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Mus spicilegus Endogenous Retrovirus HEMV Uses Murine Sodium-Dependent Myo-Inositol Transporter 1 as a Receptor

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We sought to determine the relationship between two recent additions to the murine leukemia virus (MLV) ecotropic subgroup: *Mus cervicolor* isolate M813 and *Mus spicilegus* endogenous retrovirus HEMV. Though divergent in sequence, the two viruses share an Env protein with similarly curtailed VRA and VRB regions, and infection by both is restricted to mouse cells. HEMV and M813 displayed reciprocal receptor interference, suggesting that they share a receptor. Expression of the M813 receptor murine sodium-dependent myo-inositol transporter 1 (mSMIT1) allowed previously nonpermissive cells to be infected by HEMV, indicating that mSMIT1 also serves as a receptor for HEMV. Our findings add HEMV as a second member to the MLV subgroup that uses mSMIT1 to gain entry into cells.

The diversity of receptor usage by murine leukemia viruses (MLVs) and their close relatives reflects an active recent evolutionary history. All MLV subgroups identified to date have related *env* genes and use structurally similar but unrelated small-molecule transporters as receptors (18). M813, an exogenous MLV isolated from *Mus cervicolor*, represents a novel MLV subgroup that uses murine sodium-dependent myo-inositol transporter 1 (mSMIT1) for entry (10). *Mus spicilegus* endogenous retrovirus HEMV (20) is one of the few gammaretroviruses that has yet to have its receptor identified (for a review, see reference 18).

HEMV was initially identified in our laboratory by a comprehensive analysis of endogenous MLV sequences from various *Mus* subspecies (21). Analysis of the long terminal repeats (LTRs) of multiple endogenous MLV-related proviruses from wild-type-derived mice revealed differences exceeding the scope of the previous standard classification of proviruses found in inbred strains (4–7, 17, 21). The provirus containing an unusual LTR found only in the genome of *M. spicilegus* was named HEMV, and further analyses showed that the locus represented an intact provirus (21). HEMV bears hallmarks of an ancient endogenous retrovirus, in that it is fixed in its host species, the sequences of *gag* and *pol* occupy positions near the root of the MLV phylogenetic tree, and the LTR has the simplest structure of all known MLVs (21). However, further study revealed that it is a recent insertion into the *M. spicilegus* genome and that the cloned HEMV provirus is capable of producing infectious virus (20).

The coding sequence of the HEMV *env* gene is substantially shorter than that of most other gammaretroviruses, due to differences in the VRA and VRB regions of SU that encode the receptor-binding domain. Among other known gammaretroviruses, this property was seen only in M813, whose VRA and VRB regions are up to 18 and 8 amino acids shorter than those of other gammaretroviruses, respectively. Though highly divergent in other regions of the genome, including TM, HEMV and M813 share VRA and VRB regions similar in length (Fig. 1). Overall, HEMV and M813 *env* genes show 69% identity at the nucleotide level, corresponding to 72% at the protein level, comparable to the degree of amino acid identity in the VRA (76%) and VRB (70%) regions. The most prominent differences between the two Env proteins are a three-residue gap in HEMV relative to M813 in the proline-rich region and a single mismatched cysteine at residue 181 in M813 (Fig. 1). Closer examination of the SU phylogenetic tree revealed that, although the two viral SU regions share a branch in the tree, the branches leading to the viruses from their last common node are quite long, suggesting a distant relationship (20).

The species tropism of HEMV is best described as strictly ecotropic, reflecting its ability to infect only cells of mouse origin, including *M. spicilegus* (Table 1) (20). M813 has been reported to infect mouse and rat cells, although infection of the latter is 3 orders of magnitude less efficient (16). To better understand the receptor usage of HEMV and M813, we examined the species tropism of both viruses.

The M813 *env* gene was constructed from the published sequence (16) by linking regularly staggered 120-bp oligonucleotides in sequential PCRs and cloning into pCR2.1-TOPO (Invitrogen) to create pM813-TOPO. Mismatches from the published sequence (GenBank accession no. AF327437) were repaired by site-directed mutagenesis (Stratagene). The HEMV *env* gene from pHEMV18 (21), the M813 *env* gene from pM813-TOPO, and the 10A1 *env* gene from pB6 (13) were cloned into pSV-psi-minus-E-MLV (12) to create constructs psi-HEMV, psi-M813, and psi-10A1, respectively. Cotransfection of each construct with pLac-Puro (15) or MLV-green fluorescent protein (MLV-GFP) (14) into 293T cells resulted in the production of single-round infectious LacZ-positive ($LacZ^+$) $Puro^R$ (puromycin-resistant) or GFP^+ viruses bearing the specified *env* genes. Following infection of target cells, titers of the virus inocula were determined either by counting $LacZ^+$ (staining blue with X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]) cells under a light microscope or by quantification of GFP expression via flow cytometry.

