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Mus duni endogenous virus (MDEV)

by

Gregory M. Wolgamot

A dissertation submitted in partial fulfillment of the requirements for the degree of

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1998

Approved by

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Department of Pathology

Date

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Doctoral Dissertation

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Abstract

MUS DUNNI ENDOGENOUS VIRUS (MDEV)

By Gregory M. Wolgamot

Chairperson of the Supervisory Committee:
Affiliate Professor A. Dusty Miller
Department of Pathology

Retroviral vectors are being increasingly used to deliver genes to human cells which are then returned to a patient’s body, both for medical research and direct therapeutic goals. One requirement in such ex vivo gene transfer protocols is that the transduced human cells be screened for contaminating replication-competent retroviruses (RCR). RCR was detected using a vector-rescue assay employing Mus dunni cells. We determined that the RCR was being activated from the dunni cells themselves by hydrocortisone present in the patient cell culture medium, and therefore named the novel virus Mus dunni endogenous virus (MDEV). Retroviral interference experiments indicate that MDEV uses a different receptor for cell entry than those used by previously-known mouse retroviruses. The MDEV receptor is widely expressed, as judged by the ability of MDEV to infect many cell types from many species. We molecularly cloned MDEV to further study its envelope and receptor usage. Southern blot analyses demonstrate that MDEV is endogenous to Mus dunni wild Asian mice rather than being a contaminant of the dunni cells, but is not present in other species tested, including other Asian wild mice. The sequence of the entire genome revealed novel features in addition to a distinct envelope. First, MDEV is a chimeric virus, with interior sequences derived from a virus similar to gibbon ape leukemia virus (GALV) and long terminal repeats (LTRs) derived from a virus-like 30 (VL30) retrotransposon(s). MDEV is the first example of a replication-competent retrovirus with VL30 sequences. Second, the enhancer region of the LTR contains more than six 80 bp
repeats. We provide evidence that the native MDEV provirus has only one repeat, and that these repeats are multiplied upon activation or passage of the virus through a process we call LTR expansion. This repeat number varies tremendously within an MDEV population, with viruses containing 3.15 to 11.15 repeats. We show that an expanded LTR has a stronger promoter, which likely offers MDEV a replicative advantage. Finally, we have constructed and evaluated packaging cells expressing the MDEV envelope (PD223 cells) to take advantage of the broad distribution of the MDEV receptor.
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DEDICATION

This work is dedicated to

Bonnie Harding Wolgamot (June 1, 1945 - May 31, 1996),
a great believer in education and a loving mother.
INTRODUCTION

Retroviruses have had a profound impact on research and medicine. They are known to cause fatal diseases in many species, including leukemia and AIDS in humans. However, due to their life cycle and genomic structure, retroviruses can be engineered into powerful tools that will be increasingly used to combat disease.

Many types of retroviruses have been identified, and various means have been employed to organize them into groups. These include particle morphology, antigenicity, cation preference of reverse-transcriptase, tRNA preference for priming reverse-transcription, host range, and receptor usage. Partly as a consequence of improved sequencing technology, classification based on reverse-transcriptase sequence and genomic structure is the most important for placing a retrovirus into a major group or genus. The currently recognized genera of vertebrate retroviruses (which are often referred to by their prototype members, indicated in parentheses) are avian C-type (ASLVs, avian sarcoma and leukemia viruses), mammalian C-type (MLVs, murine leukemia viruses), B-type (MMTV, mouse mammary tumor virus), D-type (MPMV, Mason-Pfizer monkey virus), HTLV (human T-cell leukemia virus), lenti (HIV, human immunodeficiency virus), spuma (HFV, human foamy virus), and fish retroviruses (WDSV, walleye dermal sarcoma virus). Retrovirus biology does not necessarily obey this nomenclature; for example, the mammalian C-type viruses contain retroviruses not only of mammals but also of ducks and snakes. Additional genera will be required as novel classes of vertebrate retroviruses or nonvertebrate retroviruses (such as the gypsy elements of Drosophila) are identified.

Despite the many groups, all retroviruses share fundamental characteristics. They have a genome bordered by long terminal repeats (LTRs) that contain promoters, enhancers, and many cis-acting sequences. Between the LTRs are the genes gag, which encodes structural proteins, pro and pol, which encode enzymatic proteins, and env, which
encodes surface proteins that mediate entry into a target cell. The simple retroviruses (ASLV, MLV, MPMV, and gypsy groups) have only these genes, while complex retroviruses (MMTV, HTLV, HIV, HFV, and WDSV groups) have additional genes. The basic retrovirus replication cycle consists of infecting a cell, reverse-transcribing to produce a DNA copy of the genome, integrating the DNA copy into a host chromosome to form a provirus, transcribing the provirus by host polymerases to produce retroviral RNA, translating some of the RNA to produce retroviral proteins, assembling new virions, and budding from the cell. Retroviruses are one of the few types of viruses that integrate into host chromosomes.

The integration of retroviruses into host DNA sets the stage for endogenous viruses, which are passed on by Mendelian inheritance. A retrovirus will become endogenous if it infects a germline cell and it does not kill the new host (derived from the germ cell) by reproductive age. Most endogenous viruses are defective, which may in part reflect the latter requirement of being benign to the host. Yet some endogenous retroviruses are replication-competent. Some of these are expressed during part of the animals’ life cycle, and others appear to be quiescent but can be induced to replicate by treating the animal or cultured cells with radiation or a wide variety of chemical agents. All endogenous retroviruses observed thus far have been simple retroviruses.

The development of concepts that lead to the use of retroviruses as tools for gene transfer has its roots in the observation of acute transforming viruses, which are viruses that rapidly induce tumors. It was discovered that these acute transforming viruses are defective for replication (except for Rous sarcoma virus of the ASLV group), and require the presence of a replication-competent “helper” virus to spread. This is because acute transforming viruses have some critical retroviral genes replaced by cellular genes. Despite these alterations in the genome, the cis-acting sequences are intact, enabling the recombinant viruses to be spread if the missing gene products are provided in trans. The
cellular genes within the acute transforming viruses were found to encode proteins that regulate cell growth, which catalyzed the research field of cell cycle, growth, and differentiation. The acquisition of oncogenes appears to be a rare event, but one which is selected at two levels. First, the spread of an oncogenic virus may be enhanced because it induces a replicating population of cells that is susceptible to infection by the helper virus, which subsequently packages additional copies of the transforming virus. Second, selection occurs at the level of research, in which there is a tremendous bias towards studying observable phenotypes such as tumors produced by acute transforming viruses. Interestingly, retroviruses carrying oncogenes have only been observed with simple retroviruses, such as the MLV and ASLV groups. This may in part reflect consolidation of cis-acting sequences, such as the packaging signal, which would have to remain intact after recombination for successful spread of a recombinant virus. Thus, acute transforming viruses are natural examples of retroviruses acting as gene vectors that indicate that other genes could be engineered into retroviruses as well.

The two features that have made retroviruses particularly attractive as gene transfer tools are that retroviruses integrate into the genome, providing opportunity for stable expression of the genes, and that the cis functions can be effectively separated from the trans functions in the genome, enabling the replacement of all retroviral genes by foreign genes. The latter also meant that the helper functions could be provided in trans, eliminating the need for a replication-competent “helper” virus to package the vector. Cells that express Gag, Pol, and Env proteins can also package retroviral vectors and are appropriately called packaging cells. Retroviral vectors and packaging cells based on MLV-type retroviruses are currently in wide use for gene transfer applications, some of which involve human patients.

Ex vivo gene transfer experiments involve removing cells from the patients’ bodies, transducing them in vitro with retroviral vectors, and returning the transduced cells to the
patients. Before the transduced cells can be returned to the patients, however, they must be tested for the presence of contaminating replication-competent retrovirus(es) (RCR) which can potentially be transferred to the patient cells from the packaging cells. RCR can be generated in the packaging cells through recombination between the vector sequences and the gag, pol, and env genes engineered into the cells, recombination between the engineered sequences and endogenous retroviruses, or by activation of endogenous retroviruses.

Contaminating RCR has traditionally been detected by a variety of S\(^+\)L\(^-\) assays, which involve placing the test medium on cells that contain an acute transforming virus (murine sarcoma virus, MSV, hence S\(^+\)) but no helper virus (such as a murine leukemia virus, MLV, hence L\(^-\)). Infection of S\(^+\)L\(^-\) cells by RCR would result in packaging and transfer of the MSV vector to neighboring nontransformed cells, inducing transformed foci. Such an assay would only be able to detect retroviruses that can 1) replicate in the cells used in the assay, and 2) package and transfer the MSV vector. This is likely to be the case since the cells used in the assay are selected for infectibility by many MLV-type viruses, and the packaging cells used in many of the human gene transfer trials are based on mouse cells that contain engineered sequences from MLV type viruses. However, there are major drawbacks to the S\(^+\)L\(^-\) assays, which involve safety (the assays involve packaging of oncogenic vectors within viruses capable of infecting human cells) and difficulty in scoring.

New assays for RCR have been developed due to the drawbacks of the S\(^+\)L\(^-\) assays. One such assay is the dunni/N2 assay. This is also a vector-rescue assay, but instead of depending on rescue of an oncogenic MSV vector, it depends on the rescue of the N2 vector which has been engineered to carry a neomycin phosphotransferase gene. Mus dunni cells were chosen for this assay because they are infectible by a wide variety of murine retroviruses, which are those most likely to contaminate the human cells, and were thought
to be free of endogenous viruses, which could cause false-positives in the assay. This dissertation project originated from positive results obtained using the dunni/N2 assay during testing for RCR during human gene transfer trials.

