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Molecular Cloning of *Mus dunni* Endogenous Virus: an Unusual Retrovirus in a New Murine Viral Interference Group with a Wide Host Range

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***Mus dunni* endogenous virus (MDEV) is activated from cells of the Asian wild mouse *M. dunni* (also known as *Mus terricolor*) in response to treatment with either 5-iodo-2′-deoxyuridine or hydrocortisone. MDEV represents a new murine retrovirus interference group and thus appears to use a different receptor for entry into cells than do other murine retroviruses. Here we show that MDEV is also not in the gibbon ape leukemia virus or RD114 virus interference groups. A retroviral vector with an MDEV pseudotype was capable of efficiently infecting a wide variety of cells from different species, indicating that the MDEV receptor is widely expressed. We isolated a molecular clone of this virus which exhibited no hybridization to any cloned retrovirus examined, suggesting that MDEV has an unusual genome. One copy of a possible retrovirus element that weakly hybridized with MDEV was present in the genomes of laboratory strains of mice, while no such elements were present in other species examined. A virus activated by 5-iodo-2′-deoxyuridine from cells of a BALB/c mouse, however, was not related to MDEV by either hybridization or interference analyses.**

Many endogenous retroviruses have been identified in both laboratory and wild mice (6, 15, 48). Much of the interest in these murine viruses has been due to their association with the development of leukemia in laboratory strains of mice (9, 48, 55) and the transformation of cells derived from these mice in vitro (5, 58). Although there are numerous copies of endogenous retroviruses in wild mice, they are rarely associated with disease, possibly because their expression is more strongly repressed than in inbred laboratory strains (14, 15). In addition, the study of endogenous retrovirus-related sequences in wild mice has indicated that some of these elements play a role in preventing infection by exogenous viruses and consequently in providing resistance to virus-induced disease (15, 16, 24, 29).

In addition to their involvement in disease, endogenous viruses have evolutionary importance. After initial infection of germ line tissue, a retrovirus becomes part of the normal genetic complement of a mouse and is transmitted vertically. By examining the retroviral sequences in the genomes of various strains of mice, it is possible to gain insight into the evolutionary relationships among mice as well as among viruses (3, 6, 53).

Many endogenous viruses, from both laboratory and wild mice, have been identified after their activation following exposure of cells to halogenated pyrimidines (1, 3, 27, 44, 49, 58) as well as after spontaneous activation during cell culture (5, 50). These viruses have been characterized by various combinations of analyses including particle morphology, host range, neutralization, interference, hybridization, and nucleotide sequence. Five classes of endogenous viruses have been identified in the genomes of laboratory mice: ecotropic, xenotropic, polytropic, modified polytropic, and mouse mammary tumor viruses (MMTV) (9, 21, 42, 48, 55, 56). In wild mice, envelope gene sequences related to the ecotropic, xenotropic, and polytropic classes are present in the germ lines (16, 25), although only an endogenous ecotropic virus has been induced in a replication-competent form and characterized (8, 54). Although some evidence indicated the presence of endogenous amphotropic viruses in wild mice (18, 46), these were later shown to be absent from the germ line and to have spread as exogenous viruses in one particular population of mice (41). In addition to these classes of virus, four groups from the genomes of Southeast Asian species of *Mus* have been activated and classified as C-I, C-II, M432, and MMTV related (3, 5, 6, 27, 38).

We have recently described the isolation of a novel retrovirus, *Mus dunni* endogenous virus (MDEV), which was activated from the cells of this Asian wild mouse during testing of human cells for replication-competent virus in human gene transfer trials (33). MDEV does not appear to match the descriptions of previously characterized viruses. Here we describe the molecular cloning of MDEV, its characterization with respect to host range, viral interference, and relationship by nucleic acid hybridization to other retroviruses.

MATERIALS AND METHODS

Nomenclature. Cells that contain a retrovirus or a retroviral vector are indicated by the cell name followed by a slash and the name of the virus, e.g., *M. dunni*/AM-MLV (for *M. dunni* cells containing amphotropic murine leukemia virus). The pseudotype of a retroviral vector refers here to the viral envelope used to make vector virions. The virus or packaging cells used to produce a retroviral vector are denoted in parentheses after the vector name, e.g., LAPSN(MDEV).

Cell culture. The following cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g of glucose per liter and 10% fetal bovine serum (FBS): *M. dunni* tail fibroblasts (a gift from Bruce Chesebro, originally described in reference 7), PA317 amphotropic retrovirus packaging cells (32), PG13 gibbon ape leukemia virus (GALV)-based packaging cells (35), NIH 3T3 thymidine kinase-negative cells (32), C2C12 cells (ATCC CRL 1772), HeLa cells (ATCC CCL 2), D17 cells (ATCC CCL 183), CCC-81 feline cells transformed with Moloney murine sarcoma virus (13), 5637 human bladder carcinoma cells (ATCC HTB 9), IB3 cells (61), HT1080 cells (ATCC CCL 121), NRK cells (11), and BALB/c 3T3 (B77/OTG) ouabain-resistant HPRT$^-$ cells transformed by Rous sarcoma virus (a gift from H. L. Ozer). Primary human dermal fibroblasts (HDF) (17) were grown in DMEM with 15% FBS. G355 feline embryonic brain cells and G355 cells infected with RD114 endogenous cat virus (12) were grown in McCoy's medium with 15% FBS. CHO-K1 cells (ATCC CCL

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61) were grown in α minimal essential medium (αMEM) with 10% FBS. HUT 78 cells (ATCC TIB 161) were grown in RPMI 1640 plus 10% FBS. QT35 cells (39) were grown in Ham's F10 medium with 10% tryptose phosphate broth, 1% chicken serum, 1% dimethyl sulfoxide, 5% FBS, and 2% sodium bicarbonate (7.5% solution).