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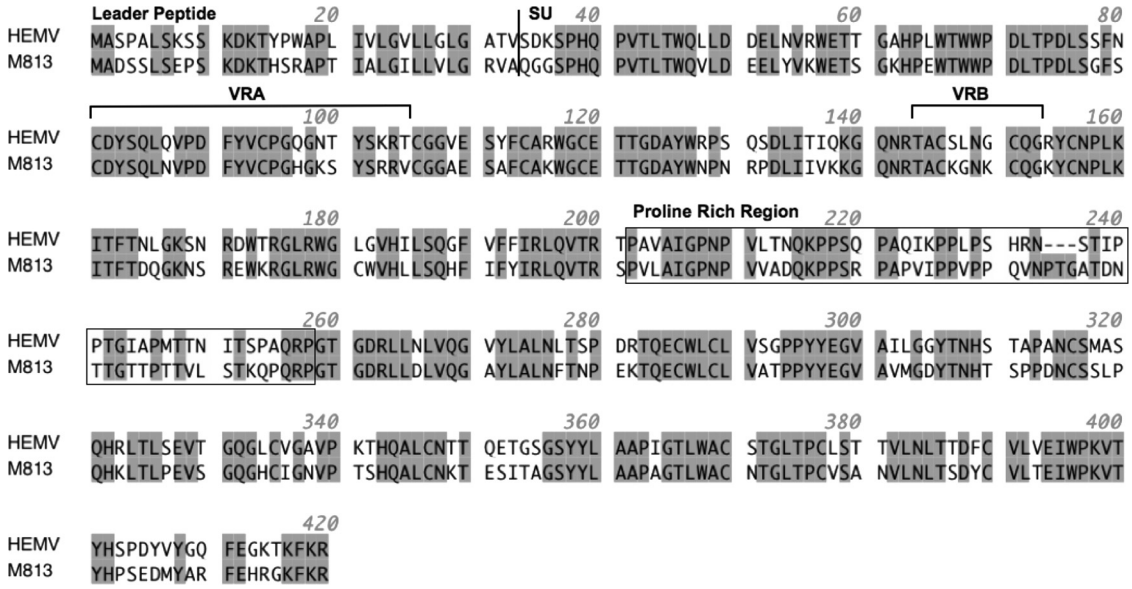

FIG 1 Relationship between HEMV and M813 Env proteins. A comparison of the SU regions of HEMV and M813 Env proteins is shown. The leader peptide, VRA and VRB domains, and the proline-rich region are indicated; the sequence represents the region upstream of the furin cleavage site.

As previously reported, both HEMV and M813 were able to infect all mouse cell lines tested, all derived from several different *Mus* species, whereas nonmurine cells, including human, simian, feline, canine, avian, and hamster cells, were not infected by either virus (Table 1) (16, 20). In contrast to published data, we found M813 to be incapable of infecting rat cells, despite using at least one cell line identical to a cell line used in the previous studies (Rat1 cells; Table 1). Even though the published infection efficiency was very low in this species, it was within the sensitivity of the described assay as set by infection levels of truly resistant target cells. The reason for this discrepancy is unknown, but we believe it unlikely to be due to low M813 titers, since an infection with several orders of magnitude less efficiency would still yield detectable titers. 10A1 pseudotypes were able to infect all cell lines tested, with the exception of CHO-K1, while those with the ecotropic (Moloney) MLV Env were restricted to mouse and some rat cell lines, as expected (Table 1).

The similarities in the SU regions of the *env* proteins of HEMV and M813, as well as the identical host ranges seen in our experiments, prompted us to use interference assays to test whether the two viruses were members of the same subgroup; i.e., used the same receptor for infection. HEMV and M813 *env* genes were cloned into pNCS (3) to generate replication-competent recombinant viruses with the specified Env proteins. NIH 3T3 cells chronically infected with these viruses, as well as viruses with a variety of other receptor specificities, were then challenged with the $LacZ^+$ $Puro^R$ HEMV, M813, 10A1, or ecotropic pseudotyped virions. M813 exhibited the same interference pattern as reported in the literature (16), completely interfering with itself. No significant drop in titers of either M813 or HEMV was observed when the cells were preinfected with members of other MLV subgroups, including ecotropic, polytropic, amphotropic, and 10A1 MLVs (Table 2), implying that both M813 and HEMV use a receptor different from that used by any of the tested subgroups. When NIH 3T3 cells were infected with replication-competent M813, HEMV was completely blocked from infection. Likewise, chronic infection of NIH 3T3 cells with HEMV abolished M813 infection (Table 2). This type of reciprocal interference is often seen with viruses that use the same domain(s) of a common entry receptor. Prior infection with HEMV or M813 did not have any effect on infection by 10A1 or ecotropic virus, suggesting that they use a separate receptor for entry.