Chapter 1 describes the detection of RCR by using the dunni/N2 helper assay and our studies that reveal that the RCR was originating from the dunni/N2 cells themselves that were used in the assay. Accordingly, we name the novel virus *Mus dunni* endogenous virus (MDEV), and demonstrate that MDEV has a wide host range and unusual receptor usage. Chapter 2 describes the molecular cloning of MDEV and experiments that show that MDEV is endogenous to the wild Asian mouse *Mus dunni* rather than a cell line contaminant. It also shows that that MDEV is a relatively unique virus that is not endogenous to other tested species. Chapter 3 demonstrates that there are six receptor groups of MLVs when assayed on dunni cells: ecotropic, amphotropic, 10A1, xenotropic, polytropic, and MDEV. This is in contrast to previous results obtained on dunni cells, and has implications for receptor usage. Chapter 4 describes the sequence analysis of MDEV, which reveals some surprising features, including a recombinant structure containing critical retroelement sequences that have not previously been observed in a replication competent virus. In addition, we find that there are more than six 80 bp repeats in the LTR. Chapter 5 shows that the endogenous MDEV provirus does not likely contain multiple repeats, and that the repeats were most likely generated upon activation or passage of MDEV. We show that an MDEV population has a tremendously variable number of repeats, from 3.15 to 11.15, and provide evidence that the repeats offer the virus a selective advantage. Chapter 6 describes the construction and evaluation of packaging cells based on MDEV to take advantage of its broad receptor distribution. Finally, Chapter 7 summarizes our current understanding of MDEV and points to new directions for future research.
Chapter I

A NOVEL MURINE RETROVIRUS IDENTIFIED DURING TESTING FOR HELPER VIRUS IN HUMAN GENE TRANSFER TRIALS

Summary

An important requirement for the use of retroviral vectors in human gene transfer experiments is the avoidance of human exposure to replication-competent (helper) retroviruses. To meet this requirement, we used a sensitive marker rescue assay for helper virus to screen vector-transduced cells prior to reinfusion into patients. This assay utilized *Mus dunni* cells harboring a retroviral vector that can be rescued by helper retroviruses. The assay indicated the presence of helper virus in medium exposed to hematopoietic cells from all patients tested, including 6 patients with various cancers and 1 patient with Gaucher's disease, whether or not the patient cells were exposed to retroviral vectors. All of the helper viruses were in a single interference group. We have now shown that treatment of the *Mus dunni* marker rescue assay cells with 5-ido-2’-deoxyuridine or hydrocortisone can activate production of an apparently identical helper virus, which we have named MDEV (*Mus dunni* endogenous virus). Thus production of virus in the assays of patient materials is likely due to exposure of the marker rescue assay cells to the hydrocortisone present in the hematopoietic cell growth medium. MDEV does not belong to any of the known murine leukemia virus groups by interference analysis, and we have called the new group multistropic due to the wide range of cells from different species MDEV will infect.
Introduction

Viral vectors are currently used in the majority of human gene transfer trials, in large part due to efficient mechanisms that viruses have evolved to transfer genetic material into cells. An important issue in experiments utilizing viral vectors is the possibility of generation of replication-competent (helper) virus due to recombination of viral components during vector production. While testing can provide a statistical measure of the likelihood of helper virus in a vector preparation, the possibility of human exposure to helper virus cannot be excluded. Because the health consequences of recombinant viruses in humans cannot be accurately predicted, there has been a constant effort by investigators and regulatory agencies to increase the stringency of helper virus testing to reduce the risk of human exposure to helper virus.

We have employed a sensitive marker rescue assay to screen for helper virus in retroviral vector preparations and in vector-transduced hematopoietic cells prior to reinfusion into patients as part of two clinical gene transfer trials (Fig. 1.1). One trial is designed to study the contribution of G-CSF-mobilized peripheral blood hematopoietic cells to long term hematopoietic reconstitution by marking the cells with a retroviral vector containing the neo gene prior to reinfusion into patients. The other trial is designed to treat Gaucher's disease by transfer of the glucocerebroside gene into hematopoietic cells to correct the metabolic defect responsible for the disease. Both trials involve harvest of G-CSF-mobilized peripheral blood hematopoietic cells followed by transduction of the cells by daily exposure to retroviral vectors during 5 days of culture. After transduction, the cells are cryopreserved for patient administration pending safety testing, including testing for helper retroviruses.

Because screening of large amounts of the retroviral vector preparations did not reveal helper virus, we were surprised to see positive assay results from testing of medium
from hematopoietic cell cultures for all of the patients tested to date. Initial experiments indicated that the viruses did not come from the individual components of the hematopoietic culture medium, but from the human hematopoietic cells. Further characterization of the viruses has shown that they are all similar if not identical, and that they likely derive from a previously uncharacterized endogenous virus in the *Mus dunni* cells used in the marker rescue assay. By interference analysis, the new virus represents a new mouse retrovirus group.

**Materials and Methods**

**Nomenclature.** Cells that contain a virus or vector are indicated by the cell name followed by a slash and the name of the virus, e.g., NIH 3T3/10A1 or PA317/LXSN. A retroviral vector in its viral form is indicated by the vector name followed, in parentheses, by the name of the helper virus or packaging cells used to pseudotype the vector, e.g., LAPS(N10A1) or LAPS(NPA317). Throughout this paper, the pseudotype of a retroviral vector refers only to the viral envelope proteins present on the vector virions. For example, vectors produced by PA317 cells will be referred to as having an amphotropic pseudotype due to the presence of amphotropic Env proteins in the virions, even though the Gag and part of the Pol proteins in these virions are derived from Moloney murine leukemia virus (74).

**Cell culture.** The following cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum: *Mus dunni* tail fibroblasts (gift from Bruce Chesebro, originally described in refs. 20,64), NIH 3T3 (TK<sup>−</sup>) cells (74), PA317 amphotropic retrovirus packaging cells (74), PM571 polytropic retrovirus packaging cells (82), PE501 ecotropic retrovirus packaging cells (78), HeLa human cervical carcinoma
cells (46), NRK rat kidney cells (32), and CCC-81 cat cells transformed with Moloney murine sarcoma virus (41). G355 (G355-5) feline embryonic brain cells, G355 cells infected with RD114 endogenous cat virus, and PG-4 Moloney murine sarcoma virus-infected G355 cells (all gifts from Donald Blair, ref. 48) were grown in McCoy's medium with 15% fetal bovine serum.

In helper virus assays, special care was taken to prevent cross-contamination of cultures. In particular, when aerosols might be generated during trypsinization and passaging of the cells, only one culture was manipulated in the laminar flow hood at a time. Culture medium was not exposed to aerosols, and once a sterile pipet had been used to withdraw medium from the bottle for distribution to cultures, it was never inserted into the bottle again.

**Marker rescue assay.** *Mus dunni* cells containing the N2 retroviral vector (4) (dunni/N2) or the LAPSN retroviral vector (81) (dunni/LAPSN) were made by transducing *Mus dunni* cells with helper-free retroviral vectors generated by using PA317 retrovirus packaging cells (74) and then selecting the cells in G418 for 1 week to ensure the presence of the vectors in all cells in the populations. A similar procedure was used to generate G355 cat cells containing the LAPSN vector (G355/LAPSN). The marker rescue assay was performed by seeding dunni/N2, dunni/LAPSN, or G355/LAPSN cells at $10^6$ per 10 cm dish on day 1, exposing the cells to the test sample (medium or hematopoietic cells) in the presence of 4 μg Polybrene per ml on day 2, passaging the cells 1:10 or 1:20 every two or three days, feeding confluent dishes of the cells on day 15 or later, harvesting the medium the day after feeding, and assaying the medium for the presence of the N2 or LAPSN vector by using *Mus dunni* cells as targets for infection. During passage, the cells were kept at high density to facilitate virus spread. Cells were passaged for the first week (at least) in 10 cm dishes and later in 6 cm dishes. Polybrene was only present during the
initial exposure of the marker rescue assay cells to test samples and was not added during passage of the cells.

**Retroviral vector assay.** The LAPS N vector was assayed by seeding target cells at $5 \times 10^4$ or $10^5$ per 6 cm dish on day 1, feeding the cells with fresh medium containing 4 µg Polybrene (Sigma) per ml and adding the LAPS N vector on day 2, and fixing and staining the cells for alkaline phosphatase expression encoded by the LAPS N vector as described (40) on day 4. Assay for the N2 virus was identical except that instead of staining for AP, the medium was changed to medium containing 0.75 mg/ml G418 (active) on day 3 and colonies were stained with Coomassie Brilliant Blue G (Sigma; 1 g/liter in 40% MeOH-10% acetic acid) on day 8.

**Human hematopoietic cell transduction.** Details of the human clinical trials have been published (111,112). Briefly, patients received G-CSF to mobilize hematopoietic stem cells into the circulation, peripheral blood cells were collected by leukapheresis, and CD34+ hematopoietic cells were isolated. CD34+ cells were transduced with the LN gene marking vector (78) produced by using PA317 cells (74), or with the LgGC retroviral vector that encodes glucocerebrosidase (unpublished results) produced by using PG13 cells (76), by cultivation of the cells in Iscove's modified Dulbecco's medium containing 50% retroviral vector stock, 12.5% heat-inactivated (56°C for 30 min) fetal bovine serum, 12.5% heat-inactivated (56°C for 30 min) horse serum, 90 µM hydrocortisone sodium succinate, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.4 mg/ml L-glutamine, 0.01 mM 2-mercaptoethanol, 4 µg/ml protamine sulfate and recombinant human IL-1β, IL-3, IL-6 and SCF (each at 50 ng/ml). Every day one half of the culture medium was replaced with fresh vector-containing medium containing protamine sulfate but without fresh cytokines. After 5 days of cultivation, the
hematopoietic cells and culture medium were harvested and the cells were cryopreserved for patient administration after safety testing.

**Endogenous virus activation.** In an initial experiment, dunni/LAPSN cells were plated at $2 \times 10^5$ cells per 34 mm well of 6-well dishes on day 1. On day 2, the cells were fed with medium containing 4 μg of Polybrene per ml and various concentrations of 5-iodo-2'-deoxyuridine (IdU) (30 μM-3 mM), 5-bromo-2'-deoxyuridine (3 μM-300 μM), 5-azacytidine (300 nM-30 μM), or no drug. Crystals of IdU were present at 3 mM IdU, so the medium was filtered with a 0.45 μm cellulose acetate filter to remove the crystals. From day 3 through week 3, the cells were passaged in medium containing Polybrene but without continued addition of nucleosides. The cells were split in the dark for the first 2 weeks.

In the second experiment, dunni/LAPSN cells were plated at $4 \times 10^5$ cells per 6 cm dish in Dulbecco's modified Eagle's medium with 10% calf serum (heat-inactivated at 56°C for 30 min) on day 1. On day 2, 10 dishes were fed with the same medium containing 4 μg of Polybrene per ml and saturated with IdU, while 5 dishes were fed with medium containing Polybrene but no drug. The medium saturated with IdU was first mixed at a concentration of 3 mM and vortexted periodically during incubation at 37°C for 30 minutes. After the remaining IdU crystals had settled to the bottom, the cells were fed with the saturated supernatant. From day 3 to week 4, the cells were split 1:3 to 1:10 every few days in medium containing Polybrene but no IdU. Exposure of three IdU-treated plates to fluorescent light or passage of the other 7 dishes in the dark for 1 week prior to light exposure had no apparent effect on virus activation.
Results

Detection of helper virus during testing of clinical samples. We used a marker rescue assay (Fig. 1.2) to screen for helper virus in medium from human hematopoietic cell cultures that had been exposed to retroviral vectors. Half of the medium in the hematopoietic cultures was replaced with vector-containing medium each day for 5 days, and the spent medium from days 1 and 5 was assayed for helper virus. Helper virus was detected in assays of culture medium from cells of all patients tested, while control assays were uniformly negative (Table 1.1). The majority of the patients had various cancers, including multiple myeloma, breast cancer, Hodgkin's lymphoma and non-Hodgkin's lymphoma, while patient 7 had Gaucher's disease, a lysosomal storage disorder. In what follows, patient 1 virus, patient 2 virus, etc. will refer to the helper virus produced following exposure of the marker rescue assay cells to culture medium from the respective patient's cells.

