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In vitro construction of a B-tropic virus by recombination: B-Tropism is a cryptic phenotype of xenotropic murine retroviruses

(murine leukemia virus/cryptic B-tropism/peptide maps/structural markers)

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ABSTRACT **A B-tropic virus was isolated *in vitro* from the progeny of mouse cells doubly infected with N-tropic and xenotropic murine leukemia viruses. Biological and structural evidence is presented suggesting that the phenotypically silent structural marker for B-tropism, expressed by the xenotropic virus p30, was transferred to an N-ecotropic virus via recombination, thus resulting in the expression of a B-ecotropic murine leukemia virus.**

Recent peptide mapping studies of the p30 major core protein from murine type C retroviruses showed that these viruses can be subclassified according to fine structural differences in the molecule (1). Furthermore, the structural differences in murine leukemia virus (MuLV) p30 were correlated with susceptibility to growth restriction imposed by the *Fv-1* locus of the mouse. Thus, a reciprocal relationship was established between the structure of a particular region of p30 and its possible function as a direct or indirect "target" of *Fv-1* restriction (2–5). The structural component of MuLV p30 that correlated with *Fv-1* tropism was found to be a single tryptic peptide, which we will designate "p30-T12." This peptide varied in its position according to several phenotypic criteria, including *Fv-1* tropism. This structure–function relationship was most clearly defined in the case of B-tropic viruses which, in all instances, displayed the p30-T12B marker (1). Furthermore, careful structural analysis of several NB-tropic variants, derived from a B-tropic virus, indicated that the NB-tropic virus p30-T12 peptide had shifted slightly from the B position to a unique position (unpublished data). Thus, together, these studies strongly implicate a direct involvement of the p30-T12B peptide in conferring B-tropism to MuLV. In the former study (1) it was found that one of two subgroups of xenotropic MuLV also displayed the p30-T12B marker. Among the xenotropic MuLV that display the p30-T12B marker are those of NZB, NZW, NIH Swiss, and wild mice (1). The NZB and NIH viruses have been defined by immunological and biochemical criteria as class III or X^b xenotropic MuLV (6–8). If one assumes that the p30-T12B marker is a structural correlate of B-tropism, then xenotropic viruses that display this marker are likely to be cryptic for B-tropism. The predicted B-tropic phenotype of these xenotropic viruses would remain silent due to their inability to infect mouse cells. Expression of the B-tropic phenotype would occur only upon the temporary or permanent loss of their xenotropic characteristics—i.e., only if the viruses were able to infect mouse cells that express an $Fv\text{-}1^n$ allele of the *Fv-1* locus.

In this report we show that a xenotropic virus that carries the p30-T12B marker does indeed carry the phenotypic viral determinant(s) for B-tropism. This is demonstrated by the *in vitro* construction of a B-tropic virus that resulted from recombination between an N-ecotropic MuLV and a xenotropic virus possessing the p30-T12B p30 peptide. Peptide mapping of the viral gene products shows that the B-tropic recombinant expresses the parental-type N-ecotropic *env* gene product gp70, the *gag* gene product p30 of the parental xenotropic virus that carries the p30-T12B structural marker for B-tropism, and the p15 of the N-tropic parent.

MATERIALS AND METHODS

Cells and Viruses. The parental N-ecotropic virus used in these studies was Akv-2-603 (9). This virus was isolated from an Akv-2 congeneic NIH Swiss mouse (10). B-Tropic viruses have never been isolated from AKR or NIH Swiss mice, and our stock of Akv-2 N-tropic virus was determined to be free of B-tropic virus because no B-tropic MuLV could be detected after successive passages on $Fv\text{-}1^b$ cells. The xenotropic MuLV was isolated from wild mouse embryo cells by J. W. Hartley (1). This wild mouse xenotropic-like virus (WM-X) will productively infect the wild mouse cell line SC-1 in addition to cells of certain heterologous species. The tryptic and chymotryptic peptide fingerprints of the WM-X gp70 clearly indicate that it is a member of the xenotropic MuLV category (11). The tryptic peptide fingerprints of WM-X p30 are not distinguishable from those of prototype MuLV p30s that belong to the 12B subclass (1); the WM-X p30 map displays the tryptic peptide marker associated with B-tropism, p30-T12B, and does not have any additional differences that distinguish it from a prototype p30 tryptic peptide map. The WM-X isolates are the only wild mouse MuLVs tested that exhibit a p30 of the 12B subclass. Most other wild mouse N-ecotropic or amphotropic MuLV belong to the p30-12A subclass; however, several isolates from Bouquet Canyon mice (12) display unique p30-T12 subclass structures.

