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Generation and Characterization of a Stable Full-Length Ecotropic Murine Leukemia Virus Molecular Clone that Produces Novel Phenotypes to Fv1 Restriction

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Retrovirus tropism can be restricted by host cell factors such as Fv1, TRIM5α, and Lv1 that inhibit infection by targeting the incoming viral capsid. The Fv1 gene inhibits murine leukemia virus infection in mice, but the precise mechanism of Fv1-mediated restriction is poorly understood. Our previous studies had demonstrated that Fv1-mediated viral tropism can be determined within the capsid protein at position 114. To study the interaction between Fv1 and CA, we introduced amino acid substitution and deletion at this site in the N-tropic AKV capsid gene. The mutated two-LTR proviral DNAs were introduced into SC-1 cells by transfection. After transfection, cell supernatants collected from transfected cells were tested for host range susceptibility. The result indicated that substitution of amino acids did not alter tropism, but the deletion of 114His produced a virus with unusual tropism. The novel phenotype produced here failed to replicate in Fv1-expressing cells. This mutant virus showing such an extreme restriction pattern would be useful for studying the mechanism of Fv1-mediated restriction.

Keywords: Retrovirus, Fv1-mediated restriction, capsid, two-LTR

During mammalian evolution, a variety of mechanisms have arisen to limit retroviral replication [2, 6, 26]. For many years, the existence of retrovirus restriction factors was thought to be unique to the mouse. The Fv1 (Friend virus susceptibility-1) gene inhibits MLV (murine leukemia virus) infection in mice [15]. However, recent findings suggest the existence of similar restriction factors in primates including humans. These factors, termed Lv1 (Lentivirus susceptibility factor-1) and Ref1 (Restriction factor-1), can inhibit a range of retroviruses, including HIV-1 and its relatives [9, 13, 25]. Ref1 is now known as TRIM5α (Tripartite motif). Human TRIM5α was identified as an intracellular restriction factor capable of blocking N-tropic MLV infection [22, 23, 34].

The virus resistance gene Fv1 is one of a number of genes that affect the susceptibility of mice to infection by retroviruses. Fv1 is present in mice as four alleles ($Fv1^n$, $Fv1^b$, $Fv1^{nr}$, $Fv1^0$), the $Fv1^n$ allele, originally found in NIH Swiss mice, allowing replication of N-tropic strains of virus and blocking B-tropic strains, whereas the $Fv1^b$ allele in BALB/c mice allows replication of B-tropic viruses and blocks N-tropic type. A third allele, $Fv1^{nr}$, restricts not only B-tropic but also some N-tropic isolates. A fourth allele, $Fv1^0$, is present in wild mice that are fully sensitive to all strains of viruses [4, 8, 32, 33].

Early studies had demonstrated that the responsible sequences of Fv1 restriction are found in CAgag, and that there are only two amino acids (CA109 and CA110) that distinguished N- and B-tropic viruses [19]. Alteration of only one of these amino acids, arginine 110, is sufficient for conversion of an N-tropic virus to a B-tropic virus. Interestingly, arginine 110 of the N-MLV capsid also determines sensitivity to TRIM5α-mediated restriction. An additional modulation of susceptibility of N ecotropic MLV to Fv1 has been mapped to position 114 [16]. A number of amino acids that are in close proximity to position CA110 (CA 82, 92–95, 105, and 117) are involved in the specification of a different host range, thus indicating a more complex interaction pattern between Fv1 and CA [19, 31]. However, a direct interaction between Fv1 and CA has never been demonstrated.

The TRIM5α restriction blocks viral replication before the completion of reverse transcription, whereas the Fv1 blocks after reverse transcription. A recent study suggests that premature disassembly of the viral capsid contributes to the restriction of N-MLV infection by TRIM5α.

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However, the exact mechanism by which Fv1 restricts MLV infection is currently unknown.