The medium from the patient cell cultures appeared to contain low levels of helper virus because occasional tests of 5 ml medium samples were negative (Table 1.1, patients 1 and 2). However, after exposure of dunned/LAPSN marker rescue assay cells to the culture medium from the patient cells and passage of the cells for 2 weeks, the LAPSN vector was produced at high titer (up to $10^5$ FFU/ml), suggesting production of the helper virus at high titer. We could not detect the helper viruses by using standard focus-induction assays, including S$^+$L$^-$ assays utilizing PG-4 cat cells (48), CCC-81 cat cells overlayed with NRK cells (77), Mv1Lu mink cells (26), or SC-1 feral mouse embryo cells overlayed with Mv1Lu mink lung cells (26) (data not shown; mink S$^+$L$^-$ assays performed by Microbiologic Associates Inc., Rockville, Maryland). Therefore the helper virus titer was estimated by using limiting dilution analysis and the Mus dunnii rescue assay for virus detection. By this technique, medium from one of the dunned/LAPSN cell cultures 2 weeks
after exposure to medium from patient cells was estimated to contain between $10^6$ and $5 \times 10^6$ infectious units of helper virus per ml.

Detection of helper virus in assays of hematopoietic cell culture medium was not related to exposure of the hematopoietic cells to retroviral vectors because parallel cultures that had not been exposed to vectors also tested positive (Table 1.1, patients 3-6). This result is consistent with the fact that we have never found helper virus in the vector stocks by marker rescue or other helper virus assays.

In all experiments we assayed standard culture medium (DMEM with 10% FBS) in parallel with the patient samples to act as a negative control, and all of these assays were negative (Table 1.1). In one experiment, hematopoietic culture medium that had not been exposed to human cells was analyzed in the marker rescue assay and found to be negative in 5 replicate assays of 5 ml medium samples each (data not shown). We also analyzed the FBS, horse serum and cytokines used in the medium in independent assays, and never found helper virus in these components. In addition, the fetal bovine serum and horse serum used were heat-inactivated at $56^\circ$C for 30 min, and we have found that the titer of the patient virus pseudotype of the LAPSN vector is reduced by $>5 \times 10^4$-fold by heating briefly to $56^\circ$C.

**All recovered helper viruses are in the same interference group.** We used interference analysis to compare the different patient viruses. Viruses from the first two patients were initially isolated by using dunni/N2 cells. To test for interference between these viruses, we transferred virus from the dunni/N2 cells to dunni/LAPSN cells and then passaged the cells for 2 weeks to allow helper virus spread. These cell populations produce the LAPSN vector with a patient 1 or patient 2 virus pseudotype, and may also produce some N2 virus. We used virus from the patient virus-infected dunni/LAPSN cells to infect the original patient virus-infected dunni/N2 cells and measured
AP+ foci to determine the infection rate. Table 1.2 shows that LAPS N pseudotyped by either patient 1 or patient 2 virus can efficiently infect dunni/N2 cells, but is inhibited from infecting dunni/N2 cells previously infected with either patient 1 or patient 2 virus. In contrast, amphotropic pseudotype LAPS N virus infects dunni/N2 cells or dunni/N2 cells infected with patient 1 virus or patient 2 virus equally well. This analysis shows that patient 1 and patient 2 viruses are in the same interference group, and that amphotropic murine virus is in a different interference group.

Since dunni/LAPS N cells were used in the marker rescue assay for patients 3-7, we tested the ability of the isolated viruses to transfer AP to dunni/N2 cells or dunni/N2 cells infected with patient 1 virus. The transduction rate of all of the patient virus isolates was reduced by >250 to 20,000-fold in dunni/N2 + patient 1 virus cells in comparison to dunni/N2 cells (data not shown). Some of the viruses had a low titer on dunni/N2 cells and did not detectably transduce dunni/N2 + patient 1 virus cells, so only a lower limit for inhibition (>250-fold) could be established. All viruses detected in medium from patient cells that had not been exposed to retroviral vectors were in the same interference group as those from patient cells that had been exposed to retroviral vectors. Thus the interference data shows that all of the viruses isolated during testing of medium samples from the human hematopoietic cells were in the same interference group and thus were closely related if not identical.

**Activation of Mus dunni endogenous retrovirus (MDEV) that is in the same interference group as the patient viruses.** Endogenous retroviruses present in the cellular genome can be activated by a variety of chemicals (28). We tested whether such viruses could be activated in dunni/LAPS N cells by various concentrations of 5-iodo-2'-deoxyuridine (IdU), 5-bromo-2'-deoxyuridine, or 5-azacytidine. At the highest concentration of IdU we detected LAPS N vector production from the dunni/LAPS N cells
three weeks after exposure to the drug. This virus proved to be in the same interference group as the patient viruses (data not shown).

In a second experiment, 10 dishes of dunni/LAPSN cells treated with a high dose of IdU and 5 dishes of untreated dunni/LAPSN cells were passaged independently and assayed for virus production. Virus production was not detected in any of the cultures at 1 week (<1 AP⁺ FFU/ml), but at time points ≥2 weeks virus production was detected in 10 of 10 IdU-treated cultures (6 x 10³ to 5 x 10⁴ AP⁺ FFU/ml when assayed on Mus dunni cells at week 4). The five untreated cultures of dunni/LAPSN cells were negative for virus production over the 4 week course of the experiment (<1 AP⁺ FFU/ml). Infection by the IdU-activated virus was blocked in cells infected with patient virus 1 (500- to 3,000-fold inhibition), showing that all of the independently-activated viruses were in the same interference group as the patient viruses, and probably derive from one endogenous provirus which we will call MDEV (Mus dunni endogenous virus).

Given that the viruses detected during screening of medium from human hematopoietic cells were all identical by interference analysis and matched the endogenous Mus dunni virus activated by IdU, we reexamined the ability of the hematopoietic culture medium to activate MDEV from Mus dunni cells. In two independent experiments, we detected virus after cultivation of dunni/LAPSN cells with the hematopoietic culture medium in 4 of 4 replicate assays in each experiment (data not shown). In prior screens of components of the medium, we had ignored chemically defined components as being unlikely sources of virus. However, the medium contained 90 μM hydrocortisone (to inhibit growth of contaminating lymphoid cells in the hematopoietic cell cultures), and steroids can induce expression of some retroviruses, thus the hydrocortisone might activate expression of MDEV. Indeed, treatment of dunni/LAPSN cells with 90 μM hydrocortisone in regular culture medium activated virus in 2 of 2 assays. Hydrocortisone
at 1 μM could also induce virus production, but less effectively. Infection by all viruses induced by hydrocortisone was completely blocked by the presence of patient 1 virus in the target cells (Table 1.4). Amphotropic virus from PA317/LAPSN cells infected dunni and dunni/N2 + patient 1 virus cells equally well, showing that there was not a general block to retrovirus infection in the dunni/N2 + patient 1 virus cells. Thus the presence of hydrocortisone in the hematopoietic culture medium can explain the frequent production of helper virus in marker rescue assays of medium from the human hematopoietic cultures.

A G355 cat cell-based marker rescue assay fails to detect helper virus in medium exposed to human hematopoietic cells. While we have shown that the hematopoietic culture medium can activate production of MDEV from *Mus dunni* cells, we have not ruled out the possibility that the human hematopoietic cells also produce a related virus. To address this possibility, we used a marker rescue assay based on G355 cat cells to test samples of the culture medium from the human hematopoietic cells, in the hope that endogenous viruses might not be activated in G355 cells. Five 5 ml hematopoietic medium samples from day 5 of the patient 6 hematopoietic cell culture were all negative for helper virus in the G355 cat cell-based marker rescue assay (Table 1.5), while five 5 ml samples from the same culture were all positive in the *Mus dunni*-based marker rescue assay (Table 1.1). In addition, the virus isolated in the *Mus dunni* assay of culture medium from patient 6 cells scored positive in the G355 cat cell-based marker rescue assay (Table 1.5), showing that this assay is capable of detecting the virus if it were present in the culture medium from patient 6 cells. Thus we have good evidence that the day 5 culture medium from the patient 6 hematopoietic cell culture was helper free, and therefore that the virus produced in the *Mus dunni* assay of the same medium must be MDEV.

MDEV represents a new murine retrovirus interference group. The LAPSN vector having either amphotropic, polytropic (MCF), or xenotropic pseudotype
was used to infect *Mus dunni* cells that were previously infected with patient 1 virus, patient 2 virus, AM-MLV, or that were uninfected with helper virus (Table 1.6). Amphotropic, polytropic and xenotropic pseudotype LAPSN vectors transduced dunni/N2 cells or dunni/N2 cells that were infected with viruses from patient 1 or patient 2 equally well, showing that the patient viruses do not interfere with entry of these murine viruses. In all cases the presence or absence of the N2 retroviral vector in the *Mus dunni* cells had no effect on the AP transduction rate, as expected since the N2 vector only encodes Neo and makes no viral proteins. As a control for viral interference, dunni/N2 cells infected with amphotropic virus were efficiently infected by polytropic or xenotropic LAPSN vectors, but not by the amphotropic LAPSN vector, as expected. Since MDEV and the patient virus isolates are in the same interference group, these results show that MDEV is not in the amphotropic, polytropic, or xenotropic interference groups.

Since *Mus dunni* cells are poorly infected by some ecotropic retroviruses, including MoMLV, we used NIH 3T3 mouse cells to conduct additional interference tests. The presence of ecotropic, amphotropic, or 10A1 virus in the mouse cells did not inhibit infection by the LAPSN vector with a patient 1 virus pseudotype (Table 1.7), indicating that MDEV and the patient viruses are not in these mouse virus classes. Control experiments showed that infection by ecotropic LAPSN vector (produced by using PE501 cells) was blocked by ecotropic (MoMLV) but not the other viruses, amphotropic LAPSN vector (produced by using PA317 cells) was blocked by amphotropic or 10A1 virus, but not by ecotropic virus, and that 10A1 pseudotype LAPSN vector was blocked by 10A1 but not the other viruses, all as expected. 10A1 virus can use both the amphotropic receptor Rab1 or the GALV receptor G1vr-1 in mouse cells (83), which is why 10A1 blocks infection by amphotropic virus but amphotropic virus does not block 10A1 virus infection.
Together with the interference data obtained in *Mus dunni* cells, these results show that MDEV is not in any of the five known murine leukemia virus interference groups (93).

The host range of MDEV is broad and includes human, mouse, rat, cat, and dog cells (Table 1.8). Additional experiments showed that MDEV also infects Chinese hamster ovary (CHO) cells efficiently (data not shown). Interestingly, MDEV poorly infected CCC-81 cat cells, which could be due to expression of the RD114 endogenous cat virus. We therefore tested whether MDEV was in the RD114 virus interference group. We detected a low level of interference (10- to 20-fold) to MDEV infection by RD114 virus in G355 cat cells, but no interference when the assays were performed in D17 dog cells (data not shown). Two other viruses shown to be in the RD114 class by interference assay in human cells (117), spleen necrosis virus and Mason Pfizer monkey virus (SRV-3), did not interfere with patient virus infection (data not shown). Thus MDEV shows a limited relationship to the RD114 endogenous cat retrovirus.