Viruses. The LAPSN retroviral vector (37) encodes human placental alkaline phosphatase (AP) and neomycin phosphotransferase. LAPSN infection was scored by histochemical staining for AP^+ foci of cells 2 days after infection as described previously (34). The replication-competent viruses used in this study included ecotropic Moloney murine leukemia virus (MoMLV) (pMLV-K [36]), amphotropic virus (AM-MLV [32]), NZB xenotropic virus (40), polytropic mink cell focus-forming virus strain 98D (10), 10A1 (43), GALV SEATO strain (60), RD114 (47), and MDEV (33). Additional viral elements used in proviral DNA form in hybridization studies included MMTV (51), GALV envelope (60), and human endogenous retroviral elements HERV-K (28) and HERV-H (59). D17 cells infected with spleen necrosis virus (SNV) or Mason-Pfizer monkey virus (MPMV) were obtained from Vineet Kewalramani (Fred Hutchinson Cancer Research Center). SNV-infected D17 cells were made by infection of D17 cells with SNV followed by cultivation of the cells to allow virus spread to all cells. MPMV-infected D17 cells were made by cocultivation of D17 cells with MPMV-producing human CEM cells to compensate for the poor infectivity of MPMV for D17 cells. SNV-pseudotype LAPSN vector was made by infecting D17/SNV cells with helper-free amphotropic LAPSN vector produced from PA317 cells, selecting the cells in G418, growing the G418-resistant cells in the absence of G418, and harvesting virus from confluent dishes of cells about 16 h after a medium change. RD114-pseudotype LAPSN vector was made in a similar manner following infection of G355/RD114 cells with the helper-free LAPSN vector.

Isolation of viral RNA and cDNA synthesis. The source of MDEV virus used for the preparation of viral RNA and for host range studies was a clone of G355/LAPSN+MDEV feline cells selected for the production of high-titer LAPSN (10^6 FFU/ml on *M. dunni* cells), called GL8c16 cells. On day 1, GL8c16 cells were seeded at 10^6 cells/10-cm plate. On day 3, cells were fed with fresh medium, and virus-containing medium was harvested 24 h later. This process was repeated on days 4 and 5. Harvested medium was filtered through 0.45-μm-pore-size bottletop filters (Nalgene) and, if not used immediately, was stored at −70°C. Filtered virus-containing medium (approximately 200 ml) was layered on top of 20% sucrose (in phosphate-buffered saline at 4 ml per tube) in six 30-ml Beckman SW28 ultracentrifuge tubes. Virus particles were pelleted at 26,000 rpm in a Beckman SW28 swinging-bucket rotor for 2 h at 4°C. After removal of the supernatant, the pellets were resuspended in a total of approximately 500 μl of ice-cold TNE buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA). Viral RNA was extracted from the pellets as described previously (30) and precipitated at 70°C overnight. RNA was pelleted in an Eppendorf microcentrifuge at 4°C for 30 min and resuspended in 1 ml of distilled water. Polyadenylated RNA was selected by using the mini-oligo(dT) cellulose spin column kit (5 Prime-3 Prime) as specified by the manufacturer. cDNA was reverse transcribed in vitro from viral RNA by using the polyadenylated RNA isolated from 400 ml of virus-containing medium per 50-μl reaction mixture. Reaction conditions were similar to those described previously (2). The reaction mixture contained viral RNA in 10 μl of distilled water, 50 mM Tris (pH 8.1), 10 mM dithiothreitol, 50 mM sodium chloride, 3 mM magnesium acetate, 0.6 mM magnesium chloride, 1 mM each dGTP, dATP, and dTTP, 100 μCi [α-^{32}P]dCTP (800 Ci/mmol), 500 μg of oligo(dT) primers, 0.5 U of Promega RNasin per μl, and 70 U of MoMLV reverse transcriptase (Stratagene). The reaction mixture was incubated at 37°C for 1 h, and cDNAs were separated from free nucleotides on a Sephadex G-50 column. This probe was then tested by Northern blot analysis for specific hybridization to MDEV transcripts and not to RNA from uninfected cells or to RNA from cells infected with amphotropic virus (data not shown). This probe was then used to screen the viral DNA library described below.

Isolation and cloning of viral DNA. On day 1, *M. dunni* cells were seeded at 2×10^6 cells per 14-cm-diameter dish in 60 dishes. On day 2, the cells were infected with MDEV plus LAPSN virus produced from GL8c16 cells (at a multiplicity of infection of 2.5 based on the LAPSN vector) with 4 μg of Polybrene per ml. At 24 h after infection, extrachromosomal DNA was isolated by the method of Hirt (19). Supercoiled DNA was further purified from contaminating chromosomal DNA by three serial centrifugations in cesium chloride (CsCl) containing ethidium bromide, as described previously (45). DNA was purified from the CsCl by multiple sodium acetate-ethanol precipitations and washed with 70% ethanol. Aliquots of the viral DNA were digested with a panel of restriction enzymes, electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane (Hybond), and analyzed by Southern blotting with the MDEV-LAPSN cDNA (described above) as a probe to determine which enzymes would be useful in cloning the MDEV DNA. Based on the results, the supercoiled DNA was digested with *Eco*RI and cloned into the Bluescript plasmid (Stratagene), generating a library with a complexity of 10^5. The library was screened by standard procedures, and positive clones were confirmed by Southern blot analysis (31).