Although the Fv1 gene was cloned a few years ago and shown to be related to the presumptive gag region of the endogenous MuERV-L elements, the mechanism by which this gene is responsible for resistance is still poorly understood [5]. Part of the difficulty in studying Fv1 restriction is the fact that resistance is not absolute; virus titers are reduced 100- to 1,000-fold in resistant cells. Furthermore, replication in restricted cells generally shows two-hit kinetics, suggesting that restriction can be overcome or abrogated by the presence of multiple infecting particles [21]. This makes restriction difficult to assess biochemically or biophysically. Creation of mutants viruses that fail to replicate in mice of specific Fv1 types should be useful for the study of the Fv1-mediated mechanism of restriction. We generated mutant viruses with more extreme patterns of restricted replication to determine the mechanism of Fv1 restriction.

Materials and Methods

Viruses and Cells

The N-tropic virus AKV, AKR-L1, and B-tropic virus WN1802B were used as reference viruses. NIH3T3 (*Fv1*^{n}), SC-1 (*Fv1*^{0}), BALB/3T3 (*Fv1*^{b}), *M. dunni* (*Fv1*^{0}), and rat XC cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics.

Two LTR Clones of AKV

A molecular clone of the AKV provirus in pBR322 was kindly provided by Dr. J. Lenz (Albert Einstein College of Medicine, New York). The AKV clone (pAKV34) had originally been cloned at the PstI site of pBR322 and was recloned into pGEM-3Z(+) (pGEM-AKV) for these experiments. However, the LTR sequence is present in two discontinuous segments in the PstI fragment.

To make one complete copy of the AKV provirus containing two LTRs, circle-junction intermediates of AKV retroviral replication were isolated by infecting SC-1 cells with wild-type AKV (1 ml). The DNA was isolated by the Hirt method as previously described [14]. Circle-juction DNA intermediates were amplified by PCR using primers U3: 5'-ACGCAAGCTTAATGAAAGACCCCTT-CATAA-3' [nt7825-7844, AKV], and U5: 5'-GAGCCCCCAAAT-GAAAGAC-3' [nt135-153, AKV]. A HindIII restriction site was introduced in the U3 primer (underlined sequences) for subcloning LTR.

The PCR reaction (20 μl final volume) contained 0.5 μg of the Hirt DNA, 20 pmols of the primers, and 2.5 U of Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems). The PCR reactions were carried out in a GeneAmp PCR system 9700 machine (PE Applied Biosystems, Foster City CA, U.S.A.). The reactions were performed for 35 cycles as follows: a 30 s DNA denaturation step at 95°C, 30 s annealing step, and 1 min extension step at 72°C. The annealing temperature in the first cycle was 63°C; each cycle was subsequently reduced by 1°C for the next 8 cycles, and the remaining 27 cycles were continued at 55°C.

The PCR products were cloned into the pCRII-Topo vector and directly sequenced.

The pCRII-Topo positive clone containing an insert was digested with HindIII and EcoRI, and ligated to HindIII/EcoRI-digested pGEM-3Z(+) (pGEM-LTR). Plasmid pGEM-AKV containing an 8.2 kb PstI fragment of AKV provirus was digested with PstI. The 8.2-kb PstI fragment of AKV was ligated to PstI-digested pGEM-LTR (pGEM-LTR-AKV). The pGEM-LTR-AKV was digested with HindIII and EcoRI and ligated to HindIII/EcoRI-digested pcDNA3.1(+) (pcDNA-AKV).

Mutagenesis

AKV mutants in the CA region (amino acid number 114) were generated by PCR using the STRATAGENE (La Jolla, CA, U.S.A.) QuickChange Site-Directed Mutagenesis kit. PCR products of the CA gene were subcloned into pGEM-T vector. Amino acid change was introduced into the pGEM-T vector containing the CA gene, using oligonucleotide designed to incorporate specific base changes.