**Discussion**

*Mus dunni* cells were adopted by the United States Food and Drug Administration for helper virus testing of materials to be used in human gene transfer trials because they are susceptible to infection and allow replication of members of all previously characterized murine retrovirus classes (64). A notable exception to this generalization is that ecotropic Moloney MLV poorly infects these cells (64), but since ecotropic virus does not infect human cells, this is not a significant concern for human gene transfer trials. Since retrovirus packaging cells in common use are typically based on the NIH 3T3 mouse cell line, and the retroviral vectors and defective helper virus components present in the packaging lines are typically derived from murine retroviruses, the choice of *Mus dunni* cells allows detection of all anticipated helper viruses in these cases. However, we have
found that an endogenous virus can be activated and spread in *Mus dunni* cells following treatment with hydrocortisone or IdU, thus steroids or other factors present in test samples can give false positive results in helper virus assays. We have also observed an apparent example of spontaneous activation of virus from dunni/LAPSN cells cultured under standard conditions (data not shown), and the virus was in the same interference group as the chemically-activated viruses described in this report. In the context of a clinical gene transfer trial, such false positives can be very costly and time consuming, and may delay therapeutic treatment. Marker rescue assays that are less prone to such false positive results would thus be desirable.

Another problem with the *Mus dunni*-based marker rescue assay is that the assay is incapable of detecting some nonmurine retroviruses, including gibbon ape leukemia virus (GALV). We have developed retrovirus packaging lines based on GALV (76) that promote more efficient infection of some myeloid and lymphoid cells (8,16,125), and are currently using a GALV-based packaging cell line in our attempt to treat Gaucher's disease by gene therapy (patient 7). In this case, we have employed a marker rescue assay based on a human cell line, in addition to the *Mus dunni* marker rescue assay, to detect potential helper viruses having a GALV pseudotype.

Several factors complicated our progress in resolving the source of the helper virus detected in screening of patient materials. We initially focussed on testing of components of the hematopoietic culture medium that were likely sources of virus, such as the horse serum, and could not detect virus in these materials. Chemical medium components, such as the hydrocortisone, were judged unlikely virus sources. In addition, an initial test of five 5 ml aliquots of the complete hematopoietic culture medium was negative for helper virus, and since the clinical grade components of the medium were expensive, we did not routinely test additional medium samples. We cannot explain this initial result in light of
later experiments in which multiple samples of the hematopoietic culture medium all tested positive. Other misleading evidence included results from an experiment where peripheral blood hematopoietic cells from one of the patients (prior to CD34+ purification or cultivation in hematopoietic medium) scored positive for helper virus when cultivated with the Mus dunnii assay cells in standard culture medium (DMEM plus 10% FBS), and the recovered virus was in the same interference group as the other patient isolates (data not shown). This appeared to be good evidence that the source of the virus was the patient cells. We still cannot explain why these cells tested positive, but the result may be due to spontaneous activation of MDEV or activation of MDEV by factors secreted by the hematopoietic cells.

In summary, the virus isolates produced by IdU or hydrocortisone treatment of Mus dunnii cells, or produced by exposure of the Mus dunnii cells to human hematopoietic cells or medium from human hematopoietic cell cultures, are all in the same interference group and probably derive from a single endogenous virus in the Mus dunnii cells (MDEV). MDEV represents a new murine retrovirus interference group, for which we suggest the name multitropic due to the wide range of cells from different mammalian species that can be infected by MDEV. Efforts are underway to derive molecular clones of the virus to help understand its relationship to other retroviruses.
Fig. 1.1: The *ex vivo* gene marking trials and the *Mus dunnii* marker rescue assay for helper virus.
Fig. 1.2: The *Mus dunni* marker rescue assay for helper virus.
Table 1.1: Medium samples from all patient hematopoietic cell cultures tested positive for helper virus in the *Mus dunni* marker rescue assay\textsuperscript{a}

<table>
<thead>
<tr>
<th>Patient</th>
<th>+vector day 1</th>
<th>+vector day 5</th>
<th>-vector day 5</th>
<th>Control medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>nd</td>
<td>+ (2/2)*</td>
<td>nd</td>
<td>- (0/1)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (4/5)*</td>
<td></td>
<td>- (0/5)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (3/3)</td>
<td></td>
<td>- (0/2)</td>
</tr>
<tr>
<td>2</td>
<td>nd</td>
<td>+ (2/2)*</td>
<td>nd</td>
<td>- (0/1)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (2/5)*</td>
<td></td>
<td>- (0/5)*</td>
</tr>
<tr>
<td>3</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
<td>+ (1/1)</td>
<td>- (0/2)</td>
</tr>
<tr>
<td>4</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
<td>+ (1/1)</td>
<td>- (0/3)</td>
</tr>
<tr>
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<td>nd</td>
<td>+ (5/5)</td>
<td>+ (1/1)</td>
<td>- (0/3)</td>
</tr>
<tr>
<td>6</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
<td>+ (1/1)</td>
<td>- (0/3)</td>
</tr>
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<td>7</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
<td>nd</td>
<td>- (0/3)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Medium (5 ml samples) harvested from patient hematopoietic cells or control medium (DMEM plus 10% FBS) was assayed for helper virus using the dunni/LAPSN or dunni/N2 marker rescue assays. Independent experiments are listed in separate rows. Assay results are given as + (vector titer of 20 to 10\textsuperscript{5} FFU/ml) or - (no LAPSN or N2 vector detected, <1 FFU/ml) followed in parentheses by the number of positive assays/total assays performed. Patients 1-6 had various cancers and patient 7 had Gaucher's disease. nd, not done.

\textsuperscript{b} Samples listed as +vector were from patient cells that had been exposed to a retroviral vector, either LN(PA317) for patients 1-6 or LgGC(PG13) for patient 7, while samples listed as -vector were never exposed to a retroviral vector. Medium assayed was harvested at the indicated time after initiation of the hematopoietic cell cultures (day 1 or day 5 after initiation).
Table 1.2: Patient 1 and 2 viruses interfere with each other.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>LAPSN titer (FFU/ml) for indicated pseudotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient 1</td>
</tr>
<tr>
<td>dunni/N2</td>
<td>$6 \times 10^4$</td>
</tr>
<tr>
<td>dunni/N2 + patient 1 virus</td>
<td>20</td>
</tr>
<tr>
<td>dunni/N2 + patient 2 virus</td>
<td>80</td>
</tr>
</tbody>
</table>

Target cells were plated at $5 \times 10^4$ per 6 cm dish and infected one day later with LAPSN vectors pseudotyped by the patient viruses or by LAPSN produced by using PA317 cells (amphotropic pseudotype). Cells were stained two days after infection for alkaline phosphatase-positive foci and titer in focus-forming units (FFU) per ml of virus calculated. Additional experiments showed that 4 of 4 independent patient 1 virus isolates in dunni/N2 cells, and 1 of 1 independent patient 2 virus isolates in dunni/N2 cells interfered with patient 1 pseudotyped LAPSN virus, confirming results from the experiment shown.
Table 1.3: 5-iodo-2'-deoxyuridine (IdU) activates a virus from *Mus dunnii* cells that uses the same receptor as the virus detected in the helper assays.

<table>
<thead>
<tr>
<th>Item</th>
<th>dunni/N2</th>
<th>dunni/N2+&quot;patient virus&quot;</th>
<th>Fold reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAPSN(PA317)</td>
<td>$2.0 \times 10^5$</td>
<td>$1.4 \times 10^5$</td>
<td>1.4</td>
</tr>
<tr>
<td>LAPSN(pt. virus)</td>
<td>$2.8 \times 10^3$</td>
<td>8</td>
<td>400</td>
</tr>
<tr>
<td>No IdU 1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
</tr>
<tr>
<td>IdU 1</td>
<td>$4 \times 10^4$</td>
<td>30</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>$2 \times 10^4$</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>$1 \times 10^4$</td>
<td>20</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>$5 \times 10^4$</td>
<td>30</td>
<td>2000</td>
</tr>
<tr>
<td>5</td>
<td>$1 \times 10^4$</td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>$3 \times 10^4$</td>
<td>10</td>
<td>3000</td>
</tr>
<tr>
<td>7</td>
<td>$5 \times 10^4$</td>
<td>40</td>
<td>1000</td>
</tr>
<tr>
<td>8</td>
<td>$6 \times 10^3$</td>
<td>10</td>
<td>600</td>
</tr>
<tr>
<td>9</td>
<td>$2 \times 10^4$</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>10</td>
<td>$1 \times 10^4$</td>
<td>10</td>
<td>1000</td>
</tr>
</tbody>
</table>

10 dishes of dunni/LAPSN cells were exposed to medium saturated with IdU for 24 hours and passaged in parallel with 5 dishes that had not been exposed to IdU. The titers shown are those obtained after 4 weeks of passage.
Table 1.4: Hydrocortisone (HC) can activate the endogenous virus in *Mus dunni* cells\(^a\)

<table>
<thead>
<tr>
<th>Virus harvested from</th>
<th>Culture</th>
<th>dunni</th>
<th>dunni/N2 + patient 1 virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>dunni/LAPSN</td>
<td>#1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>dunni/LAPSN + 1 (\mu)M HC</td>
<td>#1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>dunni/LAPSN + 90 (\mu)M HC</td>
<td>#1</td>
<td>(2 \times 10^4)</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>(5 \times 10^4)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>dunni/LAPSN + patient 1 virus</td>
<td></td>
<td>(3 \times 10^4)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PA317/LAPSN</td>
<td></td>
<td>(4 \times 10^6)</td>
<td>(3 \times 10^6)</td>
</tr>
</tbody>
</table>

\(^a\) Dunni/LAPSN cells were seeded at \(10^6\) cells per 10 cm dish on day 1. The medium was replaced with DMEM plus 10% FBS and the indicated concentrations of hydrocortisone on day 2. The cells were passaged every 2 or 3 days for 2 weeks. and LAPSN vector production was measured by using *Mus dunni* cells as targets for infection. Cultures #1 and #2 refer to independent cultures passaged and assayed in parallel for LAPSN production.
Table 1.5: Day 5 medium from the patient 6 hematopoietic cell culture tests negative for helper virus in a G355 cat cell-based marker rescue assay

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Replicates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LAPSN vector titer (FFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No virus</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5 ml patient 6 culture medium</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1 ml dunu/LAPSN + patient 6 virus</td>
<td>1</td>
<td>4 x 10⁴</td>
</tr>
<tr>
<td>10 µl dunu/LAPSN + patient 6 virus</td>
<td>1</td>
<td>4 x 10⁴</td>
</tr>
<tr>
<td>100 nl dunu/LAPSN + patient 6 virus</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>1 µl AM-MLV</td>
<td>1</td>
<td>5 x 10⁷</td>
</tr>
</tbody>
</table>

<sup>a</sup> Replicates indicates the number of independent assays performed with identical results.
Table 1.6: Patient viruses do not interfere with infection by vectors having amphotropic, polytropic, or xenotropic murine virus pseudotypes.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>LAPS N titer (FFU/ml) for indicated pseudotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amphotropic</td>
</tr>
<tr>
<td>dunni</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>dunni/N2</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>dunni/N2 + patient 1 virus</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>dunni/N2 + patient 2 virus</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>dunni/N2 + AM-MLV</td>
<td>20</td>
</tr>
</tbody>
</table>