Endogenous virus activation. BALB/c 3T3, NIH 3T3, or C2C12 cells were seeded in six-well plates at 4×10^5 cells per 3-cm-diameter well on day 1. On day 2, duplicate wells were treated with culture medium saturated with 5-iodo-2′-deoxyuridine (IdU), medium containing 90 μM hydrocortisone 21-phosphate (Sigma), or medium alone for 24 h. On day 3, the cells were trypsinized and cocultivated with D17/LAPSN cells to allow for replication of viruses that might be unable to replicate in the mouse cells and to allow detection of replication-competent retroviruses by measurement of LAPSN vector production. At weekly intervals following IdU treatment, medium exposed to the cell mixtures for 24 h was collected, filtered (0.45 μm pore size), and tested for the presence of LAPSN on *M. dunni* cells.

TABLE 1. Interference properties of MDEV, GALV, AM-MLV, 10A1, and RD114 measured in human diploid fibroblasts[a]

Interfering virus	LAPSN titer (FFU/ml) for pseudotype:			
	MDEV	PG13[b]	PA317[c]	RD114
None	3×10^4	3×10^5	9×10^4	5×10^5
MDEV	200	5×10^5	6×10^4	5×10^5
GALV	2×10^4	200	4×10^4	2×10^5
10A1	5×10^4	9×10^4	1	2×10^5
RD114	4×10^4	8×10^4	1×10^5	2×10^3

[a] Uninfected HDF cells and HDF cells infected with the indicated interfering viruses were exposed to LAPSN vectors with the indicated pseudotypes and were stained for AP^+ foci of cells 2 days after infection. Values are means of duplicate assays in a single experiment which varied by no more than 33% from the mean (except for the value 1, which represents the mean of 0 and 2 FFU/ml). The experiment was repeated with similar results.

[b] PG13 refers to a packaging cell line which produces GALV-pseudotype virus.

[c] PA317 refers to a packaging cell line which produces amphotropic-pseudotype virus.

RESULTS

MDEV is not in the GALV or RD114 retrovirus interference groups. We have previously shown by interference analysis that MDEV uses a different receptor for entry than those used by other murine leukemia viruses (33). Here we have tested for interference between MDEV and viruses from two additional groups, the GALV and RD114 cat endogenous virus groups. These viruses were chosen because members of the GALV interference group have been found to interfere with an endogenous retrovirus from the Asian mouse *M. caroli* (6, 27), and we have previously observed some interference between RD114 and MDEV in cat cells (33). Interference analysis in primary HDF (Table 1) showed that cells infected with MDEV inhibited transduction by MDEV-pseudotype LAPSN vector by about 100-fold but did not inhibit transduction by LAPSN with either a GALV, amphotropic, or RD114 pseudotype. Similarly, prior infection of the cells with GALV, 10A1, or RD114 virus inhibited entry by LAPSN virus with GALV, amphotropic, or RD114 pseudotypes, respectively, by 100- to almost 100,000-fold but had no effect on transduction by LAPSN virus with a different pseudotype. Note that 10A1 virus is essentially an amphotropic virus in these human cells (34). Thus, MDEV, GALV, 10A1 and amphotropic virus, and RD114 virus are in different interference groups when assayed in human cells, indicating that these virus groups use different receptors for cell entry.

Table 2 shows the results of additional interference assays performed in D17 canine cells. Prior infection of D17 cells with RD114, SNV, or MPMV (also called SRV-3) did not interfere with transduction by an MDEV-pseudotype LAPSN vector, but all three viruses interfered with transduction by LAPSN with an RD114 or an SNV pseudotype. These results are consistent with previous findings that RD114, SNV, and MPMV are in the same interference group when assayed in canine and human cells (22, 23, 52), and they show that MDEV is in a different interference group when assayed in canine cells. It was not possible to examine transduction by MPMV-pseudotype LAPSN vector in this experiment because this type D

TABLE 2. RD114, SNV, and MPMV do not interfere with transduction by a vector with an MDEV pseudotype in D17 dog cells[a]

Interfering virus	LAPSN titer (FFU/ml) for pseudotype:		
	RD114	SNV	MDEV
None	3×10^5	5×10^3	4×10^4
RD114	20	20	2×10^4
SNV	6×10^4	4	2×10^4
MPMV	2×10^4	20	2×10^4

[a] Uninfected D17 cells and D17 cells infected with the indicated interfering viruses were exposed to LAPSN vectors with the indicated pseudotypes and were stained for AP^+ foci of cells 2 days after infection. The results are means of at least two independent determinations, which varied by no more than 60% from the mean.

virus did not package the MoMLV-based LAPSN vector (reference 57 and data not shown).

