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Single Amino Acid Changes in the Murine Leukemia Virus Capsid Protein Gene

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The mouse *Fv1* genetic locus controls resistance to subgroups of ecotropic, MCF, and amphotropic murine leukemia viruses (MuLVs). In addition to the four previously defined alleles of *Fv1* (*Fv1*^{n}, *Fv1*^{b}, *Fv1*^{nr}, *Fv1*^{0}), we present evidence that the novel restriction pattern characteristic of DBA/2J mice maps to the *Fv1* locus and therefore represents a novel allele, here designated *Fv1*^{d}. Previous studies had demonstrated that the *Fv1*-mediated viral tropism is determined within the capsid protein gene, and that N- and B-tropic virus capsids differ only in two adjacent amino acids. We introduced various amino acid substitutions at these two sites in the N-tropic AKV MLV capsid gene, and typed resulting viruses for host range on cells carrying all five *Fv1* alleles as well as on cells from additional wild mouse species with *Fv1*-like differences in virus susceptibility. Results indicate that alteration of the first of the two amino acids does not alter tropism, but alteration of the second alone is sufficient to convert the N-tropic AKV MLV to a B-tropic virus. Substitution of leucine for arginine at this site produced a virus with an unusual tropism not characteristic of any of the naturally occurring or laboratory strains of MuLV.

INTRODUCTION

Alleles of the mouse autosomal gene *Fv1* control the relative sensitivity of cells to infection by subgroups of the mouse-tropic MuLVs: ecotropic, amphotropic, and dualtropic or mink cell focus inducing MuLVs (Pincus *et al.,* 1971). These host range subgroups use different cell surface receptors to infect mouse cells, but can be further subclassified as N-tropic if they replicate best in *Fv1*^{n} cells, B-tropic if they replicate best in *Fv1*^{b} cells, or NB-tropic if they grow equally well in *Fv1*^{n} or *Fv1*^{b} cells (Hartley *et al.,* 1970). A third restriction allele termed *Fv1*^{nr} restricts replication of some types of N-tropic and all B-tropic viruses (Pincus *et al.,* 1971; Steeves and Lilly, 1977). In all cases, the *Fv1*-mediated restriction is not absolute, but is detected as a 100- to 1000-fold reduction in the efficiency of virus replication in the resistant cells. Studies on wild mice identified a fourth allele, *Fv1*^{0}, which fails to restrict N- or B-tropic viruses (Hartley and Rowe, 1975; Kozak, 1985).

Closer examination of susceptibility to standard N-, B-, and NB-tropic viruses in wild mouse species and inbred strains suggests that additional allelic variants may exist. In some of these mice, small but reproducible differences have been observed in susceptibility to the prototype viruses used to distinguish the known *Fv1* alleles. Atypical resistance patterns in wild mice such as *M. pahari* and *M. m. domesticus* (WSA) differ from those of any of the four known alleles (Kozak, 1985). Among inbred mice, unusual patterns have also been described in at least two inbred strains, DBA/2 and NZB/N (Pincus *et al.,* 1971, 1975). For DBA/2 mice, these differences have been particularly well documented. Various studies have reported that, in comparison to cells from other mice classed as *Fv1*^{n}, DBA/2 cells produce higher than expected titers of B-tropic virus (Pincus *et al.,* 1971). Also, a much higher proportion of B-tropic virus infected DBA/2 cells become virus producers (Pincus *et al.,* 1975; Benjers *et al.,* 1979), and B-tropic virus DNA production is not inhibited in DBA/2 mice (Yang *et al.,* 1980). The genetic basis for the differences observed in these inbred and wild mice has not been determined, so although multiple *Fv1*-like restriction phenotypes exist, only four (*Fv1*^{n}, *Fv1*^{b}, *Fv1*^{nr}, *Fv1*^{0}) have been formally attributed to allelic variation at the *Fv1* locus.

The molecular basis for the *Fv1* restriction is unknown. Use of MuLV pseudotypes of vesicular stomatitis virus showed that this restriction involves a step in the viral replication cycle after virus adsorption and penetration of nonpermissive cells (Krontiris *et al.,* 1973; Huang *et al.,* 1973). Analysis of viral nucleic acids in infected cells demonstrated that *Fv1*-mediated restriction ultimately prevents formation of proviral DNA (Jolicoeur and Rassart, 1980; Sveda and Soeiro, 1976; Prysiak and Varmus, 1992). Thus, the *Fv1* gene product affects replication at an early stage in the life cycle of the restricted virus.