Target cells were infected with the LAPS N vector (carrying alkaline phosphatase) produced by using PA317 cells (amphotropic pseudotype), PM571 cells (polytropic (MCF) pseudotype), or by using dunni/LAPS N cells infected with NZB xenotropic virus (89) (xenotropic pseudotype). Cells were stained two days after infection for alkaline phosphatase-positive foci and the vector titer in focus-forming units (FFU) per ml of virus is shown.
Table 1.7: Moloney, amphotropic, and 10A1 murine leukemia viruses do not interfere with transduction by a vector having a patient 1 virus pseudotype

<table>
<thead>
<tr>
<th>Target cells</th>
<th>LAPSN titer (FFU/ml) for indicated pseudotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient 1 virus</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>200</td>
</tr>
<tr>
<td>NIH 3T3/MoMLV</td>
<td>200</td>
</tr>
<tr>
<td>NIH 3T3/AM-MLV</td>
<td>400</td>
</tr>
<tr>
<td>NIH 3T3/10A1</td>
<td>200</td>
</tr>
</tbody>
</table>

Target cells were seeded at $10^5$ per 6 cm dish on day 1, infected with LAPSN virus with the indicated pseudotypes on day 2, and stained for AP$^+$ foci on day 4. Helper virus-infected NIH 3T3 cells were generated by transfection of the cells with molecular clones of MoMLV (pMLV-K, ref. 79), AM-MLV (pAM, ref. 77), or 10A1. The permuted 10A1 virus DNA clone (pB6, ref. 92, gift from Alan Rein, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland) was cut with SalI restriction endonuclease and religated to generate intact 10A1 provirus circles prior to transfection into NIH 3T3 cells. Cells were passaged for several weeks to allow complete virus spread. Viruses used for infection were derived from dunni/LAPSN + patient 1 virus cells (patient 1 virus pseudotype), PE501/LAPSN cells (ecotropic pseudotype), PA317/LAPSN cells (amphotropic pseudotype), or NIH 3T3/LAPSN + 10A1 virus cells (10A1 pseudotype). The experiment shown was repeated two more times with similar results.
Table 1.8: The patient viruses have a wide host range\textsuperscript{a}

<table>
<thead>
<tr>
<th>Target cells</th>
<th>species</th>
<th>Virus titer (CFU/ml or FFU/ml) for indicated pseudotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>human</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>mouse</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>\textit{Mus dunnii}</td>
<td>wild mouse</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>NRK</td>
<td>rat</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>CCC-81</td>
<td>cat</td>
<td>5</td>
</tr>
<tr>
<td>G355</td>
<td>cat</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>D17</td>
<td>dog</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>Patient 1 virus</td>
<td>Patient 2 virus</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^4$</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^4$</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$&lt;5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^3$</td>
<td>$7 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The LAPS\textsuperscript{N} vector was used for infection of the G355 and D17 cells, while the N2 vector was used for all other cell types. Results are expressed in CFU/ml for the N2 vector and in FFU/ml for the LAPS\textsuperscript{N} vector.
Chapter II

MOLECULAR CLONING OF MUS DUNNI ENDOGENOUS VIRUS, AN UNUSUAL RETROVIRUS IN A NEW MURINE VIRAL INTERFERENCE GROUP WITH A WIDE HOST RANGE

Summary

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. A molecular clone of this virus was isolated, which exhibited no hybridization to any cloned retrovirus examined, suggesting that MDEV has an unusual genome. One copy of a possible retroviral 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.
**Introduction**

Many endogenous retroviruses have been identified in both laboratory and wild mice (19, 44, 107). Much of the interest in these murine viruses has been due to their association with the development of leukemia in laboratory strains of mice (22, 107, 120) and the transformation of cells derived from these mice *in vitro* (17, 124). 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 (43, 44). In addition, the study of endogenous retrovirus-related sequences in wild mice have indicated that some of these elements have a role in preventing infection by exogenous viruses, and consequently, providing resistance to virus-induced disease (44, 45, 60, 69).

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 (10, 19, 119).

Many endogenous viruses, from both laboratory and wild mice, have been identified after their activation following exposure of cells to halogenated pyrimidines (1, 10, 66, 96, 109, 124) as well as after spontaneous activation during cell culture (17, 110). 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 MMTV (22, 57, 91, 107, 120, 121). In wild mice, envelope gene sequences related to the ecotropic, xenotropic, and polytropic classes are present in the germ lines (49, 61), although only an
endogenous ecotropic virus has been induced in a replication-competent form and characterized (21, 118). Although some evidence indicated the presence of endogenous amphotropic viruses in wild mice (50, 101), these were later shown to be absent from the germ line, and to have spread as exogenous viruses in one particular population of mice (90). 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 (10, 17, 19, 66, 84).

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 (73). 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., HDF/MDEV or *M. dunni*/AM-MLV. 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., LAPS(N(MDEV)).

**Cell Culture.** The following cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g glucose per liter and 10% fetal bovine serum (FBS): *M. dunni* tail fibroblasts (gift from Bruce Chesebro, originally described in ref. 20), PA317 amphotropic retrovirus packaging cells (74), PG13 gibbon ape leukemia virus (GALV)-based packaging cells (76), NIH 3T3 thymidine kinase-negative cells (74), C2C12 cells (ATCC CRL 1772), HeLa cells (ATCC CCL 2), D17 cells (ATCC CCL 183), CCC-81 cat
cells transformed with Moloney murine sarcoma virus (41), 5637 human bladder carcinoma cells (ATCC HTB 9), IB3 cells (131), HT1080 cells (ATCC CCL 121), NRK cells (30), and Balb/c 3T3 (B77/OTG) ouabain-resistant HPRT− cells transformed by Rous sarcoma virus (gift from H. L. Ozer). Primary human dermal fibroblasts (HDF) (49) were grown in DMEM with 15% and 10% FBS, respectively. G355 feline embryonic brain cells and G355 cells infected with RD114 endogenous cat virus (33) were grown in McCoy's medium with 15% FBS. CHO-K1 cells (ATCC CCL 61) were grown in αMEM with 10% FBS. HUT 78 cells (ATCC TIB 161) were grown in RPMI 1640 plus 10% FBS. QT35 cells (85) were grown in Ham's F10 medium with 10% tryptose phosphate broth, 1% chick serum, 1% DMSO, 5% FBS, and 2% sodium bicarbonate (7.5% solution).

Viruses. The LAPSN retroviral vector (81) encodes human placental alkaline phosphatase (AP) and neomycin phosphotransferase. LAPSN infection was scored by histochemical staining for AP+ foci of cells two days after infection as described (75). The replication competent viruses used in this study included ecotropic Moloney murine leukemia virus (MoMLV) (pMLV-K, ref. 79), amphotropic (AM-MLV, ref. 74), NZB xenotropic (89), polytropic (MCF) virus strain 98D (23), 10A1 (92), gibbon ape leukemia virus (GALV) SEATO strain (129), RD114 (102), and M. dunni endogenous virus (MDEV) (73). Additional viral elements used in proviral DNA form in hybridization studies include: mouse mammary tumor virus (MMTV) (114), GALV envelope (129), and human endogenous retroviral elements (HERV-K) (68) and HERV-H (126). 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 LAPS+ 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 LAPS+ vector was made in a similar manner following infection of G355/RD114 cells with the helper-free LAPS+ 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/LAPS+MDEV cat cells selected for the production of high titer LAPS+ (10⁶ FFU/ml on *M. dunni* cells), called GL8c16 cells. On day one, GL8c16 cells were seeded at 1×10⁶ cells/10 cm plate. On day three 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 using 0.45 μm bottle top 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; 4 ml per tube) in 6 X 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 removing the supernatant, pellets were resuspended in a total of approximately 500 μl 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 (70) and precipitated at 70°C, overnight. RNA was pelleted in an Eppendorf microcentrifuge at 4°C for 30 minutes, and resuspended in 1 ml distilled water. Polyadenylated RNA was selected using the Mini-Oligo (dT) Cellulose Spin Column Kit (5 prime-3 prime) according to the manufacturer’s instructions. cDNA was reverse transcribed in vitro from viral RNA using the polyadenylated RNA isolated from 400 ml of virus-containing medium per 50 μl reaction. Reaction conditions were similar to those described (5). The reaction mix contained viral RNA in 10 μl 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 of dGTP, dATP, and dTTP, 100 μCi [α-32P]-dCTP (800 Ci/mmol), 500 μg/ml oligo dT primers, 0.5 units/μl Promega RNasin, and 70 units of Moloney murine leukemia virus reverse transcriptase (Stratagene). The reaction was incubated at 37°C for one hour and cDNAs were separated from free nucleotides using 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 one, *M. dunni* cells were seeded at 2 x 10^6 cells per 14 cm-diameter dish in 60 dishes. On day two, the cells were infected with MDEV plus LAPSN virus produced from GL8c16 cells (at an MOI of 2.5 based on the LAPSN vector) with 4 μg/ml Polybrene. 24 hours after infection, extrachromosomal DNA was isolated by the method of Hirt (52). Supercoiled DNA was further purified from contaminating chromosomal DNA by three serial centrifugations in cesium chloride (CsCl) containing ethidium bromide, as described (100). 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 0.8% agarose gel, transferred to a nylon membrane (Hybond) and analyzed by Southern blot using the MDEV plus LAPSN cDNA (described above) as a probe to determine which enzymes would be useful in cloning the MDEV viral DNA. Based on the results, the supercoiled DNA was digested with EcoRI and cloned into the Bluescript plasmid (Stratagene), generating a library with a complexity of 10^5. The library was screened using standard procedures, and positive clones were confirmed by Southern blot analysis (72).

**Endogenous virus activation.** Balb/c 3T3, NIH 3T3, or C2C12 cells were seeded in 6 well plates at 4 x 10^5 cells per 3 cm-diameter well on day one. On day two, duplicate wells were treated with culture medium saturated with IdU, medium containing
90 μM hydrocortisone 21-phosphate (Sigma), or medium alone for 24 h. On day three, 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.

Results

**MDEV is not in the GALV or RD114 retrovirus interference groups.** We have previously shown that MDEV uses a different receptor for entry than those used by other murine leukemia viruses by interference analysis (73). Here we have tested for interference between MDEV and viruses from two additional groups, the gibbon ape leukemia virus (GALV) and the 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* (19,66), and we have previously observed some interference between RD114 and MDEV in cat cells (73). Interference analysis in primary human dermal fibroblasts (HDF) (Table 1.1) 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 inhibited entry by LAPSN virus having 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 (75). 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 1.2 shows the results of additional interference assays performed in D17 canine cells. Prior infection of D17 cells with RD114, spleen necrosis virus (SNV), or Mason Pfizer monkey virus (MPMV, SRV-3) did not interfere with transduction by an MDEV-pseudotype LAPS vector, but all three viruses interfered with transduction by LAPS 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 (58, 59, 117), and show that MDEV is in a different interference group when assayed in canine cells. It was not possible to examine transduction by MPMV-pseudotype LAPS vector in this experiment because this type D virus did not package the MoMLV-based LAPS vector (data not shown, see also ref. 122).