Because of our prior finding that MDEV-pseudotype vectors appeared to infect feline CCC-81 cells poorly and because feline cells contain and could potentially express the cat endogenous virus RD114, we also tested for interference between MDEV and RD114 in G355 feline embryonic cells that are permissive for MDEV infection. In this case, prior infection of the G355 cells with RD114 inhibited transduction by the MDEV-pseudotype LAPSN vector by 5- to 500-fold (Table 3 and data not shown). We do not know the reason for this target cell-dependent interference pattern or for the high variability in interference between RD114 and MDEV observed in feline cells. Perhaps one receptor on cat cells is primarily used by both MDEV and RD114, but additional receptors on other cells can serve as independent receptors for the two viruses.

These results show that MDEV is in a different interference group from those of GALV, amphotropic virus, 10A1, and RD114, although there appears to be some overlap for receptor utilization with the RD114 group. These findings extend our previous results showing MDEV does not belong to the ecotropic, xenotropic, amphotropic, 10A1, or polytropic interference groups (33).

MDEV has a wide host range. MDEV-pseudotype LAPSN virus was able to infect a variety of cell types from all species tested, including mouse, rat, hamster, quail, cat, dog, and human cells (Table 4). The titer of LAPSN(MDEV) was between 1×10^5 and 5×10^6 on all cells tested, except for BALB/c 3T3 cells, for which the titer was only 200 FFU/ml. No phenotypic changes were observed in cells infected with MDEV, except in experiments with the HUT 78 human T-cell line, in which an increase in doubling time and very large multinucleated cells were observed. The ability of MDEV-pseudotype vector to infect different cell types from multiple species indicates that the receptor used by this virus is widely distributed.

TABLE 3. RD114 interferes with transduction by a vector with an MDEV pseudotype in G355 cat cells[a]

Interfering virus	LAPSN titer (FFU/ml) for pseudotype:		
	RD114	MDEV	PA317
None	2×10^5	2×10^3	6×10^5
RD114	3	90	5×10^5

[a] Uninfected or RD114-infected G355 cat cells were exposed to LAPSN vectors with the indicated pseudotypes and were stained for AP^+ foci of cells 2 days after infection. The results are means of at least two independent determinations, which varied by no more than 67% from the mean, except for the LAPSN(MDEV) infection of G355/RD114 cells, where the LAPSN titer varied from <2 to 300 FFU/ml ($n = 6$, mean = 90 FFU/ml).

TABLE 4. Host range of MDEV[a]

Species	Cell type	LAPSN (MDEV) titer (FFU/ml)
Mouse (wild)	*M. dunni*	4×10^5
Mouse (laboratory)	NIH 3T3	6×10^5
	BALB/c 3T3	200
	C2C12	1×10^5
Rat	NRK	2×10^5
Hamster	CHO-K1	5×10^6
Quail	QT35	8×10^5
Cat	CCC-81	5×10^5
	G355	4×10^6
Dog	D17	2×10^6
Human	HT1080	5×10^6
	IB3	4×10^6
	5637	5×10^5
	HDF	9×10^5
	HeLa[b]	5×10^5

[a] The indicated cells were exposed to MDEV-pseudotype LAPSN and were stained for AP^+ foci of cells 2 days after infection. Values are means of duplicate assays in a single experiment which varied by no more than 25% from the mean. The experiment was repeated with similar results.

[b] The LAPSN(MDEV) titer on HeLa cells was measured by production of G418-resistant colonies (CFU per milliliter) rather than AP^+ foci, because HeLa cells have very high levels of endogenous AP.

A virus produced from laboratory mouse-derived cell lines by activation with IdU is distinct from MDEV. There are several reports of activation of endogenous retroviruses in cells from laboratory strains of mice, both spontaneously and after exposure to chemicals such as IdU (6, 48). It was of interest to determine whether these viruses were similar to MDEV. We therefore exposed NIH 3T3, C2C12, and BALB/c 3T3 laboratory mouse cells to either IdU or hydrocortisone. The exposed cells were then cocultivated with D17/LAPSN cells to allow for replication of viruses that might be unable to replicate in the mouse cells and to allow the detection of replication-competent retroviruses by measurement of LAPSN vector production. Beginning at 1 week, duplicate wells of BALB/c 3T3 cells treated with IdU were found to be positive for production of LAPSN. The replication-competent virus that was activated is referred to below as BALB/c 3T3 IdU-induced retrovirus (BIRV). Duplicate wells of BALB/c 3T3 cells which were treated with hydrocortisone or were untreated remained negative for the production of virus for the 7-week duration of the experiment. In addition, we were unable to detect replication-competent virus after identical treatment of either NIH 3T3 or C2C12 cells.

Interference assay results showed that MDEV does not interfere with transduction by LAPSN(BIRV) but that both polytropic and xenotropic viruses do interfere with LAPSN (BIRV) (Table 5). These results indicate that BIRV is not in the same interference group as MDEV but is a member of either the polytropic or xenotropic group of viruses, which are closely related and have been shown to interfere with each other in some cell types (10). The strains of polytropic and xenotropic virus used here show some degree of nonreciprocal

TABLE 5. Endogenous virus induced from BALB/c 3T3 cells uses a different receptor than MDEV in *M. dunni* cells[a]

Interfering virus	LAPSN titer (FFU/ml) for pseudotype:			
	MDEV	BIRV	Polytropic	Xenotropic
None	6×10^5	8×10^4	1×10^5	4×10^6
MDEV	3	6×10^4	ND[b]	ND
Polytropic	4×10^5	400	<1	1×10^5
Xenotropic	4×10^5	<2	1	6
Amphotropic	4×10^5	5×10^4	ND	ND

[a] Uninfected *M. dunni* cells and *M. dunni* cells infected with the indicated interfering viruses were exposed to LAPSN vectors with the indicated pseudotypes and were stained for AP^+ foci of cells 2 days after infection. Values are means of duplicate assays in a single experiment which varied by no more than 17% from the mean (except for the value 3, which was the mean of 6 and 0 FFU/ml). The experiment was repeated with similar results.