The selection of the WM-X MuLV for these studies was based on the presence of the p30-T12B marker and the ability of the virus to infect SC-1 cells. The latter property enables one to infect a virus-negative mouse cell, SC-1, with a xenotropic virus and thus to initiate *in vitro* the envisioned recombination sequence that occurs in the mouse. When that occurs, a cell that is expressing all or part of the proper xenotropic virus is superinfected with, or expresses, an endogenous N-ecotropic MuLV, thereby providing the proper parental viruses for the appearance of a B-tropic recombinant.

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Abbreviations: MuLV, murine leukemia virus; WM-X, wild mouse xenotropic virus; XC, rat tumor cells that fuse in the presence of ecotropic but not xenotropic MuLV.

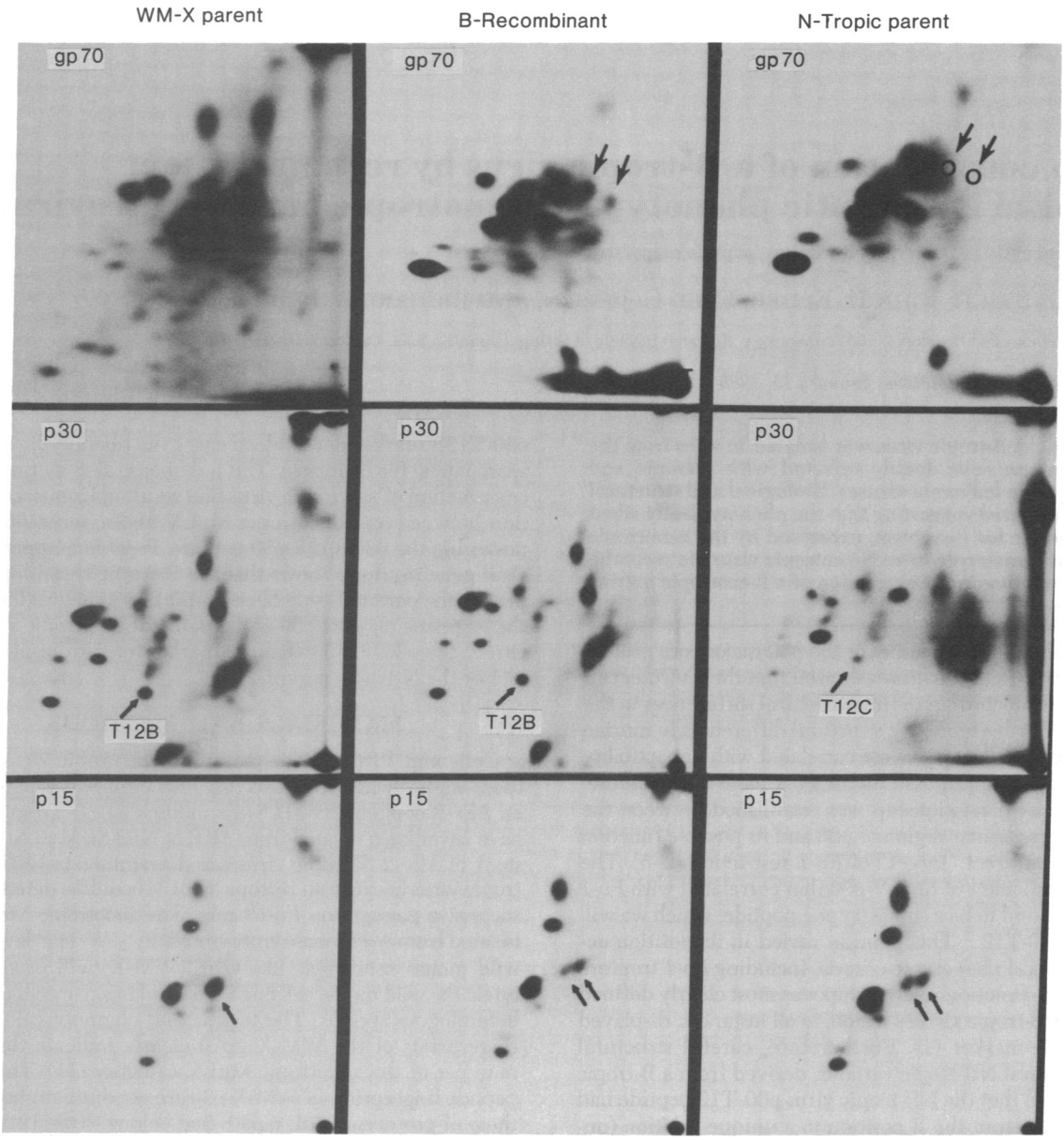

FIG. 1. Two-dimensional separations of ^{125}I-labeled tryptic peptides of p30 and chymotryptic peptides of gp70 and p15 from the wild mouse xenotropic (WM-X), Akv-2-603 N-tropic parental, and B-tropic recombinant MuLVs. The peptide maps were obtained as described (1, 11, 16). The additional spots indicated in the B-tropic recombinant gp70 map are the only apparent differences between this gp70 and the gp70 of the N-tropic parental MuLV. The p30s of the WM-X and B-recombinant viruses each contain the B-tropic-specific T12B peptide. The N-tropic parental virus p30 displays the N-tropic-specific T12C peptide (1). The p15 of the WM-X is distinguishable from those of the N-tropic parent and B-recombinant MuLVs. The p15 fingerprints of the latter two viruses are not distinguishable from one another.

B-Tropic Recombinant Isolation. Subconfluent WM-X-producing SC-1 cells (18081) were superinfected with Akv-2 at approximately 0.01 plaque-forming unit per cell. Controls consisted of 18081 cells not superinfected with Akv-2 and uninfected SC-1 cells that were infected at the same low multiplicity with Akv-2. The cultures were split 1:10 on days 5 and 10 after superinfection. After 2 weeks the cultures were fed, the medium was harvested after an additional 24 hr and filtered through 0.45-μm-diameter Millipore