Because of our prior finding that MDEV-pseudotype vectors appeared to infect feline CCC-81 cells poorly, and cat 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 LAPS vector by 5- to 500-fold (Table 1.3 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 (73).

**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 1.4). The titer of LAPSN(MDEV) was between $10^5$ and $6 \times 10^6$ on all cells tested, except for Balb/c 3T3 cells, for which the titer was only 200 FFU/ml. Of the different cell types infected with MDEV, no pathology was observed 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.

**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 (19, 107). 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 detection of replication-competent retroviruses by measurement of LAPSN vector production. Beginning at one 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 in the text 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 seven 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 BIRV (Table 1.5). 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 (23). The strains of polytropic and xenotropic virus used here show some degree of nonreciprocal interference in M. dunnii cells, in that xenotropic virus strongly inhibits polytropic vector transduction (10^3-fold), while polytropic virus much less severely inhibits transduction by xenotropic virus (~40-fold). BIRV is capable of infecting M. dunnii, NIH 3T3, G355, and primary human fibroblasts (Table 1.6), 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 employed 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 using ecotropic Moloney murine leukemia virus (MoMLV), NZB xenotropic virus, gibbon ape leukemia virus (GALV), 98D polytropic virus, mouse mammary tumor virus (MMTV), amphotropic virus (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. dunnii 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 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 Mus dunnii 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 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 (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.

To test whether the retroviral sequence in 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 blot, using the insert from pMDEV9 as a probe (Fig. 2.2). 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.
MDEV is endogenous to the *M. dunni* mouse genome, and related sequences are present in the genome of laboratory strains of mice. To determine whether MDEV was actually present in the germline of the *M. dunni* mouse and not an acquired contaminant of the *M. dunni* cell line, we performed Southern analysis using the viral DNA insert from pMDEV9 as a probe to examine genomic DNA from the *M. dunni* cell line and from 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) were the same in the various samples (Fig. 2.3). Two strongly hybridizing bands were visible in DNA from the *M. dunni* cells and these same bands were present in DNA from both *M. dunni* mice, indicating that MDEV is present in the germline 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 may not have 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. 2.3). 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 kbp. 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. 2.3, data not shown). These data suggest that the 3 and 6 kbp bands may comprise a second, MDEV-related element with a Xhol site in each LTR and an additional, internal Xhol site. This element may also be responsible for the large, somewhat indistinct >23 kbp band seen on the blot of EcoRI-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. 2.3). Bands of identical sizes (approximately 9 kbp) and with the same relative intensities are visible in 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. 2.4). 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, 2X SSC, 0.1% SDS wash). Sequences related to MDEV were only seen 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 germlines.

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 low 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 this virus 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 cat 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 *Mus caroli* interferes with the infectious primate viruses GALV and SSAV (19, 66). 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 *Mus caroli* virus. Some endogenous murine viruses isolated from laboratory mice have neither been cloned nor 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 for many of these murine virus studies (1, 96, 124). Interference analysis showed that BIRV does not interfere with MDEV, and that it most likely belongs to the polytropic group of viruses. These interference results indicate that MDEV uses a novel receptor for cell entry.

Beyond utilizing a unique receptor, MDEV is unusual with regard to its exceptionally large 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 1.4) 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. dunnii* cells using either a reverse-transcriptase-polymerase chain reaction with primers to various parts of the env
gene, which could detect xenotropic, ecotropic, polytropic and modified polytropic viruses (54), or by hybridization using probes for several MLV DNAs (64). Here we have demonstrated that MDEV does not hybridize to the genomes of any of the other viruses tested, including amphotropic, ecotropic, xenotropic, polytropic, 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 3 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.
Table 2.1: Interference properties of MDEV, GALV, amphotropic virus, 10A1, and RD114 measured in human diploid fibroblasts

<table>
<thead>
<tr>
<th>Interfering virus</th>
<th>LAPS N titers (FFU/ml) for the following pseudotypes:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDEV</td>
</tr>
<tr>
<td>none</td>
<td>3x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDEV</td>
<td>200</td>
</tr>
<tr>
<td>GALV</td>
<td>2x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>10A1</td>
<td>5x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>RD114</td>
<td>4x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Uninfected HDF cells and HDF cells infected with the indicated interfering viruses were exposed to LAPS N vectors with the indicated pseudotypes and were stained for AP<sup>+</sup> foci of cells two 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 a second time with similar results.

<sup>b</sup> PG13 refers to a packaging cell line which produces GALV-pseudotype virus.

<sup>c</sup> PA317 refers to a packaging cell line which produces amphotropic-pseudotype virus.
Table 2.2: RD114, SNV, and MPMV do not interfere with transduction by a vector having an MDEV pseudotype in D17 dog cells\(^a\)

<table>
<thead>
<tr>
<th>Interfering virus</th>
<th>LAPSN titer (FFU/ml) for the following pseudotypes:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD114</td>
</tr>
<tr>
<td>none</td>
<td>3x10^5</td>
</tr>
<tr>
<td>RD114</td>
<td>20</td>
</tr>
<tr>
<td>SNV</td>
<td>6x10^4</td>
</tr>
<tr>
<td>MPMV</td>
<td>2x10^4</td>
</tr>
</tbody>
</table>

\(^a\) 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 two days after infection. Results are means of at least two independent determinations, which varied by no more than 60% from the mean.
Table 2.3: RD114 interferes with transduction by a vector having an MDEV pseudotype in G355 cat cells

<table>
<thead>
<tr>
<th>Interfering virus</th>
<th>LAPS N titer (FFU/ml) for the following pseudotypes:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD114</td>
<td>MDEV</td>
</tr>
<tr>
<td>none</td>
<td>$2 \times 10^5$</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>RD114</td>
<td>3</td>
<td>90</td>
</tr>
</tbody>
</table>

* G355 or RD114-infected G355 cat cells were exposed to LAPS N vectors with the indicated pseudotypes and were stained for AP$^+$ foci of cells two days after infection. Results are means of at least two independent determinations, which varied by no more than 67% from the mean, except for the LAPS N(MDEV) infection of G355/RD114 cells, where the LAPS N titer varied from $<2$ to 300 FFU/ml (n=6, mean=90).
Table 2.4: Host range of MDEV

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell Type</th>
<th>LAPSN(MDEV) titer (FFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (wild)</td>
<td><em>Mus dunnii</em></td>
<td>4 X 10^5</td>
</tr>
<tr>
<td>Mouse (laboratory)</td>
<td>NIH 3T3</td>
<td>6 X 10^5</td>
</tr>
<tr>
<td></td>
<td>Balb/c 3T3</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>C2C12</td>
<td>1 X 10^5</td>
</tr>
<tr>
<td>Rat</td>
<td>NRK</td>
<td>2 X 10^5</td>
</tr>
<tr>
<td>Hamster</td>
<td>CHO-K1</td>
<td>5 X 10^6</td>
</tr>
<tr>
<td>Quail</td>
<td>QT35</td>
<td>8 X 10^5</td>
</tr>
<tr>
<td>Cat</td>
<td>CCC-81</td>
<td>5 X 10^5</td>
</tr>
<tr>
<td></td>
<td>G355</td>
<td>4 X 10^6</td>
</tr>
<tr>
<td>Dog</td>
<td>D17</td>
<td>2 X 10^6</td>
</tr>
<tr>
<td>Human</td>
<td>HT1080</td>
<td>5 X 10^6</td>
</tr>
<tr>
<td></td>
<td>IB3</td>
<td>4 X 10^6</td>
</tr>
<tr>
<td></td>
<td>5637</td>
<td>5 X 10^5</td>
</tr>
<tr>
<td></td>
<td>HDF</td>
<td>9 X 10^5</td>
</tr>
<tr>
<td></td>
<td>HeLa^b</td>
<td>5 X 10^5</td>
</tr>
</tbody>
</table>

^a The indicated cells were exposed to MDEV-pseudotype LAPSN and were stained for AP^+ foci of cells two 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/ml) rather than AP^+ foci because HeLa cells have very high levels of endogenous alkaline phosphatase.
Table 2.5: Endogenous virus induced from Balb/c 3T3 cells uses a different receptor than MDEV in *M. dunni* cells*

<table>
<thead>
<tr>
<th>Interfering virus</th>
<th>LAPS N titer (FFU/ml) for the following pseudotypes:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDEV</td>
</tr>
<tr>
<td>none</td>
<td>6x10^5</td>
</tr>
<tr>
<td>MDEV</td>
<td>3</td>
</tr>
<tr>
<td>Polytropic</td>
<td>4x10^5</td>
</tr>
<tr>
<td>NZB</td>
<td>4x10^5</td>
</tr>
<tr>
<td>Amphotropic</td>
<td>4x10^5</td>
</tr>
</tbody>
</table>

*Uninfected *M. dunni* cells and *M. dunni* cells infected with the indicated interfering viruses were exposed to LAPS N vectors with the indicated pseudotypes and were stained for AP*+* foci of cells two 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 a second time with similar results.
Table 2.6: Host range of BIRV

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell Type</th>
<th>LAPS(N(BIRV)) titer (FFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (wild)</td>
<td><em>Mus dunni</em></td>
<td>4x10^4</td>
</tr>
<tr>
<td>Mouse (laboratory)</td>
<td>NIH 3T3</td>
<td>6x10^3</td>
</tr>
<tr>
<td>Cat</td>
<td>G355</td>
<td>5x10^4</td>
</tr>
<tr>
<td>Human</td>
<td>HDF</td>
<td>3x10^3</td>
</tr>
</tbody>
</table>

*The indicated cells were infected with BIRV-pseudotype LAPS(N and were stained for AP foci of cells two days after infection. Shown here are the titers of a bulk population of cells producing BIRV plus LAPS(N. Similar results were obtained using a second, independently IdU-induced bulk population of cells producing BIRV.*
Fig. 2.1: The MDEV cloning strategy.
Fig. 2.2: 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, 0.2X SSC, 0.1% SDS wash) using 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. dunnii cells transduced with the LAPSN vector (lane 1), M. dunnii cells infected with AM-MLV and LAPSN (lane 2), and uninfected G355 cells (lane 4). Cells producing MDEV included M. dunnii cells infected with MDEV plus LAPSN vector (lane 3) and G355 cells infected with MDEV plus LAPSN vector (lane 5).
Fig. 2.3: 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 *EcoRI* (which cuts once within MDEV, left panels) or *XhoI* (which cuts once within each LTR, right panel) and were analyzed by Southern blot hybridization at high stringency (68°C, 0.1X SSC, 0.1% SDS wash) using the 9.4 kbp insert from pMDEV9 as a probe. The first four lanes of the left panels represent a two day film exposure, while lanes 5 and 6 (containing mouse tail DNA) were from the same blot but were overloaded, so a one 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 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 *XhoI* Southern blot.
Fig. 2.4: Zoo blot analysis for the presence of MDEV-related sequences. Genomic DNA samples (10 μg per lane) were digested with EcoRI, which cuts once within MDEV, and were analyzed by Southern blot hybridization at low stringency (40°C, 2X SSC, 0.1% SDS) using the 9.4 kbp insert from pMDEV9 as a probe. Cells not defined in materials and methods: RM2, primary rat myoblasts (14); BBM, primary baboon bone marrow (gift from J. Potter).
Chapter III