The sequences of the primers were as follows:

Leu-a: 5'-GGTAGGAACCTCCTAGTTCTC-3'
Leu-b: 5'-GAGAACTAGGAGGTTCCTACC-3'

Trp-a: 5'-GGTAGGAACTGGCTAGTTCTC-3'
Trp-b: 5'-GAGAACTAGCCAGTTCCTACC-3'

D114a: 5'-AAGAGGTAGGAACCTAGTTCTCTATC-3'
D114b: 5'-GATAGAGAACTAGGTTCCTACCTCTT-3'

The mutated fragment was removed as a 723-bp *Bsi*WI-*Psh*AI fragment corresponding to nucleotides 1,126–1,849 of the viral genome. This fragment was ligated to pGEM-AKV from which the corresponding 723-bp fragment had been deleted. Plasmid pGEM-AKV containing an 8.2-kb PstI fragment was recloned to pcDNA-AKV. All mutants were confirmed by DNA sequencing.

Transfection

To test the effects of these mutations on viral tropism, mutated proviral DNA was introduced into SC-1 cells and NIH3T3 cells using the QIAGEN (Valencia, CA, U.S.A.) SuperFect Transfection Kit. After 3 days in culture, supernatants were harvested every 3 days up to 21 days and assayed for the presence of reverse transcriptase (RT).

Fv1 Typing

Culture supernatants were collected from SC-1 transfected cells and used for further analysis.

To characterize the replication patterns of mutant viruses, subconfluent cultures of SC-1 (*Fv1*^{0}), *M. dunni* (*Fv1*^{0}), NIH3T3 (*Fv1*^{n}), and BALB/c (*Fv1*^{b}) cells were infected with 10-fold dilutions of virus-containing medium in the presence of polybrene (4 mg/ml; Aldrich, Milwaukee, WI, U.S.A.). Reference virus and mutants virus replication in these cells were scored by the XC test 4–5 days after infection [17, 18].

Cloning of the *Fv1*^{n} and Making Stable Cell Line Containing *Fv1*^{n}

To obtain a full-length *Fv1*^{n} ORF, PCR was performed using the forward primer Fv1a and the reverse primer Fv1b from the

published sequence (GenBank Accession No. X97720). The sequences of the *Fv1*^{n} specific primers were as follows:

Fv1a: 5'-TAGCCGAGTTCTAGGGAAAC-3'
Fv1b: 5'-TGTATTTGGCAACCTTTGGA-3'

This 1.4-kb full-length *Fv1*^{n} was cloned into pCRII-Topo (Invitrogen) and transferred to pcDNA3.1(-) (Invitrogen, Frederick, MD, U.S.A.) following XbaI plus HindIII digestion. The *Fv1*^{n}-containing clone was introduced into SC-1 cells, and individual G418-resistant cell clones were selected and tested for expression of *Fv1*^{n} by infection with B-tropic MLV and mutant virus.

RESULTS

Construction of Two-LTR Molecular Clone

To characterize the replication patterns of mutant viruses, we constructed a two-LTR molecular clone of AKV (Fig. 1). The AKV clone (pAKV34) had originally been cloned at the PstI site of pBR322. However, the LTR sequence is present in two discontinuous segments in the PstI fragment. In our previous transfection method, full-length AKV DNA was released from pAKV34 by PstI digestion, following concatemerization *in vitro* to recreate the two-LTR configuation, and was transfected into NIH3T3 cells. This method takes time to generate a virus stock and produces small amounts of progeny of virions. Compared with pAKV34, the pcDNA-AKV produces high titers of virus in transfected SC-1 cells. Progeny viruses released into the supernatant medium of these pcDNA-AKV-transfected SC-1 cells were detected by RT assay.

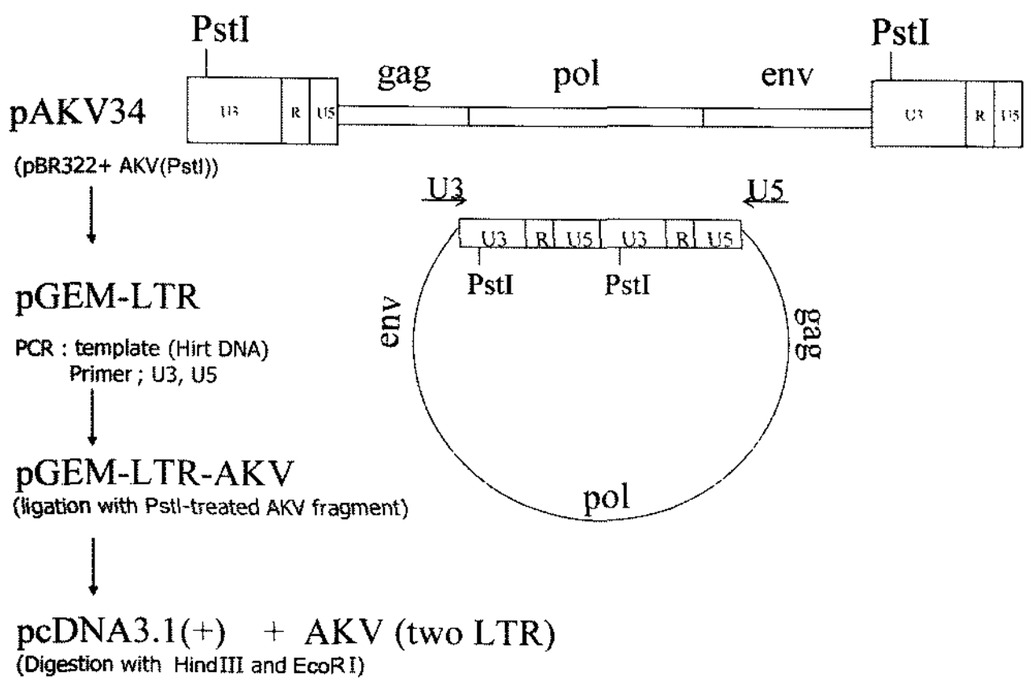

Fig. 1. Diagrammatic representation of the two-LTR full-length molecular clone.
The two-LTR gene fragment was amplified from unintegrated circular viral DNA present in SC-1 cells infected with wild-type AKV and ligated with pGEM-3Z(+) after both were digested with HindIII and EcoRI (pGEM-LTR). Full-length AKV DNA was released from pAKV34 plasmid by PstI digestion and cloned using PstI-digested pGEM-LTR (pGEM-LTR-AKV). After digestion of pGEM-LTR-AKV with HindIII and EcoRI, the resultant fragment was ligated to the HindIII to EcoRI fragment from pcDNA3.1(+) (pcDNA-AKV).