filters to eliminate all cells, and aliquots were stored at −70°C. The harvested viruses were titered on BALB/c secondary mouse embryo fibroblasts (*Fv-1^b* cells) by the standard UV/XC plaque assay (13). Five days after infection of *Fv-1^b* cells with dilutions of the viruses, the cultures were fed and the medium was harvested, filtered, and stored at −70°C. The cultures were then UV-irradiated and XC cells were added. When the results of the XC assay were determined, the frozen supernatants from the dilution plates that had contained less than 10 plaques were used to infect subconfluent *Fv-1^b* cells in the presence of polybrene. At this point there were XC plaques induced from supernatants from the Akv-2/WM-X doubly infected cells and the Akv-2 infected SC-1 culture. As expected, no plaques were evident in plates from the WM-X infected SC-1 culture. These new *Fv-1^b* cultures and the uninfected control were subcultured when confluent (about every 5 days) and tested for reverse transcriptase activity (14) at weekly intervals. Fresh *Fv-1^b* cells were added after several subcultures when the original cells showed signs of entering crisis. After several weeks, the *Fv-1^b* cells, infected with the XC plaque limiting-dilution supernatant from the WM-X/Akv-2 infected SC-1 assay plate, showed evidence of particle-associated reverse transcriptase activity. The *Fv-1^b* cell culture from the original Akv-2 infected SC-1 control and the *Fv-1^b* cell flask

Table 1. Growth of MuLV on cells of different *Fv-1* genotypes

Virus*	p30-T12†	$\log_{10}$ pfu/ml‡				Syncytial plaque ratios§			
		Fv-1^b	*Fv-1*^{n}	SC-1	Foci on S^+L^- mink	SC-1/*Fv-1*^{n}	SC1/*Fv-1*^{b}	*Fv-1*^{n}/*Fv-1*^{b}	*Fv-1*¶ tropism
Akv-2	C	1.7	3.8	4.4	<1	6.4	862.8	135.3	N
WM-X	B	<1	<1	<1	4.7	—	—	—	?
B-recomb.	B	4.7	1.1	4.9	<1	6909	1.6	.0003	B
WN1802B	B	4.8	1.2	4.9	<1	5063	1.4	.0003	B

* Akv-2-603 N-ecotropic MuLV. WM-X, wild mouse xenotropic MuLV (1). B-recomb., B-tropic virus isolated from progeny virus from SC-1 cells doubly infected with N-tropic and WM-X viruses after several limiting dilution passages on *Fv-1*^{b} cells. WN1802B, B-tropic virus isolated from BALB/c (18).

† p30-T12, p30 tryptic peptide number 12 positions as described (1). Position C is a marker for N-tropic MuLV of inbred strains of mice. Position B is a marker for B-tropic MuLV of inbred strains of mice and of certain xenotropic viruses.

‡ pfu, XC-syncytial plaque-forming units per ml as determined by SXC (15). *Fv-1*^{b} cells and *Fv-1*^{n} cells are secondary embryo fibroblasts from BALB/c and NIH mice and are homozygous for *Fv-1*^{b} and *Fv-1*^{n}, respectively (17, 18). SC-1 cells are a wild mouse cell line that is *Fv-1*$^-$ (19). S^+L^- mink cells have been described (20). Infection of S^+L^- mink cells with a MuLV helper rescues the defective mouse sarcoma virus, causing a morphological transformation (focus) quantitatively proportional to the number of input helper viruses.

§ Syncytial plaque ratios are based on plaque-forming units per ml.

¶ *Fv-1* tropism of the appropriate virus as determined by relative growth on *Fv-1*^{n}, *Fv-1*^{b}, and SC-1 cells. N, N-tropic; B, B-tropic.

alone remained negative for reverse transcriptase for at least 2 additional weeks, at which time they were terminated. The supernatants from the culture that showed reverse transcriptase activity were harvested, filtered, and assayed for XC plaque-forming viruses on *Fv-1*^{b} cells. Two additional cycles of *Fv-1*^{b} cell infection with supernatants from XC limiting-dilution plates were performed before an XC plaque-inducing virus arose that was able to grow well on B-cells. Clones of this virus were picked from syncytial plaques on agarose-covered SXC (15) assay plates. These virus clones were grown and titered on *Fv-1*^{b} cells. The virus clone that displayed the best growth and induction of SXC plaques was grown in sufficient quantities for peptide mapping studies. Numerous attempts to generate B-tropic recombinants by the same procedure, but infecting at the various steps with higher multiplicities of virus per cell, invariably resulted in overgrowth by the N-tropic Akv-2 parent.

Peptide Maps. ^{125}I-Labeled tryptic and chymotryptic peptide maps of the gp70, p30, and p15 products of sucrose gradient-purified viruses were derived as described (1, 11, 16).

RESULTS

B-Tropic Recombinant. Superinfection of wild mouse xenotropic virus-producing SC-1 cells with an N-tropic AKR virus resulted in progeny viruses that segregated a stable B-tropic MuLV after several passages at low multiplicities of infection of *Fv-1*^{b} cells. The *Fv-1*^{b} cells are restrictive to infection by N-tropic and xenotropic MuLV (17, 18). Progeny of parallel cultures of SC-1 cells infected only with the N-tropic or xenotropic parent MuLV did not produce a B-tropic virus after passage on the *Fv-1*^{b} BALB embryo fibroblasts. Table 1 shows that the B-tropic recombinant was ecotropic and XC positive and displayed virtually the same host range properties on *Fv-1*^{n}