MURINE RETROVIRUSES USE AT LEAST SIX DIFFERENT RECEPTORS FOR ENTRY INTO MUS DUNNI CELLS

Summary

Murine retroviruses have been divided into 6 interference groups that use different receptors for cell entry; the ecotropic, xenotropic, polytropic, amphotropic, 10A1, and Mus dunni endogenous virus (MDEV) groups. Some interference is observed between xenotropic and polytropic viruses, and between amphotropic and 10A1 viruses, indicating some overlap in receptor specificity between these groups, but otherwise these interference groups appear completely independent. In contrast, one study found interference among many of these groups when assayed in Mus dunni wild mouse cells using an immunofluorescence assay to detect infection by the challenge virus. Here we have used a more direct assay for cell entry using pseudotyped retroviral vectors to measure interference in M. dunni cells, and find no evidence for extensive interference between members of different murine retrovirus groups. Indeed, our results in M. dunni cells are consistent with interference results observed in other cell types, and indicate that the anomalous interference results previously observed in M. dunni cells using the immunofluorescence assay were most likely due to factors other than those that affect receptor-mediated virus entry. In summary, our results show that murine retroviruses use at least six different receptors for entry into M. dunni cells.
Introduction

Retroviruses can be divided into interference groups based on their ability to inhibit infection by other retroviruses. Interference is due to synthesis of retroviral envelope protein in infected cells, which binds to specific receptor proteins and blocks infection by retroviruses that use the same receptors for cell entry. The murine retroviruses fall into six interference groups, the ecotropic, xenotropic, polytropic, amphotropic, 10A1, and Mus dunni endogenous virus (MDEV) groups (27,73,103,104). The 10A1 and amphotropic groups are not completely independent, but display a pattern of nonreciprocal interference; 10A1 virus blocks entry by amphotropic virus, but amphotropic virus does not block entry of 10A1 virus (104). The basis for this phenomenon is that amphotropic viruses use a single receptor for cell entry, designated Pit2 (Ram1), while 10A1 can use both Pit2 and the related receptor Pit1 (Glvr1) (83). The xenotropic and polytropic viruses also display nonreciprocal interference and thus have overlapping receptor specificity (27). The receptors for these viruses are apparently encoded by different alleles of the same locus on mouse chromosome 1 (62), but the receptors have not yet been molecularly cloned.

While interference analysis in mink, human, and laboratory mouse cells yields a similar interference grouping for the mouse retroviruses, results reported for Mus dunni wild mouse cells using immunofluorescence assay are quite different, and show interference between viruses that do not interfere when tested in other cells (23). For example, entry of an ecotropic retrovirus was reduced by 100-fold by prior infection of M. dunni cells with an amphotropic retrovirus, in contrast to results in other cell types where these viruses show no interference. It is therefore possible that these viruses use similar receptors in M. dunni cells, even though the receptors for ecotropic and amphotropic retroviruses have been cloned from laboratory mice and are unrelated. Alternatively, the recent discovery of coreceptors required for HIV entry into cells (6,38) suggests that these viruses may share a coreceptor required for entry into M. dunni cells, and the effect is not observed in other
cells because of the presence of different coreceptors or an excess of the required coreceptor. Thus the unique interference properties of murine retroviruses in *M. dunni* cells might provide important insights into factors affecting retrovirus entry into cells. We have therefore reinvestigated interference of murine retroviruses in *M. dunni* cells using retroviral vectors.

**Materials and Methods**

**Nomenclature.** Cells that contain a retroviral vector are indicated by the cell name followed by a slash and the name of the vector, e.g., *dunni/LN*. A retroviral vector in its viral form is indicated by the vector name followed, in parentheses, by the name of the helper virus or packaging cells used to pseudotype the vector, e.g., LAPSN(10A1) or LAPSN(PA317). In this report, the pseudotype of a retroviral vector refers only to the viral envelope protein present on the vector virions that determines the cell-surface receptor utilization pattern of the vector, irrespective of the origin of the Gag and Pol proteins. For example, vectors produced by PA317 cells will be referred to as having an amphotropic pseudotype due to the presence of amphotropic Env protein in the virions, even though the Gag and part of the Pol proteins in these virions are derived from Moloney murine leukemia virus (MoMLV) (74).

**Retroviruses.** The following replication-competent retroviruses were obtained from Bruce Chesebro (23): 1387, MoMLV strain 1387; AKR623, AKR MLV strain 623; 1E, Friend MCF strain 1E; 4070A and 1504A, amphotropic MLV strains 4070A and 1504A (23). The MLV-K strain of MoMLV was obtained from NIH 3T3 cells transfected with the pMLV-K clone of MoMLV (79). NZB xenotropic virus was obtained from *M. dunni* cells transfected with the NZB molecular clone NZB 9-1 (89) after cutting the plasmid with *EcoRI* and religating the DNA to generate intact NZB provirus circles. 10A1 virus was obtained from NIH 3T3 cells transfected with the permuted 10A1 virus DNA
clone pB6 (92) after cutting the plasmid with SalI and religating the DNA to generate intact 10A1 provirus circles. The LN retroviral vector encodes neomycin phosphotransferase (78), and the LAPSN vector encodes neomycin phosphotransferase and human placental alkaline phosphatase (AP) (81).

**Cell culture.** *M. dunnii* tail fibroblasts (dunni cells) (64) were grown in Dulbecco’s modified Eagle’s medium with 4.5 g/l glucose and 5% fetal bovine serum at 37°C in a 10% CO₂/air atmosphere. PE501 ecotropic (78), PM571 polytropic (82), and PA317 amphotropic (74) retrovirus packaging cell lines were grown under similar conditions except that 10% fetal bovine serum was used. Dunni/LN and dunni/LAPSN cells were made by transduction with helper-free vector stocks produced by PA317 retrovirus packaging cells containing the vectors. The transduced cells were selected in G418 to ensure that all cells expressed the vectors. Dunni/LN and dunni/LAPSN cells were infected with replication-competent viruses by seeding the cells the day before infection at 10⁵ per 6 cm dish, infecting the cells with 100 μl virus stock in the presence of 4 μg/ml Polybrene (Sigma), and passaging the cells for ≥11 days in the absence of Polybrene to allow complete virus spread. Virus was harvested from confluent layers of cells 16 h after a medium change and frozen at -70°C. Helper-free retroviral vectors were generated from retrovirus packaging cells as previously described (78). The envelope protein in PE501 cells is from MoMLV molecular clone pMLV-K, that in PM571 is from Friend MCF strain 98D, and that in PA317 cells is from amphotropic MLV strain 4070A.

**Interference assays.** Interference assays were performed as follows. On day one, infected and uninfected dunni/LN cells were seeded at 5 x 10⁴ per 3 cm-diameter well of 6 well culture trays. On day two, the medium was replaced with 2 ml medium containing 4 μg Polybrene per ml and the pseudotyped LAPSN vectors were added. On day 4 the cells were stained for AP⁺ foci as described (40). Results are expressed as the
log₁₀ of the fold interference, or log₁₀[(LAPSN vector titer on uninfected dunnī/LN cells)/(LAPSN vector titer on dunnī/LN cells infected with the interfering virus)].

Results

Use of retroviral vectors or immunofluorescence assays show different results in interference experiments in dunnī cells. Table 3.1 depicts key results from the previous experiments (23) in which viruses assigned to different interference groups exhibit interference in M. dunnī cells (underlined values). Results are displayed as the log₁₀ of the fold-interference, and values of 2 or greater were considered by the authors to indicate interference. Ecotropic virus 1387, but not ecotropic virus AKR623, interfered with polytropic viruses 98D and 1512, and both ecotropic viruses interfered with infection by xenotropic virus AKR6. Amphotropic virus 4070A, but not amphotropic virus 1504A, interfered with xenotropic virus AKR6 and ecotropic virus 1387. Polytropic virus 1E interfered with ecotropic virus 1387 and with xenotropic virus AKR6. Challenge by amphotropic viruses was not analyzed in this report because of the lack of amphotropic virus-specific antibodies, which were required for virus detection in the immunofluorescence assay used.

We obtained the M. dunnī tail fibroblasts used in the previous report (23), and transduced the cells with the LN retroviral vector (78), that expresses neomycin phosphotransferase, or with the LAPSN vector (81), that expresses neomycin phosphotransferase and human placental alkaline phosphatase (AP), by using helper virus-free vector stocks made from PA317 retrovirus packaging cells (74) containing the vectors. As expected, the dunnī/LN and dunnī/LAPSN cells did not produce the vectors in the absence of added replication-competent virus (data not shown). We also obtained the original retroviruses used in the previous study (23) and used these to infect the dunnī/LN and dunnī/LAPSN cells. The presence of replication-competent retrovirus was confirmed
in all cases by measurement of the production of the LN or LAPS N vectors. Interference assays were performed by exposing infected or uninfected dunni/LN cells to pseudotyped LAPS N vector produced from infected dunni/LAPS N cells and by measuring transduction (gene transfer and expression) by the LAPS N vector by staining for foci of AP+ cells. The presence of the LN vector in the target cells does not interfere with transduction by the LAPS N vector or with detection of AP expression, and was only included to allow detection of the presence of the interfering viruses by measurement of LN vector production.

The results of our interference analysis (Table 2.2) are inconsistent with the prior results obtained in M. dunni cells (Table 2.1), but are consistent with previously determined interference groupings in other cell types. Interference between viruses in the same group ranged from 20- to 300,000-fold, while interference between viruses from different groups was <10-fold. Disregarding the results obtained when the interfering virus was 1387 (see Discussion), and the results showing partial interference between polytropic and xenotropic viruses, which were expected based on previous work in cells other than M. dunni cells (27), the difference between intragroup interference (160- to 300,000-fold) and intergroup interference (<2-fold) is even more dramatic.

Interference analyses with additional pseudotypes of the LAPS N vector confirmed the interference group assignments of the replication-competent viruses used in this study (Table 2.2). LAPS N virus produced by PE501 ecotropic packaging cells (78) behaved like the ecotropic LAPS N(1387) virus. PE501 cells were constructed by using the pMLV-K clone of MoMLV (79), and 1387 virus represents a different isolate of MoMLV. LAPS N produced by PM571 Friend MCF strain 98D-based packaging cells (82) behaved like the other polytropic-pseudotype LAPS N vectors, although the interference observed for LAPS N(PM571) in M. dunni cells infected with 1E virus was not as great as observed for the other polytropic viruses. LAPS N pseudotyped with the xenotropic NZB virus (89)
behaved like the xenotropic AKR6 pseudotype. LAPSN(PA317) behaved like the other amphotropic pseudotypes of the vector, but the best correlation was with LAPSN(4070A), which was expected since PA317 cells were constructed by using env from a molecular clone of 4070A.