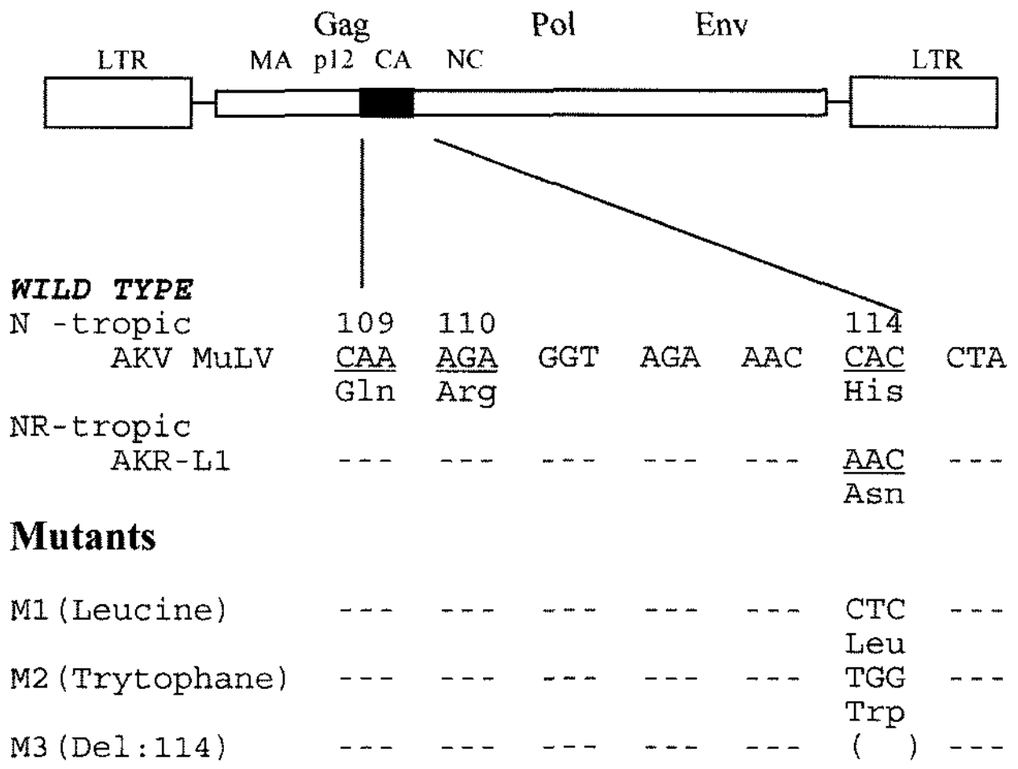

Fig. 2. Site-directed mutagenesis in the Fv1-sensitive region of the AKV MuLV CA gene. Nucleotide and amino acid sequences are given for the prototype virus, for AKR-L1, and for three mutants.

Analysis of Tropisms of AKV Mutants

In our previous mutagenesis studies, residue 114 of MLV CA was identified as a Fv1-modulating determinant. Therefore, three mutant viruses containing Leu, Trp, and deletion of His in residue 114 were produced, and all were replication competent in SC-1 cells (Fig. 2). Unexpectedly, clone pcDNA-AKV (M3; D114) did not yield infectious virus in the transfected NIH3T3 cells (Table 1). To increase the transfection efficiency, stable NIH3T3 cell lines expressing the mutant provirus pcDNA-AKV (M3; D114) were generated by stable transfection. Stable cell lines also did not produce replication-competent virus (data not shown). This result suggests that Fv1 restriction is also likely to involve the late stages of infection. Further study will be required to completely rule out the possibility of Fv1 action being involved late in the viral replication cycle. To determine the effect of alteration of 114His, cell culture supernatants collected from transfected SC-1 cells were tested for viral tropism (Table 2). The viruses containing 114Leu and 114Trp resembled AKR-L1, whereas the virus with deletion of 114His was restricted by NIH3T3 cells and BALB/3T3 cells. Therefore, M3

Table 1. Virus replication after transfection of cells with proviral DNAs containing CA mutations.

DNA	NIH3T3(n)	SC-1 (o)
AKR-L1	+	+
M1 (leucine)	+	+
M2 (tryptophan)	+	+
M3 (Del: 114)	–	+

Virus was monitered by release of virion-associated RT into the culture medium.
+, High levels of RT; –, no RT.

Table 2. Replication of wild-type and mutant viruses in cells of various laboratory strains.

	Log_{10} virus titer (*Fv1* type)[a]			
Virus	SC-1(o)	Mus dunni(o)	NIH3T3(n)	BALB/ 3T3(b)
AKR-L1	5.4	4.6	4.7	3.7
M1 (leucine)	5.1	4.9	4.5	2.6
M2 (tryptophan)	5.0	4.8	4.4	3.2
M3 (Del: 114)	4.9	3.0	0.3	1.0

[a]Measured as the number of XC pfu in 0.2 ml.
The *Fv1* tropism of each cells is given in parentheses.

mutant(D114) could not be clearly classified as either N- or B-tropic.

Transfection Assay for Fv1 Restriction

There are many genetic differences between SC-1 cells and NIH3T3 cells besides Fv1, and therefore, we determined whether this novel phenotype was an Fv1-mediated phenomenon. To test the possibility of the *Fv1* gene product to restrict this M3 mutant (D114) virus, we prepared a pcDNA3.1(−) construct expressing the n-alleles of Fv1. This was introduced into SC-1 ($Fv1^0$) cells by stable transfection. Introduction of $Fv1^n$ showed the expected pattern: B-tropic viruses were patially restricted and also showed strong restriction of M3 (D114) virus (Fig. 3). Supernatants from $Fv1^n$ expressing SC-1 cells contained lower levels of virus than SC-1 cells, as estimated by RT assay (data not shown).