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Biochemical and genetic studies (Benade *et al.,* 1978; Hopkins *et al.,* 1977; Gautsch *et al.,* 1978) implicated the viral *gag*-encoded capsid protein (CA) as the target of *Fv1* restriction. Sequence analysis of the viral DNA segment containing the allelic genetic determinants of N- and B-tropism identified two consecutive amino acid differences in CA at positions 109 and 110 which distinguished N- and B-tropic viruses (DesGrosseillers and Jolicoeur, 1983; Ou *et al.,* 1983). Although these two amino acid differences clearly differentiate N- and B-tropic viruses, the relative contributions of the two amino acids to the phenotypic difference between N- and B-tropic viruses is unknown. Here we describe experiments designed to address this issue, and to determine whether additional alterations at these sites also alter virus tropism. In these studies, we attempted to increase our chances of identifying *Fv1*-mediated phenotypic change by typing mutant viruses on a panel of cells which included the prototypical $Fv1^{n,b,nr,0}$ types supplemented with cells from various wild mouse species of undefined *Fv1* type. In this report, we define the viral target of the *Fv1* gene product and describe a novel resistance pattern associated with a substitution at this site.

MATERIALS AND METHODS

Viruses

The viruses were grown from stocks obtained from J. Hartley (NIAID, Bethesda, MD) and included the NB-tropic Moloney MuLV, the N-tropic viruses AKV and AKRL1, and the B-tropic virus WN1802B (Pincus *et al.,* 1975).

Cells

The mouse cell lines NIH 3T3 ($Fv1^n$) and SC-1 ($Fv1^0$) (Hartley and Rowe, 1975), mouse embryo fibroblasts, cultures derived from mouse tail biopsies, and rat XC cells (Rowe *et al.,* 1970) were grown in DMEM containing 10% fetal calf serum and antibiotics. Embryo fibroblast cells were prepared from DBA/2J, BALB/cJ, C3H/H3J, and 129/J obtained from The Jackson Laboratory (Bar Harbor, ME). NFS/N mice were originally obtained from The Small Animal Section, (NIH, Bethesda, MD). Embryo cultures were also prepared from mice obtained from random bred colonies of the wild derived mice *M. m. domesticus* (formerly *M. praetextus,* PRAE), *M. caroli* (CAR), *M. m. domesticus* (Watkins Star, WSA), and *M. m. domesticus* (Havens Farm, HAV). These mice were provided by Dr. M. Potter (NCI, NIH, Bethesda, MD) through his contract (NO1-CB-21075) with PerImmune (Rockville, MD). Cultures of tail biopsy tissue were prepared as described previously (Lander *et al.,* 1978) from individual mice of the BXD series of RI (recombinant inbred) strain mice obtained from The Jackson Laboratory.

Oligonucleotide-directed mutagenesis

A 2.8-kb *Kpn*I fragment from pAKR623 (Lowy *et al.,* 1980) containing nucleotides 32–2873 of the AKV N-tropic viral genome was subcloned into M13mp18 as a substrate for mutagenesis (M13K1). Specific amino acid changes were introduced into M13K1 using the Bio-Rad (Richmond, CA) Muta-Gene M13 mutagenesis kit, using 18-bp oligonucleotides designed to incorporate specific base changes. The mutated fragment of M13K1 was removed as a 720-bp *Oxa*N1 fragment corresponding to nucleotides 1062–1782 of the viral genome. This fragment was ligated to pAKV34 from which the corresponding 720-bp fragment had been deleted. Plasmid pAKV34 contains an 8.2-kb *Pst*I fragment of pAKR623 subcloned into pBR322 and was a gift of Dr. J. Lenz (Albert Einstein College of Medicine, New York) (Lenz *et al.,* 1982). Each recovered mutant was sequenced (Sanger *et al.,* 1977).

Transfection

DNAs digested with *Pst*I to release the viral inserts, were treated with T4 ligase overnight and transfected into NIH 3T3 cells by the calcium phosphate method (Graham and Ven der Eb, 1973). A day after transfection, cells were trypsinized and divided into two plates, which were overlaid with SC-1 cells to amplify the virus titer. At 3- to 4-day intervals, each culture was trypsinized and replated into duplicate cultures. One of each pair of cultures was assayed for virus by the XC plaque assay (Rowe *et al.,* 1970), and the other was used for continuous passage.

Fv1 typing

Culture fluid was saved from each passage after transfection, filtered, and stored at −70°. Fluid from cultures which contained virus by XC assay were further tested for their ability to grow in primary cells derived from different wild mice and laboratory strains. Subconfluent cultures of these cells were infected with 10-fold dilutions of virus-containing medium in the presence of polybrene (4 μg/ml; Aldrich, Milwaukee, WI). For comparison, the same cell cultures were used to titrate standard stocks of MuLVs. Virus replication in cultured cells was scored by the XC test 4–5 days after infection.

RESULTS

Fv1 typing panel

In order to fully characterize the replication patterns of the naturally occurring as well as mutant viruses, we collected cells representative of the four standard *Fv1* alleles, as well as cells with atypical *Fv1*-like restriction patterns. For example, although DBA/2 is usually classified as an $Fv1^n$ mouse, infectivity studies have shown that DBA/2 cells are more susceptible to B-tropic viruses

TABLE 1

Replication of Wild-Type and Mutant Viruses in Cells of Various Laboratory Strains and Wild Mouse Species

Virus	*Fv1* tropism	Log_{10} virus titer (*Fv1* type)								
		SC-1 (0)	NFS (n)	BALB/c (b)	129 (nr)	DBA/2 (d)	HAV	WSA	CAR	PRAE
AKV	N	5.5	4.5	1.9	2.8	3.6	4.4	3.6	4.8	3.3
AKR-L1		5.4	5.2	2.7	5.1	4.4	5.0	4.9	ND[a]	4.8
WN1802B	B	6.4	2.5	5.9	2.4	3.7	5.7	4.3	5.5	5.0
Moloney	NB	5.3	5.1	5.4	5.5	5.3	5.1	4.6	5.0	5.2
A3	N	4.9	3.9	1.1	1.5	2.8	3.2	2.8	3.6	ND
A19	B	4.4	—[b]	3.8	0.9	2.5	3.7	2.5	3.5	4.0
LS-5	N	5.2	4.7	2.4	ND	3.9	4.8	ND	4.8	ND
ASP-7	B	3.2	0.2	2.5	ND	ND	ND	ND	ND	ND
TRP-7	N	3.5	3.2	1.2	ND	2.7	2.0	3.5	ND	ND
LU-7		6.1	3.5	2.5	2.1	2.4	5.0	3.2	3.6	3.2

[a] ND, not done.
[b] —, Not detectable.

than other strains classed as $Fv1^n$ (Pincus *et al.,* 1975; Benjers *et al.,* 1979). At the molecular level, DBA/2J differs from NIH3T3 cells in the type and quantity of viral DNA produced following infection by B-tropic virus (Yang *et al.,* 1980). We therefore added DBA/2J cells to our typing panel (Table 1). To this panel were also added mice bred from founders trapped in widely separated geographic locations: Morocco (PRAE), Thailand (CAR), and Maryland (HAV and WSA). One of these mice (PRAE) had been previously identified as $Fv1^0$ in genetic crosses (Kozak, 1985), but repeated assays demonstrate that cells from this mouse routinely produce higher titers of B-tropic virus than N-tropic virus than observed in SC-1 cells or *M. spretus* ($Fv1^0$) (Table 1).

For DBA/2J, genetic studies had never determined whether the unusual features associated with replication could be attributed to *Fv1,* or to other, unlinked genes. It is difficult to type hybrid mice for small differences in virus titer since *Fv1* restriction is semidominant. However, recombinant inbred strains have been generated between DBA/2J and C57BL/6J mice which are homozygously fixed for alleles derived from either parent. These mice have been typed for hundreds of markers permitting genetic linkage of new genes or phenotypes to specific chromosome locations. Therefore, we examined cultured cells derived from 10 individual BXD RI strains for susceptibility to N- and B-tropic viruses (Table 2). In our hands, repeated assays using DBA/2J cells produced higher titers of B-tropic virus and lower titers of N-tropic virus than observed on NFS/N cells. These results are consistent with the replication patterns originally described for DBA/2N (Pincus *et al.,* 1971), although these patterns were, at the time, used to class DBA/2 as $Fv1^n$.