The results obtained when the interfering virus was 1387 were somewhat anomalous. The positive-control interference observed with 1387 was lower than that observed with other viruses, yet all retroviral titers were somewhat reduced on the cells infected with 1387. We noticed that the 1387 virus-infected durni/LN cells grew slowly, displayed many multinucleated cells, and exhibited high rates of cell death, a phenomenon that persisted even with prolonged cultivation. To test whether the behavior of *M. dunni* cells infected with 1387 virus was a peculiar property of this strain of MoMLV, or perhaps was due to a contaminating virus present in the virus preparation, we infected *M. dunni* cells in parallel with 1387 virus harvested from 1387 virus-infected durni/LAPSN cells, or with virus harvested from NIH 3T3 cells transfected with a DNA clone of MoMLV, pMLV-K (79), and observed the cells during several passages in culture. The presence of large multinucleated cells that increased in number with time was observed in *M. dunni* cells infected with either virus preparation, although it was most pronounced in cells infected with 1387 virus (data not shown). These results indicate that the toxicity and cell fusion observed in 1387 virus-infected *M. dunni* cells is not due to a contaminant in the 1387 virus stock, but is typical of MoMLV infection in *M. dunni* cells.

**Different strains of dunni cells are available.** Several investigators have found that *M. dunni* tail fibroblasts are poorly infected by MoMLV and by vectors with an MoMLV pseudotype, even though these cells can be efficiently infected by other ecotropic viruses (34,35,64). In contrast, we found that *M. dunni* cells were relatively efficiently infected by the LAPSN vector with a 1387 (MoMLV) pseudotype, the apparent titer on *M. dunni* cells being 10⁶ FFU/ml (Table 2.2). There are two types of *M. dunni* tail fibroblasts
in common use, and the use of different cells by different labs might explain the these results. The type used here and by Chesebro and Wehrly (23) can be recognized by the unusual characteristic that confluent layers of the cells secrete molecules that render the culture medium highly viscous, a property that is not shared with the *M. dunni* cells used in other studies (34,35), and we will refer to the strains as dunni-v and dunni-nv (to denote viscous and non-viscous medium) respectively.

We obtained some dunni-nv cells (gift from Carolyn Wilson, originally from Janet Hartley; ref. 34) for direct comparison with the dunni-v cells used here (Table 2.3). The apparent titer of the PE501 (MoMLV) pseudotype LAPSN vector was similar in both dunni cell strains, but was up to 250-fold lower in dunni cells than in NIH 3T3 cells. The titer of the 1387 (MoMLV) pseudotype LAPSN vector was also similar on both dunni cell strains, but was reduced by only about 6-fold in dunni cells compared with NIH 3T3 cells. For comparison, the titer of amphotropic LAPSN vector was similar in both *M. dunni* cell strains and in NIH 3T3 cells. The ecotropic virus AKR623, which is not an MoMLV strain, promoted LAPSN infection of dunni-nv cells at the same rate as NIH 3T3 mouse cells, but at a 20-fold lower rate in dunni-v cells. Thus, while there are virus strain- and cell strain-dependent variations in ecotropic-pseudotype vector infection of *M. dunni* cells, both *M. dunni* cell lines can be infected by virions carrying the MoMLV envelope. The markedly lower rate of infection in *M. dunni* cells in comparison to NIH 3T3 cells seen for one strain of MoMLV is consistent with prior observations (34,35,64).

To determine whether both dunni lines are properly identified as being from *Mus dunni* mice, we performed Southern analyses using a probe from MDEV, which is only known to be present in *Mus dunni*. The blot shows identical patterns of bands using DNA from dunni-nv or dunni-v cells (Fig. 3.1). The different phenotypes of the two *M. dunni* cell strains are probably due to the fact that the original isolate (64) that was distributed to many groups was not cloned before distribution (Sisir Chattopadhyay, personal
communication), and it appears that different culture conditions allowed the outgrowth of different subpopulations of *M. dunni* cells.

**Murine retroviruses use at least six different receptors in Mus dunni cells.** Finally, we have examined interference between representatives of all of the MLV interference groups in *M. dunni* (dunni-v) cells (Table 3.4). Ecotropic and MDEV viruses show no interference with any of the other groups. 10A1 and amphotropic viruses show nonreciprocal interference, but no interference with any of the other groups. Similarly, polytropic and xenotropic groups show nonreciprocal interference, but no interference with any other groups.

**Discussion**

Retroviral interference experiments have provided a useful means to examine receptor usage among retroviruses. Interference experiments have been generally consistent when performed in various cell types, but one report of interference in *Mus dunni* cells showed unusual interference patterns (23). For example, interference was demonstrated between amphotropic and ecotropic MLVs. However, both the amphotropic and ecotropic receptors have been cloned since the report was made, and the receptors are not related; the amphotropic receptor is a phosphate transporter, and the ecotropic receptor is a cationic amino acid transporter. Thus, it was possible that a previously unrecognized aspect of viral interference could be studied in dunni cells. Alternatively, these experiments in dunni cells could provide the first evidence of coreceptor usage by MLVs, as is the case for HIV. It is possible that there is overlap of coreceptor usage among the MLVs only in dunni cells, or that all MLVs use the same coreceptor in all cells but it is limiting only in dunni cells.

An indirect focal immunofluorescence assay was used in the previous report (23) to detect infection of the challenge retroviruses. This assay is dependent on spread of the
challenge virus to generate a focal area of cells reactive with antibody against the challenge virus, and therefore is dependent on multiple events in addition to receptor-mediated virus entry. To circumvent this complication, we used pseudotypes of a retroviral vector encoding human placental alkaline phosphatase (AP) to study interference in M. dunni cells. Using this more direct assay, we find that interference patterns in M. dunni cells are similar to those reported for other cells.

Somewhat anomalous results were observed with the ecotropic MoMLV strain 1387. We saw lower interference than expected for the positive ecotropic controls on dunny/LN+1387 cells, yet the infection rate was somewhat reduced for all other retroviruses. Cell death, indicating toxicity, was also observed in the dunny/LN+1387 cells. Thus the reason that infection rates observed for non-ecotropic LAPSN pseudotypes in 1387 virus-infected dunny/LN cells were low compared to those obtained in uninfected dunny/LN cells is most likely due to cell death and slow growth of the 1387 virus-infected dunny/LN cells rather than interference by the 1387 virus. In addition, the continued presence of multinucleated cells in the dunny/LN cells infected with 1387 virus indicates that the receptor used by 1387 virus is not effectively blocked by the virus, resulting in continued virus-mediated cell fusion, and provides an explanation for the poor interference of the 1387 virus with ecotropic pseudotype LAPSN viruses.

We have previously determined that dunny-v cells are most likely derived from Mus dunni (also known as Mus terricolor) animals, and are not cells from another species due to culture contamination or mistaken identification. This conclusion depends on Southern analyses of restriction enzyme-digested DNA samples from these M. dunni cells and from M. dunni mice, which show identical patterns of bands that hybridize to a probe made from the relatively unique endogenous virus MDEV. DNA samples from cell lines derived from laboratory mice show a different pattern of weakly-hybridizing bands, and DNA samples from cells of other species show no hybridization to an MDEV probe (15). Here we have
performed southern analysis on DNA from both dunni-v and dunni-nv cells, and show that both are most likely derived from *Mus dunni*.

In summary, the reason for the more complicated interference patterns previously observed among these virus groups in *M. dunni* cells (23) is likely due to the assay employed, which required spread of the challenge virus after infection to produce a signal, and virus spread might have been inhibited by the interfering virus. Alternatively, detection of the challenge virus with antibodies may have been difficult due to background staining of the interfering virus that was present in all cells. The assay used here more directly measures virus entry and does not require virus spread. Our results provide no evidence for altered receptor utilization or coreceptor usage in *M. dunni* cells, and we conclude that murine retroviruses can be divided into six interference groups that use at least six different receptors for entry into *M. dunni* cells.
Table 3.1: Interference of murine retroviruses in *M. dunni* cells measured by immunofluorescence assay

<table>
<thead>
<tr>
<th>Challenge MLV</th>
<th>Interfering MLV</th>
<th>Ecotropic</th>
<th>Polytropic</th>
<th>Amphotropic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>1387</td>
<td>AKR623</td>
<td>1E</td>
</tr>
<tr>
<td>Ecotropic</td>
<td>1387</td>
<td>nd</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Polytropic</td>
<td>1E</td>
<td>1.7</td>
<td>1.1</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>98D</td>
<td>3.0</td>
<td>0.9</td>
<td>&gt;3</td>
</tr>
<tr>
<td></td>
<td>1512</td>
<td>&gt;2.5</td>
<td>1.3</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Xenotropic</td>
<td>AKR6</td>
<td>&gt;3.2</td>
<td>&gt;3.5</td>
<td>&gt;3.4</td>
</tr>
</tbody>
</table>

\(^a\) Results are derived from Table 5 of ref. 23 and are displayed as the log\(_{10}\) of the fold interference, or log\(_{10}\) [(challenge virus titer on uninfected cells)/(challenge virus titer on cells infected with the interfering virus)]. Numbers in bold indicate interference results between viruses in the same group, and underlined results indicate interference between viruses in different groups.

\(^b\) Not determined.
Table 3.2. Interference of MLV in *M. dunni* cells measured by marker transfer assay$^a$

<table>
<thead>
<tr>
<th>LAPS pseudotype</th>
<th>Titer [log(FFU/ml)]</th>
<th>1387</th>
<th>AKR623</th>
<th>1E</th>
<th>4070A</th>
<th>1504A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecotropic</td>
<td></td>
<td>6.0</td>
<td>1.4</td>
<td>2.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Poly tropic</td>
<td></td>
<td>4.1</td>
<td>1.3</td>
<td>3.6</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>1E</td>
<td></td>
<td>7.0</td>
<td>0.3</td>
<td>nd</td>
<td>4.5</td>
<td>nd</td>
</tr>
<tr>
<td>PM571</td>
<td></td>
<td>5.0</td>
<td>0.7</td>
<td>0.0</td>
<td>2.2</td>
<td>0.0</td>
</tr>
<tr>
<td>1512</td>
<td></td>
<td>5.5</td>
<td>0.6</td>
<td>0.0</td>
<td>5.5</td>
<td>nd</td>
</tr>
<tr>
<td>Xenotropic</td>
<td></td>
<td>6.8</td>
<td>0.5</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>AKR6</td>
<td></td>
<td>6.7</td>
<td>0.5</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>NZB</td>
<td></td>
<td>6.8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>5.2</td>
</tr>
<tr>
<td>Amphotropic</td>
<td></td>
<td>6.3</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>5.3</td>
</tr>
<tr>
<td>4070A</td>
<td></td>
<td>7.0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$^a$The LAPS pseudotype with the indicated pseudotypes was used to infect dunni/LN cells or dunni/LN cells infected with the indicated interfering MLVs, and the apparent vector titers in FFU/ml were determined. The vector titer is expressed in the Table as the log$_{10}$ of the mean titer determined on dunni/LN cells, and is an arithmetic mean of at least two experiments. Interference values are expressed as the log$_{10}$ of the fold interference, or log$_{10}$[(LAPS titer on uninfected dunni/LN cells)/(LAPS titer on dunni/LN cells infected with the interfering virus)]. The fold interference was calculated as the arithmetic mean of at least two independent determinations, which varied by no more than 58% from the mean. