN cells. However, this rate is lower than the ≥95% LAPSN transduction rate observed using XMRV+LAPSN virus from HTX cells (13).

We next tested whether XMRV infection of FLYA/LAPSN cells might increase virus production from the cells and allow transformation of 208F cells. FLYA/LAPSN cells, and HTX cells as a positive control, were exposed to XMRV from 22Rv1 cells (10), the cells were passaged for 2 weeks to allow complete virus spread, and viruses harvested from the cells were assayed for transformation of 208F cells as described above. Ten milliliters of virus from FLYA/LAPSN+XMRV cells induced no foci in 4 10-cm dishes of 208F cells, while 10 ml of virus from HTX/XMRV cells induced one transformed focus in 4 10-cm dishes of 208F cells. About 70% of the 208F cells exposed to virus from FLYA/LAPSN+XMRV cells were AP^+, showing that the cells had been infected. Thus, we were unable to demonstrate transformation by virus from FLYA cells. However, the rate of 208F cell infection was lower than that previously observed with virus from HTX/XMRV+LAPSN cells, and even the rate of transformation by the latter virus is at the border of detectability (13).

Perspective. The acutely transforming virus that we found in 208F cells infected with virus from HTX/XMRV+LAPSN cells arose by recombination between Nras* and LAPSN vector sequences at short regions of sequence identity. It is generally assumed that such recombination events are generated primarily during reverse transcription, because the rate of DNA recombination at such short regions of homology is expected to be exceedingly low, while template switching during reverse transcription is common. Two general models involving template switching during reverse transcription have been proposed to explain such recombination events. Following the model proposed by Zhang and Temin (24), the LAPSN vector could have integrated upstream of the Nras* gene in the infected HTX cells and recombination could have occurred during retroviral reverse transcription after packaging of a read-through mRNA containing both LAPSN and Nras* sequences. Alternatively, following the model proposed by Hajjar and Linial (6), illegitimate packaging of an Nras* mRNA could have resulted in direct recombination between Nras* and LAPSN RNA during retroviral reverse transcription. We can clearly rule out a mechanism involving aberrant splicing of a read-through transcript because of lack of suitable signatures of splicing in the LNras*SN virus. Overall, these data demonstrate that the human retrovirus XMRV is able to mediate the generation of retroviruses carrying host cell oncogenes.

The LAPSN vector is produced at a ~6-fold-higher titer than is XMRV from HTX/XMRV+LAPSN cells (4×10^7 versus 6.5×10^6 focus-forming units [FFU]/ml, respectively) (13), indicating that LAPSN RNA is much more abundant in virions and providing an explanation for involvement of LAPSN and not XMRV in recombination with Nras*. The structure of LNras*SN is reminiscent of the well-known ras-containing transforming retroviruses, Harvey and Kirsten murine sarcoma viruses, which were generated in rats infected with murine leukemia viruses. These independently isolated viruses contain ras sequences inserted into rat virus-like 30S (VL30) elements in place of internal VL30 sequences, all flanked by murine leukemia virus sequences, including their LTRs (5). Most acutely transforming retroviruses have been isolated following infection of animals with nontransforming replication-competent retroviruses, as was the case for the Harvey and Kirsten murine sarcoma viruses. However, acutely

transforming viruses have also been generated in cultured cells using various nontransforming replication-competent retroviruses (15–17, 23), showing that XMRV is not unique in this regard. Interestingly, production of acutely transforming viruses was facilitated by growth of the nontransforming parental viruses in chemically transformed cells or in cultured tumor cells (16, 17).

LNras*SN appears to be the first example of oncogene acquisition by a retroviral vector during vector replication, and although we did not detect the production of acutely transforming virus from retrovirus vector-producing packaging cells made using HT-1080 cells, our general results indicate that this may happen at some low rate. This possibility should be considered when vectors are made from packaging cells derived from tumor cells or cells that express oncogenes, such as packaging cells based on HT-1080, 293, and 293T cells.

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