The 10 RI strains could be classed into two groups based on virus replication (Table 2). The strain distribution pattern showed a perfect correlation with the *Fv1*-linked gene *G6pd1.* A perfect correlation was also observed with *Fv1* typings reported in a previous study using these same RI strains which identified two phenotypes but which made no distinction between the DBA/2 phenotype and the prototypic $Fv1^n$ phenotype (Taylor *et al.,* 1977). In our hands, five strains closely resembled the prototype $Fv1^b$ cells in their 3–4 log resistance to N-tropic virus and were typed as $Fv1^b$. The other five strains resembled DBA/2J, in that they were more susceptible to N-tropic virus and less susceptible to B-tropic virus than C57BL/6J. If DBA/2J mice contained an unlinked gene responsible for modifying an otherwise standard $Fv1^n$ allele, then some of the mice inheriting the DBA/2J *Fv1* allele should have lacked this gene and showed the typical $Fv1^n$ phenotype. The likelihood that a second gene unlinked to *Fv1* is responsible for the DBA/2 phenotype is $(1/2)^5 = 0.03$. Thus, the more likely explanation is that

TABLE 2

Virus Replication in Cultured Tail Embryo Fibroblasts of 11 BXD RI Strains and Standard Inbred Mice

	Log_{10} virus titer		N/B ratio	*Fv1* type	*G6pd1*
	AKV	WN1802B			
NFS/N	3.9	1.8	2.1	n	
BALB/c	2.4	5.0	0.5	b	
SC-1 cells	4.4	5.5	0.8	0	
DBA/2J	3.4	3.0	1.1	d	
BXD2	0.3	3.1	0.1	b	B
BXD6	1.4	4.2	0.3	b	B
BXD9	0.1	3.1	0.03	b	B
BXD11	—	2.3	—	b	B
BXD15	0.7	4.2	0.2	b	B
BXD8	1.3	1.7	0.8	d	D
BXD12	3.3	2.9	1.1	d	D
BXD13	3.0	2.5	1.2	d	D
BXD14	3.2	3.2	1.0	d	D
BXD16	3.1	3.0	1.0	d	D