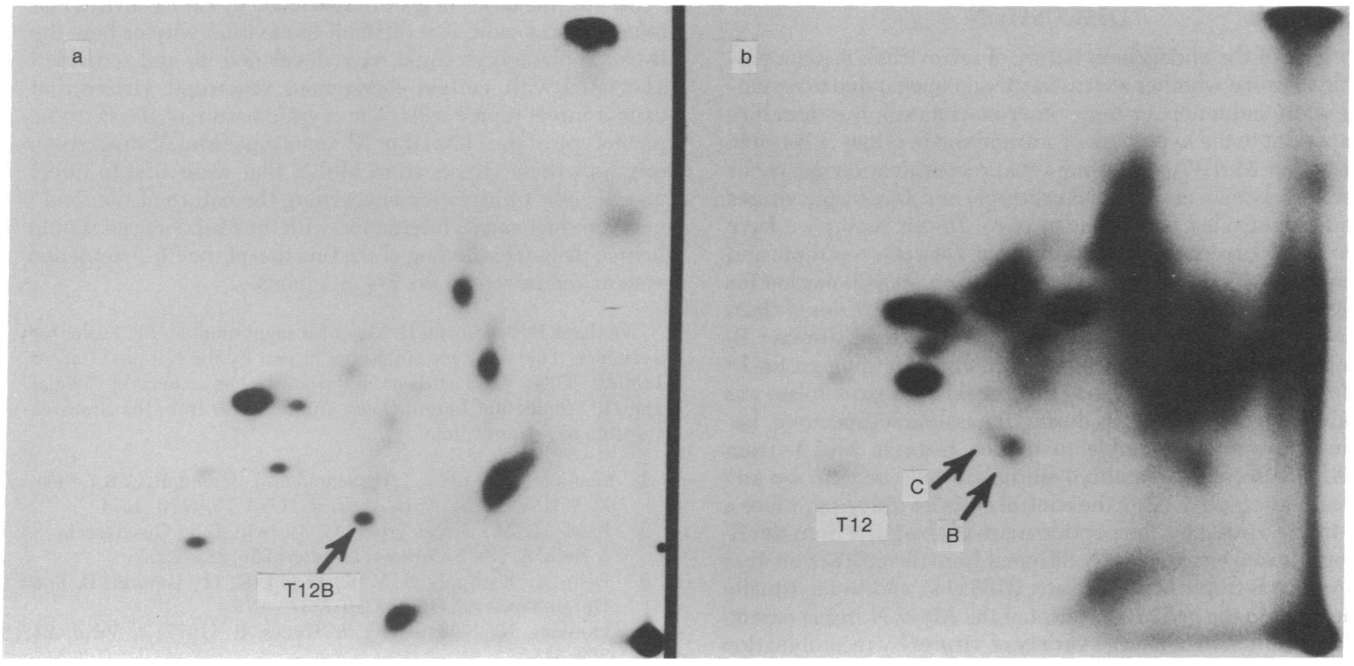

FIG. 2. Mixtures of two-dimensional ^{125}I-labeled tryptic peptides of the p30s from Akv-2-603 N-tropic and WM-X parental viruses and the B-tropic recombinant MuLV. The fingerprints in the two panels are the result of mixtures of tryptic peptides that have been co-separated. (*a*) Mixture of p30 tryptic peptides from WM-X and B-tropic recombinant viruses. (*b*) Mixture of p30 tryptic peptides from Akv-2 and B-tropic recombinant viruses at approximately 1:2.

and *Fv-1*^{b} cells as did the prototype B-tropic virus, WN1802B. Although WN1802B and the recombinant were phenotypically indistinguishable, the unique structural marker on WN1802B p30, a distinguishing feature of this virus (1), is not found in p30 fingerprints of the B-tropic recombinant virus.

Peptide Maps. Fig. 1 shows peptide maps of the gp70s, p30s, and p15s from the cloned parental and recombinant viruses. The peptide maps of the proteins of the parental N-tropic and xenotropic viruses are easily distinguishable. The gp70 fingerprint of the B-tropic recombinant is virtually identical to that of the AKR N-tropic parental virus except for two spots seen in the peptide maps of the B-tropic recombinant gp70 that are not seen in the map of the N-tropic AKR virus (arrows). This suggests that recombination might have taken place within *env*. Genetic recombination within *env* is apparently a frequent event associated with MuLV, as has been shown (9, 21).

The p30 tryptic peptide map of the B-tropic recombinant contains the *p30-T12B* marker, the same as that of the parental xenotropic virus. The N-tropic parental virus p30 differs from the p30 of the xenotropic and B-tropic viruses by containing the marker *p30-T12C*^{n} which is indicative of N-tropic viruses of inbred strains of laboratory mice (1).

To demonstrate this point, Fig. 2 shows fingerprints consisting of mixtures between p30 tryptic peptides from the B-tropic recombinant and the two parental viruses. The only differences in the position of tryptic peptides derived from the three viral p30s are in the no. 12 peptides. Other features that might appear to distinguish these maps have been determined to be caused by spot intensity differences that can vary from one preparation to another. In our experience, differences in spot intensity are inherent to the procedure, which is highly sensitive to stoichiometric and other conditions of the iodination reaction, and do not represent primary structural differences.