[b] ND, not determined.

interference in *M. dunni* cells, in that xenotropic virus strongly inhibits polytropic vector transduction (10^5-fold) whereas polytropic virus much less strongly inhibits transduction by xenotropic virus (~40-fold). BIRV is capable of infecting *M. dunni*, NIH 3T3, G355, and primary human fibroblasts (Table 6), indicating that BIRV is a polytropic virus, since xenotropic viruses do not infect cells from laboratory mice.

Isolation of a molecular clone of MDEV. To isolate a clone of MDEV, we initially used a strategy involving the use of radiolabeled DNA from a variety of cloned retroviruses as potential probes for the detection of plasmids containing MDEV sequences. However, probes made with ecotropic MoMLV, NZB xenotropic virus, GALV, 98D polytropic virus, MMTV, AM-MLV, RD114, and two species of human endogenous retroviral elements (HERV-K and HERV-H) failed to hybridize to MDEV on Northern blots of RNA extracted from MDEV-producing *M. dunni* cells, even under low-stringency conditions, and were therefore not useful as probes with which to clone the virus (data not shown).

A second strategy, which was successful in cloning MDEV, involved the construction of a plasmid library from unintegrated viral DNA and screening of the library for clones containing MDEV sequences by using a probe derived from viral RNA. Extrachromosomal DNA was harvested from *M. dunni* cells 24 h after infection with a mixture of MDEV and LAPSN virus, and supercoiled DNA, predicted to contain circular LAPSN and MDEV reverse transcription products with one and two long terminal repeats (LTRs), was purified by serial centrifugation in CsCl gradients containing ethidium bromide. The presence of the LAPSN vector provided a positive control for the cloning procedure. Aliquots of the purified DNA were digested with a panel of restriction enzymes, electrophoresed, and hybridized with radiolabeled cDNA made from RNA extracted from virus produced from G355 cat cells infected with MDEV and LAPSN. The viral RNA was isolated from virus produced from cat cells, rather than from *M. dunni* cells, to minimize the presence of sequences reactive with *M. dunni* DNA sequences that were expected to be present in the viral DNA library. Hybridization analysis revealed bands in addition to those predicted for one- and two-LTR-containing LAPSN species, and these were presumed to be MDEV (data not shown). Based on these results, a library was made with *Eco*RI-digested DNA, which appeared to cut once within MDEV. The MDEV-plus-LAPSN cDNA probe identified multiple clones of LAPSN and clones of a retrovirus presumed to be MDEV, containing both one and two LTRs, from the library. No positive clones other than LAPSN and the presumptive MDEV were identified.

TABLE 6. Host range of BIRV[a]

Species	Cell type	LAPSN(BIRV) titer (FFU/ml)
Mouse (wild)	*M. dunni*	4×10^4
Mouse (laboratory)	NIH 3T3	6×10^3
Cat	G355	5×10^4
Human	HDF	3×10^3

[a] The indicated cells were infected with BIRV-pseudotype LAPSN and were stained for AP^+ foci of cells 2 days after infection. Shown are the titers of a nonclonal population of cells producing LAPSN(BIRV). Similar results were obtained with a second, independently IdU-induced bulk population of cells producing BIRV.

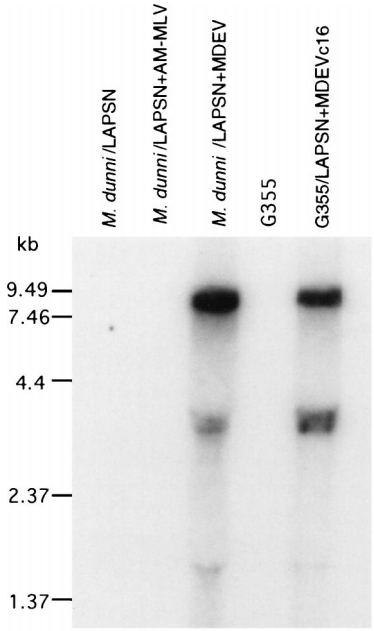

FIG. 1. pMDEV9 hybridizes to two viral RNA species in MDEV-infected cells. Total cytoplasmic RNA samples (10 μg per lane) were analyzed by Northern blot hybridization at high stringency (65°C, 0.2× SSC–0.1% SDS wash) with the 9.4-kbp insert from pMDEV9 as a probe. As negative controls for the specificity of the probe, the following RNA samples were included: RNA from *M. dunni* cells transduced with the LAPSN vector (lane 1), *M. dunni* cells infected with AM-MLV and LAPSN (lane 2), and uninfected G355 cells (lane 4). Cells producing MDEV included *M. dunni* cells infected with MDEV plus LAPSN vector (lane 3) and G355 cells infected with MDEV plus LAPSN vector (lane 5).