The reciprocal interference between HEMV and M813 implied usage of a common entry receptor. To test this possibility directly, cDNA of the mSMIT1 gene, the entry receptor for M813, was amplified from an NIH 3T3 cDNA library (11) and cloned into pLPCX retroviral expression vector (Clontech) to create pLPCX-mSMIT. mSMIT-transducing virus was made by cotransfecting 293T cells with pLPCX-mSMIT, pMLVgagpol, and a vesicular stomatitis virus G (VSV-G) expression vector. Canine Cf2 Th cells were then infected either with mSMIT-transducing virus or with virus made with "empty" pLPCX and selected with puromycin (10 μg/ml). Puromycin-resistant colonies were expanded and challenged by the use of GFP-expressing MLV pseudotypes, and the titers were determined to give matched infectious units on NIH 3T3 cells. Infection was scored by flow cytometry. The expression of mSMIT1 rendered nonpermissive Cf2 Th cells susceptible to infection by both HEMV and M813 (Table 3), confirming mSMIT1 to be a receptor for HEMV. Moloney MLV (MMLV), capable of infecting only murine cells, remained unable to infect Cf2 Th cells, whereas 10A1-MLV, which has a broad host range, was able to infect canine cells regardless of mSMIT1 expression. Infectious units were reduced for M813 due to rapid inactivation of this pseudotype over the course of the experiment (data not shown).

The possibility that HEMV and M813 might use the same receptor was suggested by the similarity and phylogenetic relationship of their SU proteins, relative to other gammaretroviruses, and by the unusually short receptor-binding regions (VRA and VRB) shared by these viruses. They also have a common host range: unlike other MLVs, they infect only cells from *Mus* species. Although only partial sequences are available for the M813 genome

TABLE 1 HEMV and M813 host ranges are restricted to mouse cells

Cell line	Titer (IU/ml) of viral pseudotype[a]				Description
	HEMV	M813	MoMLV	10A1	
Murine					
NIH 3T3	3.2×10^5	1.5×10^5	3.6×10^5	5.1×10^5	NIH—Swiss embryonic fibroblasts
MMK	7.0×10^5	1.2×10^5	4.8×10^5	3.2×10^5	*Mus musculus molossinus* kidney cells
SC1	1.7×10^5	1.9×10^5	3.0×10^5	2.3×10^5	Feral mouse embryonic cells
M. dunni	1.4×10^6	3.4×10^5	2.0×10^5	4.8×10^5	*Mus dunni* tail fibroblasts
M. spicilegus	1.7×10^5	4.1×10^4	1.7×10^5	1.3×10^5	*Mus spicilegus* tail fibroblasts
MC3T3	2.1×10^5	7.3×10^4	2.9×10^5	2.7×10^5	*Mus musculus* calvaria osteoblasts
Rat					
Rat1	<1	<1	8.5×10^4	2.8×10^5	Rat embryonic fibroblasts
REF	<1	<1	1.9×10^2	7.2×10^5	Rat embryonic fibroblasts
Hamster					
E36	<1	<1	<1	2.8×10^5	Chinese hamster lung cells
CHO-K1	<1	<1	<1	<1	Chinese hamster ovary cells
Primate					
COS1	<1	<1	<1	8.7×10^4	African green monkey kidney fibroblasts
293T	<1	<1	<1	4.4×10^5	Human kidney epithelial cells
293TmCAT1	<1	<1	4.0×10^5	2.5×10^5	293T cells expressing mCAT1 receptor
Avian					
DF1	<1	<1	<1	2.4×10^5	Chicken fibroblasts
QT6	<1	<1	<1	1.7×10^5	Quail fibrosarcoma cells
Feline					
FEF	<1	<1	<1	3.4×10^5	Feline embryonic fibroblasts
Canine					
D17	<1	<1	<1	2.3×10^5	Canine osteosarcoma cells

[a] The MLV-based pLacPuro vector construct was cotransfected with clones expressing the indicated *env* genes. Titers were determined by X-Gal staining. The standard deviations for titers on the order of 10^5 ranged between 1.25×10^5 and 3.76×10^5, those on the order of 10^4 ranged between 2.40×10^4 and 7.42×10^4, and those on the order of 10^3 ranged between 2.19×10^3 and 4.81×10^3. The data represent the averages of the results of at least two independent experiments.