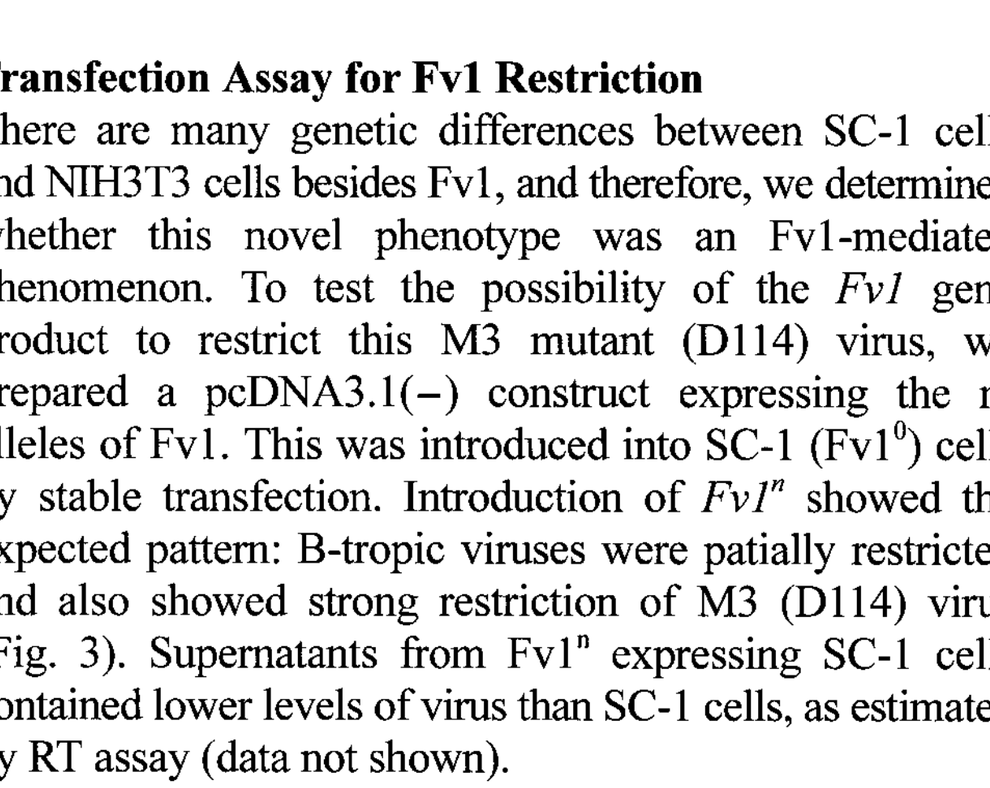

Fig. 3. Restriction of Fv1-transduced SC-1 cells after infection with B-tropic MLV and mutant (D114) virus.
The Fv1 expression plasmid was introduced into SC-1 cells by transfection, and G418-resistant cell clones were isolated. These cells were then infected with B-tropic MLV (**A**) and mutant virus (**B**). Virus replication was scored by the XC test from 4 to 5 days after infection.