To test whether the retroviral sequence in one of the presumptive MDEV clones (pMDEV9) was related to the virus which was activated from the *M. dunni* cells, cytoplasmic RNA samples isolated from *M. dunni* and G355 cells producing MDEV were analyzed by Northern blotting by using the insert from pMDEV9 as a probe (Fig. 1). The probe hybridized to 8.6-kb genomic and 3.7-kb subgenomic RNAs in both cell types, demonstrating the specificity of the probe for the MDEV transcripts present in infected cells. The pMDEV9 probe did not hybridize to RNA from cells infected with AM-MLV plus LAPSN vector, indicating limited sequence similarity between MDEV and AM-MLV or LAPSN. The probe did not hybridize to RNA from uninfected G355 cells or to *M. dunni* cells transduced with the LAPSN vector, which do not produce MDEV, indicating that MDEV is not normally transcribed in unactivated *M. dunni* cells or uninfected G355 cells.

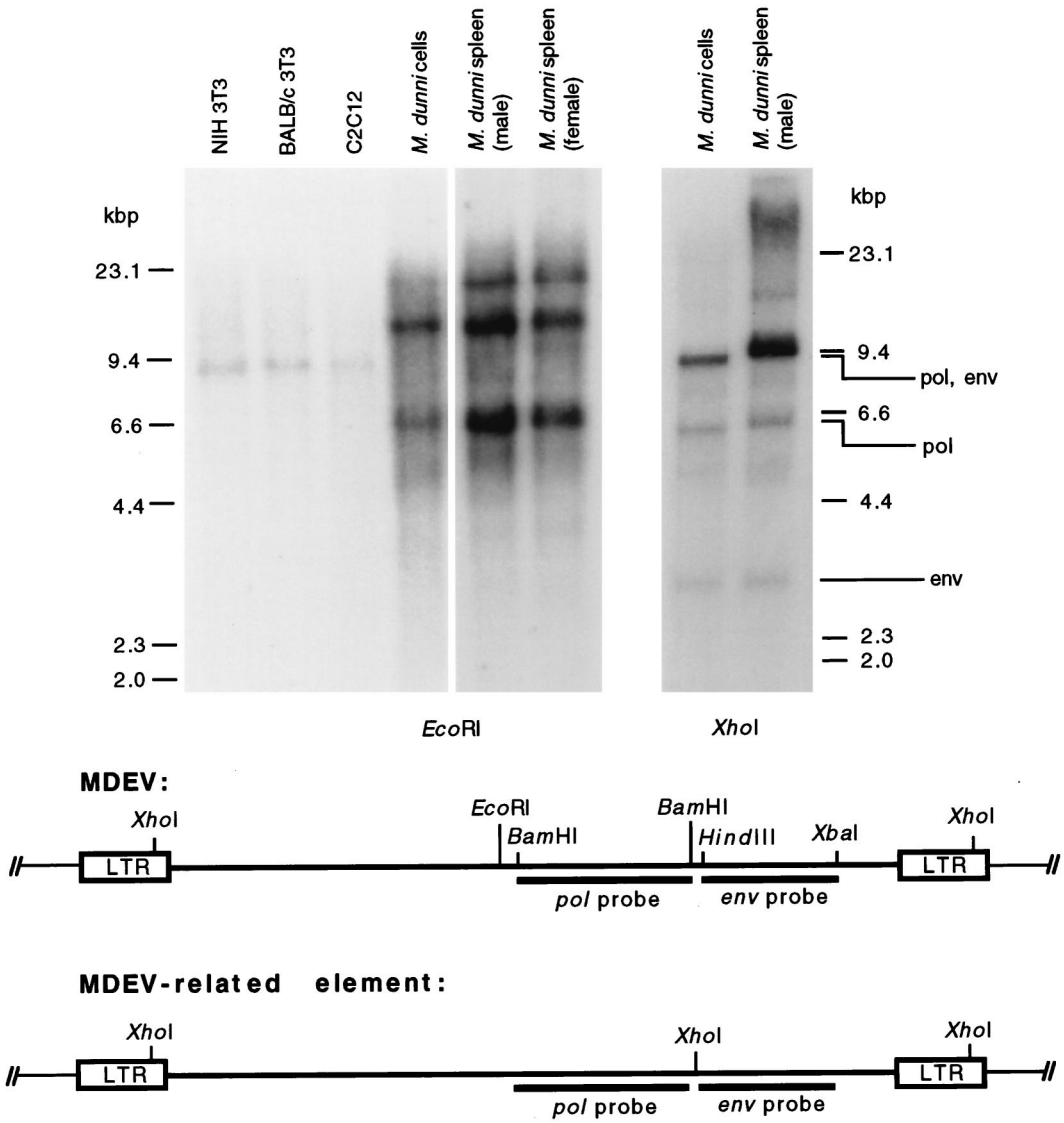

FIG. 2. MDEV is present in the *M. dunni* genome, and related sequences are present in cultured cells from laboratory strains of mice. Genomic DNA samples (10 μg per lane) were digested with *Eco*RI (which cuts once within MDEV [left panels]) or *Xho*I (which cuts once within each LTR [right panel]) and were analyzed by Southern blot hybridization at high stringency (68°C, 0.1× SSC–0.1% SDS wash) with the 9.4-kbp insert from pMDEV9 as a probe. The first four lanes of the left panels represent a 2-day film exposure, while lanes 5 and 6 (containing mouse spleen DNA) were from the same blot but were overloaded, and so a 1-day film exposure is shown to compensate for the overloading. Shown below the autoradiographs are diagrams of the MDEV provirus and a putative second provirus that is related but not identical to MDEV. Additional probes for MDEV *pol* and *env* genes used in the analysis of the blots are indicated by bars on the provirus diagrams. Bands to which the *pol* and *env* probes hybridize are shown at the right of the *Xho*I Southern blot.