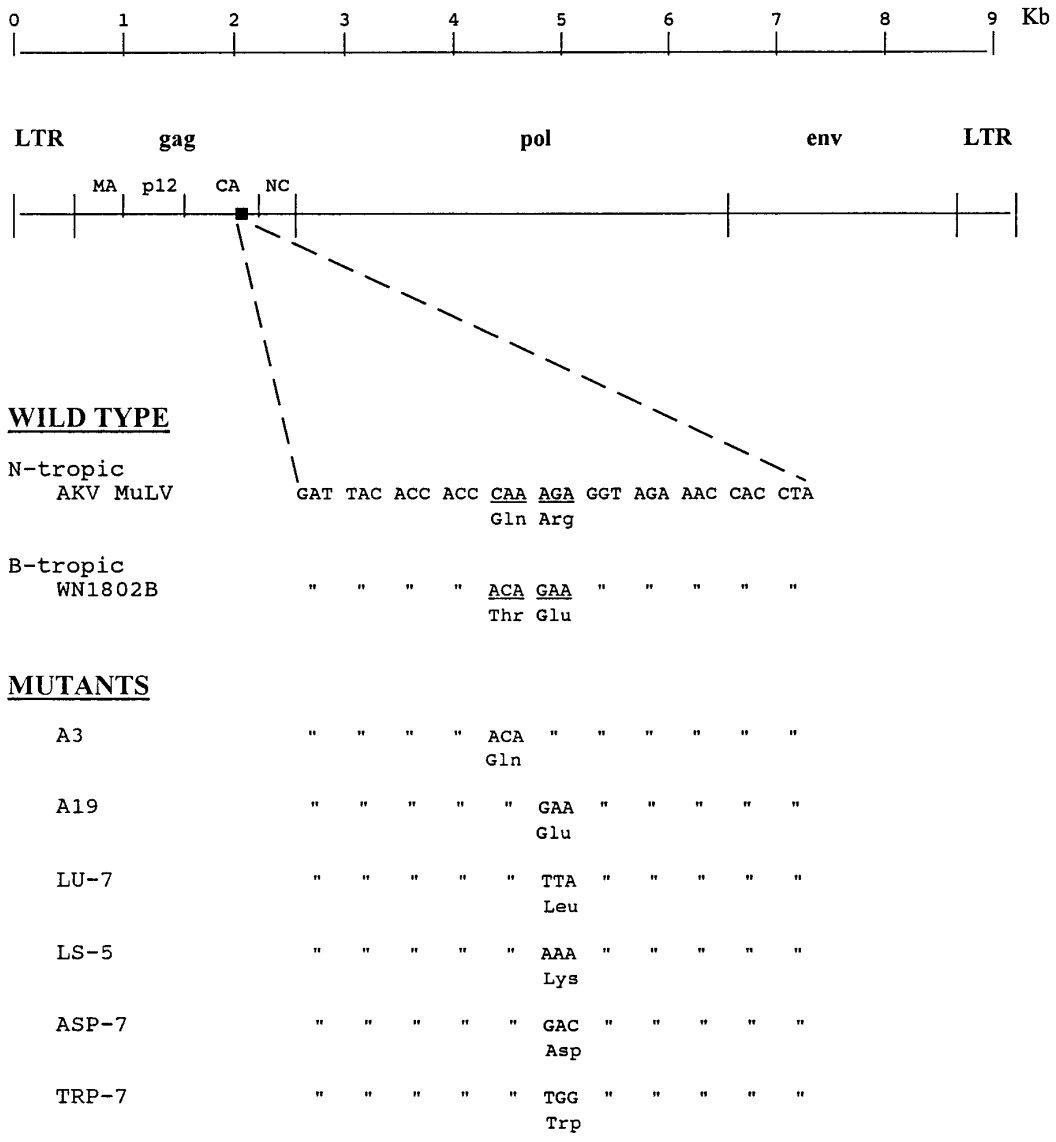

FIG. 1. Site-directed mutagenesis in the *Fv1*-sensitive region of the AKV MuLV CA gene. Nucleotide and amino acid sequences are given for the prototype viruses and six mutants in the region of CA known to differ between N- and B-tropic viruses.

the replication pattern observed in DBA/2J mice is *Fv1*-mediated. We therefore propose that DBA/2 contains a novel fifth allele at this locus, which we designate *Fv1*^{d}.

Mutagenesis

The N-tropic AKV MuLV contains Gln and Arg in positions 109 and 110 in CA, and the B-tropic virus WN1802B contains Thr and Glu in the corresponding positions (Fig. 1). Therefore, we initially produced mutants containing these substitutions: Thr for Gln at position 109 (mutant A3) and Glu for Arg at position 110 (mutant A19).

In both cases, replication competent viruses were detected in transfected NIH 3T3 cell cultures overlaid with SC-1 cells. The viral tropism of the stocks prepared from these cultures were initially determined by infecting NFS/N and BALB/c cells (Table 1). For the A3 mutant, replacement of the AKV position 109 Gln with Thr did not alter the tropism; A3 is clearly N-tropic. In mutant A19, alteration of Arg to Glu at position 110 generated B-tropic virus. These results indicate that a change in a single amino acid at position 110 is sufficient to alter viral tropism from N to B.

Additional position 110 mutants

Because of the obvious importance of amino acid 110 to *Fv1*-mediated virus restriction, we made additional substitutions at this site (Fig. 1). Four mutant viruses containing Leu, Lys, Asp, and Trp substitutions were produced, and all were replication competent. Determination of viral tropism following infection of these viruses into NFS/N, BALB/c, and SC-1 cells demonstrated that replacement of Arg with Lys or Trp or did

not alter the N-tropism (Table 1). However, substitution with Asp produced B-tropic virus. The fourth mutant, LU-7, which contains a leucine substitution, replicated to higher titers on SC-1 cells than NFS and BALB/c cells. LU-7 titers were only marginally better on NFS cells than on BALB/c; LU-7 thus could not be clearly classified as either N- or B-tropic.