(limited to *env* and portions of *pol* and the LTR), available evidence suggests that the two viruses are not closely related outside SU. Unlike SU, the TM region is relatively well conserved among gammaretroviruses and has been previously used to establish viral relationships, most often congruent with reverse transcriptase (RT) (1). Phylogenetic analysis places the M813 TM well away from that HEMV, after the branching of feline leukemia virus (FeLV) (20). The LTRs of the two viruses share about 77% sequence identity.

TABLE 2 Receptor interference between MLV Env proteins

Preinfecting virus	Relative titer of challenge virus[a]			
	HEMV	M813	Ecotropic	10A1
None	1	1	1	1
HEMV	$<3.1 \times 10^{-6}$	$<6.5 \times 10^{-6}$	0.42	1.51
M813	$<3.1 \times 10^{-6}$	$<6.5 \times 10^{-6}$	2.03	0.13
Ecotropic	0.54	0.17	$<2.6 \times 10^{-6}$	0.73
Amphotropic	0.50	0.98	0.64	0.84
Polytropic	0.15	0.26	0.20	0.13
10A1	1.08	0.24	0.32	$<1.96 \times 10^{-4}$

[a] Data were calculated as the ratio of titers on preinfected cells and on uninfected cells. The data represent the averages of the results of at least two independent experiments. The variation between experiments was no more than 64% of the mean value in each case.

Three major hypotheses as to how HEMV and M813 came to belong to the same subgroup despite the obvious divergence of most of their genomes present themselves. The first is that HEMV and M813 share an ancestor that, although distantly related, used an ancestral form of mSMIT1 and that their divergence reflects evolution as an exogenous virus infecting different host species in different parts of the world while preserving the use of the same receptor. The second hypothesis is that the two viruses independently arrived in this subgroup through convergent evolution, the two SU proteins being the result of jumping similar evolutionary hurdles. The third possibility is that the two viruses evolved separately from a distant gammaretrovirus ancestor and acquired the same receptor-binding domain by a relatively recent recombination event. It is worth noting that the discordance between the SU and TM regions suggests the possibility of at least one recombination event in the evolutionary history of these viruses. Such events are well documented to alter the host range of MLVs, such as the 10A1 virus (8), as well as being found to do so during replication of endogenous ecotropic viruses in some strains of mice (2, 9). In this case, recombination would require that all elements of the final virus be present either as exogenous or endogenous viruses in a single host. Given the rather different geographic ranges of *M. spicilegus* and *M. cervicolor* in eastern Europe and southern Asia, where these two viruses were isolated, it is likely that other, intermediate viruses exist. Identifying *pol* and TM regions intermediate to

TABLE 3 HEMV and M813 use mSMIT1 as a receptor

Target cell	% (±SD) GFP-positive cells[a]				
	HEMV	M813	Ecotropic	10A1	No envelope
NIH 3T3	75.3 ± 1.3	7.3 ± 0.8	72.5 ± 0.3	70.1 ± 1.4	0.9 ± 0.3
Cf2 Th LPCX ("empty")	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	65.0 ± 3.1	0.9 ± 0.1
Cf2 Th LPCX-mSMIT	45.4 ± 2.7	12.2 ± 1.7	0.1 ± 0.1	69.1 ± 2.7	0.9 ± 0.1

[a] Data represent percent infection of 10,000 gated viable cells of 1×10^5 cells infected, the threshold was set at 1.0% fluorescein isothiocyanate (FITC) in uninfected cells. The data represent the averages of the results of three independent experiments.

those positions might prove fruitful with respect to understanding this relationship.

Although it is common for distantly related viruses to retain the use of cognate receptors, for instance, in the case of GaLV and FeLV-B (19), and although incremental changes in receptor usage are achievable both *in vivo* and *in vitro*, as seen in coreceptor usage by HIV-1 and HIV-2, understanding how a true subgroup switch occurs remains elusive. The establishment of HEMV and M813 as members of the same subgroup may have important implications for the evolution and spread of gammaretroviruses. Their relationship represents an experimentally tractable model for a subgroup's origin and evolutionary history. Investigating how the two viruses came to be members of the same subgroup depends upon further exploration of wild mouse populations, the examination of carrier species, and, finally, the possible identification of more members of this subgroup.

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