MDEV is endogenous to the *M. dunni* genome, and related sequences are present in the genomes of laboratory strains of mice. To determine whether MDEV was actually present in the germ line of the *M. dunni* mouse and not an acquired contaminant of the *M. dunni* cell line, we performed Southern analysis with the viral DNA insert from pMDEV9 as a probe to examine genomic DNA from the *M. dunni* cell line and from the spleens of two *M. dunni* mice. The genomic DNA samples were digested with *Eco*RI, which cuts once within the MDEV provirus, to allow detection of MDEV, its copy number, and whether its integration site(s) was the same in the various samples (Fig. 2). Two strongly hybridizing bands were visible in DNA from the *M. dunni* cells, and the same bands were present in DNA from both *M. dunni* mice, indicating that MDEV is present in the germ line of the *M. dunni* mouse at the same integration site as in the cell line. Given that the entire MDEV provirus was used as a probe, these two bands probably represent the two halves of one copy of MDEV. In addition, a less intense band was visible at approximately 23 kbp in the DNA of both mice and at still lower intensity in the DNA from the *M. dunni* cell line. This third band suggests the presence of a second element closely related to MDEV.

Given the possibility that the DNA band at 23 kbp was not transferred efficiently to the filter or that the band was the result of a partially digested DNA fragment, we attempted to further clarify the number of copies of elements closely related to MDEV by Southern blot analysis of *Xho*I-digested DNA (Fig. 2). There is one *Xho*I site in each LTR of the MDEV clone. Therefore, digestion of DNA containing a provirus with the same sites would produce a fragment of approximately 9

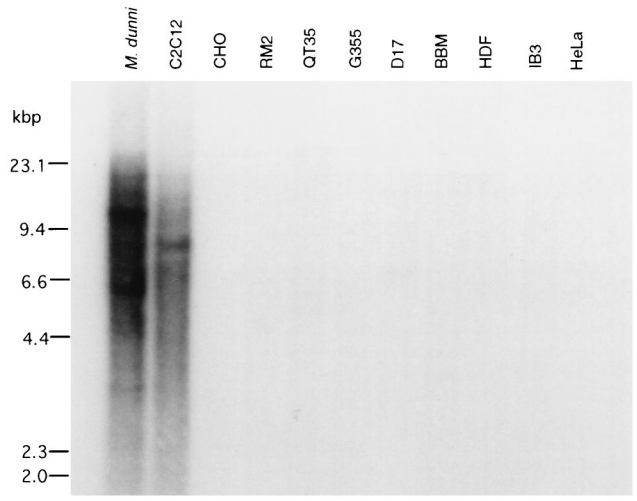

FIG. 3. Zoo blot analysis for the presence of MDEV-related sequences. Genomic DNA samples (10 μg per lane) were digested with *Eco*RI, which cuts once within MDEV, and were analyzed by Southern blot hybridization at low stringency (40°C, 2× SSC–0.1% SDS) with the 9.4-kbp insert from pMDEV9 as a probe. Cells not defined in Materials and Methods: RM2, primary rat myoblasts (4); BBM, primary baboon bone marrow (gift from J. Potter).

kbp. By using the entire MDEV provirus as a probe on this blot, a 9-kbp band was observed that coincides with that expected from MDEV in the *M. dunni* cells. *Xho*I-digested *M. dunni* DNA also produced DNA fragments of approximately 6 and 3 kbp, which hybridized less well to MDEV. Using MDEV *pol* and *env* DNA fragments as probes on this blot, we determined that the band at 6 kbp hybridized to MDEV *pol*-related sequences and the band at 3 kbp hybridized to MDEV *env*-related sequences, while the presumptive MDEV band at 9 kbp hybridized to both probes (indicated to the right of the autoradiograph in Fig. 2 [data not shown]). These data suggest that the 3- and 6-kbp bands may comprise a second, MDEV-related element with an *Xho*I site in each LTR and an additional, internal *Xho*I site. This element may also be responsible for the large, somewhat indistinct ~23-kbp band seen on the blot of *Eco*RI-digested DNA.

In addition to the two elements described above that hybridized with the MDEV probe, the high level of background hybridization in the lanes containing *M. dunni* DNA indicates that there are sequences weakly related to MDEV that are dispersed throughout the genome. In contrast, cell lines derived from laboratory strains of mice contain very little MDEV-related DNA (Fig. 2). Bands of identical sizes (approximately 9 kbp) and with the same relative intensities are visible in *Eco*RI-digested DNA from NIH 3T3, BALB/c 3T3, and C2C12 cells (derived from NIH Swiss, BALB/c and C3H mice, respectively). This result indicates that one copy of a retroviral element related to MDEV is present at the same site in these genomes, consistent with integration of the element into the genome prior to the divergence of these strains. The relatively faint hybridization of MDEV to these samples compared with *M. dunni* DNA indicates that the sequences are related but not identical to MDEV. In addition, no hybridization of the pMDEV9 probe was detected to DNA or RNA from D17 cells infected by BIRV, while a probe made from 98D polytropic virus did recognize BIRV sequences in these samples, indicating that the MDEV-related sequences present in the BALB/c 3T3 cells are not BIRV DNA (data not shown).