Peptide maps of the viral p15s show that the B-tropic recombinant acquired the p15 of the N-tropic parental virus, indicating that at least two genetic crossovers must have occurred during formation of the B-tropic virus.

DISCUSSION

Because of the endogenous nature of retroviruses it is not possible to prove whether a virus has arisen *in vivo* due to recombination, induction, or some other mechanism; it is therefore important to the hypothesis of a recombinant origin of B-tropic and other MuLV to determine that recombination can occur between known endogenous ecotropic and xenotropic viruses under controlled conditions *in vitro*. In this report we have shown that recombination does occur between ecotropic and xenotropic MuLV *in vitro* and that, when the p30 marker for B-tropism is transferred from a xenotropic virus to one with an ecotropic *env* by recombination, the virus then displays B-tropism and is restricted in mouse cells containing an *Fv-1*^{n} allele. We consider it unlikely that the B-tropic virus isolate was induced from *Fv-1*^{b} cells during the isolation procedure, because it was not possible to induce B-tropic MuLV from BALB/c secondary embryo fibroblast cells *in vitro* by any means attempted. Also, the control cultures did not produce a B-tropic virus, and the peptide maps of the gp70 from the B-tropic isolate were distinctly different from the gp70s of another BALB/c B-tropic MuLV isolate, B138 (11), and were virtually identical to the gp70 fingerprint of the Akv-2 N-tropic parent. The production of a large variety of viruses by recombination seems to be the normal situation in the mouse. It appears valid to view an MuLV as a modular assortment of viral genes, each with different functions, that together confer a particular phenotype to the virus. It should be possible to construct virtually any MuLV *in vitro* from two or more different MuLV by using the proper selective pressures. Recombinants generated *in vivo* or *in vitro* can be characterized genotypically, thereby defining the parental origins of the viral gene products and the point(s) of genetic recombination.

B-tropic MuLV isolates are rare, relative to N-tropic muLV, and have been isolated mainly from BALB and C57BL strains of mice (18, 22–26). The B-tropic viruses are of special interest, however, because their appearance is usually associated with neoplastic disease, and some of them (e.g., x-irradiation-induced MuLV and some BALB/c isolates) are oncogenic upon inoculation into mice (27–30).

The significance of the simultaneous appearance of B-tropic viruses and tumors is not known. However, it suggests that escape from stringent regulation of expression of the class III or X^b xenotropic MuLV in *Fv-1*^{b} mice can be deleterious or lethal to the mouse. Consistent with this idea, we have recently determined (unpublished results) that certain (prototype II) xenotropic viruses of the class III or X^b type are the probable parents to oncogenic or mink cell focus-inducing *env* gene recombinant MuLVs.

The origin of B-tropic MuLV in mice, such as the BALB/c and C57BL inbred lines, has not been clear. These mice are homozygous for the *Fv-1*^{b} allele, making them permissive to B-tropic viruses, but chemical induction of BALB or C57BL cells results only in the transient production of N-tropic viruses and xenotropic viruses of the class II or X^a group (31). This and immunological, structural, and sequence studies have suggested that it is likely that B-tropic viruses arise by recombination between endogenous N-tropic and class III or X^b xenotropic MuLV (1, 32, 33). Alternatively, it has been suggested that B-tropic viruses could arise by mutation of endogenous N-tropic viruses to B-tropic viruses (34). Recent sequence information related to *Fv-1* specific viral genetic determinants indicates that at least four mutational events would be required to generate a B-tropic virus from an N-tropic virus, whereas a single-step mutation will generate NB-tropic viruses from B-tropic MuLV (33).

Because the target of growth restriction by *Fv-1* is a virus that infects mouse cells, it is difficult to envision why or how the B-tropic phenotype could have developed in, and remained associated with, certain endogenous xenotropic viruses that cannot infect mouse cells. The cryptic nature of the B-tropic phenotype of the class III or X^b xenotropic MuLV suggests an origin of these viruses from MuLV that were able to infect mouse cells. Information concerning the nature of the *Fv-1*^{n} gene product and its interaction with the MuLV target should help in the understanding of the function of the *Fv-1* restriction system and its role in the life of a mouse.

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