We then examined virus replication on the full panel of cells described above. Table 1 shows virus replication patterns for standard viruses in each of these cells along with the patterns observed for various mutants. Mutants A3, LS-5, and TRP-7 resembled the AKV MuLV N-tropic virus, whereas mutants A19 and ASP-7 resembled the B-tropic WN1802B. LU7 replicated to highest titers on SC-1 and HAV. Although its titer was markedly reduced on NFS cells, titration of LU-7 by direct XC plaque assay was linear on both SC-1 and NFS. Titration of LU-7 on cells from an additional *Fv1*^{n} mouse, C3H/HeJ, gave similar results (not shown). In contrast, using BALB/c, DBA/2, WSA, and 129 cells, the number of foci counted at the lower dilutions decreased with the square of the dilution factor, i.e., with 2-hit kinetics. This indicates a requirement for dual infection for focus induction on these cells and is characteristic of *Fv1* restriction (Pincus *et al.,* 1975).

DISCUSSION

Allelic variation at the *Fv1* locus is more extensive than originally thought. Our results establish the fact that at least some of the differences which distinguish DBA/2 mice from *Fv1*^{n} mice are encoded by a gene at or closely linked to *Fv1.* These data confirm the previous observation that DBA/2 mice are unusually sensitive to B-tropic virus (Pincus *et al.,* 1971) and establish that this increased sensitivity, along with a decrease in sensitivity to N-tropic virus, is likely to be *Fv1*-mediated. Although we did not examine B-tropic virus-infected DBA/2 cells to determine the proportion of virus producing cells or to characterize the type and quantity of viral DNA produced, the reported differences previously observed in these properties for DBA/2 (Pincus *et al.,* 1975; Benjers *et al.,* 1979; Yang *et al.,* 1980) are also compatible with a novel *Fv1* allele.

The phenotypes associated with other differences in virus susceptibility observed in inbred strains such as NZB (Pincus *et al.,* 1971) and in wild mouse species may also be governed by variation at the *Fv1* locus. Since these differences are generally small and difficult to distinguish in hybrid mice using assays based on plaquing efficiency, we instead initiated an effort to generate viruses with altered host range which might help differentiate *Fv1* alleles.

Site-directed mutagenesis was used to determine that a change in a single amino acid at position 110 of CA is responsible for altering viral tropism from N to B; substitution at amino acid 109 was not found to be associated with phenotypic variation. It should be noted that the amino acids Thr and Gln found, respectively, at position 109 in WN1802B and AKV MuLV are both neutral/polar amino acids. In contrast, site 110 is occupied in AKV by the basic amino acid Arg, and in WN1802B by the acidic amino acid Glu. Substitution with another acidic amino acid (Asp) produced B-tropic virus, and substitution with either another basic amino acid (Lys) or with the neutral/polar amino acid Trp produced N-tropic virus. Replacement, however, with the neutral/hydrophobic amino acid Leu produced a virus which replicated to lower titers in both *Fv1*^{n} and *Fv1*^{b} cells relative to SC-1 cells, a pattern which did not resemble that of any of the naturally occurring or laboratory strains of MuLV. This emphasizes the importance of this single site as the target for the *Fv1* gene product.

Studies are now in progress to introduce further substitutions as well as deletions in and around this site for several reasons. First, studies on the nature of the *Fv1* restriction have been hampered since this restriction is measured as a quantitative difference in virus titers which can be overcome at high m.o.i. This makes restriction difficult to assess biochemically (Prysiak and Varmus, 1992). Creation of mutant viruses which fail to replicate in mice of specific *Fv1* types should be useful for these studies. While the novel LU-7 phenotype produced here does not show such an extreme restriction, results with this mutant do indicate that substitutions at this site can produce unusual phenotypes. Efforts to create additional mutations might produce more dramatic phenotypic differences. Second, sequence analysis of the CA gene in NB-tropic viruses indicates that substitutions at this same amino acid site cannot account for the failure of the *Fv1* gene product to restrict these viruses. Instead, these viruses are characterized by a number of amino acid differences distributed throughout CA. We are therefore in the process of using site-directed mutagenesis to assess the contribution of these specific differences to NB-tropism.

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