Sequences closely related to MDEV are present only in mice. We used the entire MDEV provirus as a probe on a Southern blot of genomic DNA from a variety of animals in addition to mice (Fig. 3). While MDEV was clearly present in *M. dunni* DNA, no hybridization to the genomic DNA of cells derived from hamsters, rats, quails, cats, dogs, baboons, or humans was detectable even under low-stringency conditions (40°C, 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate [SDS] wash). Sequences related to MDEV were seen only in the C2C12 mouse cells. These results further suggest the unusual content of the MDEV genome, as well as the rarity of this virus or viruses closely related to it. Thus, MDEV would not be useful as a taxonomic tool for examining the evolution of animals other than mice or the retroviral sequences in their germ lines.

DISCUSSION

MDEV is a replication-competent retrovirus endogenous to the Asian wild mouse *M. dunni*. The majority of endogenous murine viruses are found in many copies per genome. By Southern blot analysis, we found one copy of a provirus apparently identical to our MDEV clone in *M. dunni* DNA, another closely related provirus, and a background of other sequences with some similarity to MDEV. Following exposure of cultured *M. dunni* cells to certain chemicals, the normally quiescent MDEV provirus is transcriptionally activated, producing 8.6-kb (genomic) and 3.7-kb (subgenomic) viral RNA species. Once activated, MDEV can infect and replicate in *M. dunni* and many other cells without further chemical treatment, indicating that all of the genes required for virion production and virus replication are present and that the viral promoter can remain active. We have observed that some cell types fail to produce virus following MDEV infection but have not determined the nature of the block. MDEV shares a basic proviral structure with other retroviruses (LTR-*gag-pol-env*-LTR) but is distinctly different from other retroviruses in several respects.

The first unusual feature of MDEV is that it represents a novel murine viral interference group distinct from the amphotropic, xenotropic, ecotropic, polytropic, and 10A1 groups. In addition, MDEV does not interfere with SNV, MPMV, or GALV. Although MDEV appears to share a receptor with the RD114 virus in G355 feline cells, it does not do so in the other target cells tested. Not all of the four groups of endogenous viruses identified in Southeast Asian species of mice, as well as laboratory mouse-derived MMTV, have been tested for interference patterns, and we are unable to compare them directly against MDEV. However, it is known that the prototype group C1 virus isolated from the Asian mouse *M. caroli* interferes with the infectious primate viruses GALV and SSAV (6, 27). Although MDEV is also present in the genome of a wild Asian mouse, it does not interfere with GALV and thus is distinct from the *M. caroli* virus. Some endogenous murine viruses isolated from laboratory mice have not been cloned or characterized with respect to their interference group, and so we are again unable to directly compare them to MDEV. However, we have activated an endogenous virus (BIRV) from cells of the BALB/c mouse, a source of many of these murine virus studies (1, 44, 58). Interference analysis showed that BIRV does not interfere with MDEV and that it most probably belongs to the polytropic group of viruses. These interference results indicate that MDEV uses a novel receptor for cell entry.

Beyond using a unique receptor, MDEV is unusual with regard to its exceptionally broad host range. MDEV is able to efficiently infect every cell type that we have tested, with the

exception that BALB/c 3T3 cells were infected at a low level. We previously tested the host range of MDEV on a subset of the cell types tested here (Table 4) with similar results, except that the titer of the previously used MDEV-pseudotype vector was much lower than that used here and CCC-81 cat cells were very poorly infected. We do not know the reason for the discrepant results in the CCC-81 cells, but this may relate to the variable interference of RD114 with MDEV infection, since cat cells carry and potentially could express the endogenous RD114 virus.

Finally, MDEV appears by hybridization analysis to have an unusual genome. Previous studies were not able to detect endogenous virus in *M. dunni* cells either by a reverse transcriptase PCR with primers to various parts of the *env* gene, which could detect xenotropic, ecotropic, polytropic, and modified polytropic viruses (20), or by hybridization with probes for several MLV DNAs (26). Here we found that MDEV does not hybridize to the genomes of any of the other viruses tested, including amphotropic, ecotropic, xenotropic, and polytropic viruses, 10A1, GALV, RD114, and MMTV. Furthermore, we have used the MDEV genome as a probe to look for related sequences in the genomic DNA of several animal species and found none except in other mice. MDEV hybridization to genomic DNA of laboratory mice was limited to one faint band of about 9 kbp that was present in all three mouse strains examined, indicating a single common integration site. We are currently sequencing the MDEV genome to determine its relationship to other viruses.

In summary, MDEV was initially isolated as a contaminant in a human gene transfer trial. Our results indicate that MDEV arose from an endogenous provirus that is present in *M. dunni* mice. MDEV appears to use a different receptor for cell entry than those of other retroviruses, and it will be interesting to further characterize and clone this novel receptor to better understand the types of proteins that retroviruses can utilize for cell entry. The ability of MDEV to efficiently infect many types of human cells indicates that retrovirus packaging cells based on MDEV may ultimately prove useful for gene transfer purposes and possibly for human gene therapy applications.

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