DISCUSSION

The *Fv1* gene restricts murine leukemia virus replication *via* an interaction with the viral capsid protein; however, the precise mechanism of action of Fv1 is poorly understood. Although crystallographic studies suggested that a potential Fv1 binding domain exists in the MLV CA, there is no evidence for a direct interaction between Fv1 and CA [27]. Several mechanisms have been proposed for Fv1 restriction, including binding of Fv1 to CA, binding of Fv1 to an undetermined CA helper factor, and direct binding of Fv1 to a pre-integration complex (PIC). In the case of the third possible route, CA has been suggested to act as a transport signal for the pre-integration complex (PIC), and therefore, binding of Fv1 to CA will alter CA binding to the PIC. As a result, the PIC could not be able to migrate into the nucleus. However, this mechanism to explain the Fv1-mediated restriction presented here lacks experimental evidence [7, 10–12]. Human TRIM5α has recently been characterized to restrict N-tropic MLV. Although there is no sequence homology between Fv1 and TRIM5α, they both restrict retroviruses in a CA-dependent manner. The TRIM5α restrictions block viral replication before the completion of reverse transcription, whereas the Fv1 blocks after reverse transcription. The exact mechanism by which human TRIM5α restricts N-MLV infection is unknown; however, a recent study suggests that premature disassembly of the viral capsid contributes to the restriction of N-MLV infection by human TRIM5α [3, 24, 29]. Understanding the TRIM5α-mediated restriction mechanism may help to elucidate the restriction mechanism of Fv1.

Previous mutagenesis studies had focused on residues 109 and 110, and demonstrated that the virus is B-tropic when residue 110 contains acidic amino acids. On the other hand, when basic amino acids were substituted at this site, N-tropic viruses resulted. The possibility that mutagenesis in CAgag produces novel phenotype has been demonstrated in previous studies [19]. Studies on the mechanism of Fv1 restriction have been hampered, because natural levels of Fv1 expression appear very low and Fv1 can be abrogated by exposure to restricted virus at high multilicity of infection [1, 28, 30]. Therefore, mutant viruses with more extreme patterns of restricted replication would be potentially useful reagents in studies to determine the mechanism of Fv1 restriction. First of all, stable infectious molecular clones of MLV that generate high titers of virus infecting murine cell lines will be useful to study the mechanism of Fv1-mediated restriction. In our previous studies, we used single-LTR molecular clones to produce viruses. However, this clone produced a small amount of progeny virus and the procedure is also time-consuming. We overcame the above limitation by constructing a stable full-length molecular clone (Fig. 1). It is far easier to prepare and to analyze wild-type and mutant virus

stocks with two-LTR rather than single-LTR molecular clones. Site-directed mutagenesis was used in this two-LTR molecular clone to create mutant viruses.

Interestingly, our studies of the AKV D114 mutant carrying a deletion of histidine at CA114 showed specific restriction patterns (Table 2). This pattern of replication has not been previously described. To investigate how Fv1 mediates the restriction of retrovirus, our MLV capsid variant will be used later for fate-of-capsid assay [23] that allows to monitor the steady-state levels of particulate retroviral capsid within an infected cell.

To ensure that the failure of progeny virus production in NIH 3T3 cells was due to the effects of the Fv1 but not due to other cellular factor, we created stable SC-1 cell lines expressing the n-allele of Fv1, and confirmed Fv1 expression in SC-1 cells by using B-tropic virus. $Fv1^n$-expressing SC-1 cells showed restriction of B-tropic virus, and thus, behaved like $Fv1^n$ cells. Our present results showed that the virus with a deletion at CA 114 was restricted in both infection and replication in SC-1 ($Fv1^n$) cells. It is likely that the deletion of histidine may increase the efficiency of interaction between CA and Fv1. Additional study is required to determine whether Fv1-mediated restriction could involve prevention of capsid uncoating, acceleration of capsid uncoating, or destruction of the capsid. The elucidation of the mechanism of restriction presented above makes it possible to obtain a novel restriction protein and exploit the inhibitory properties of Fv1-like restriction factors in the context of gene therapy.

Recent work identified a number of new CA residues that differentially modulate resistance to Fv1 [31], suggesting that the Fv1-CA interaction is complex, with multiple amino acids on both sides playing important roles. Our present studies demonstrated that a certain manipulation of CA leads to generation of viruses possessing novel phenotype. These results shed light on the fundamental genetic mechanisms of retroviral restriction. In addition, our observation is potentially of considerable significance to design viral vectors that possess novel expanded or restricted host ranges by virtue of simple modification of the retroviral CA.

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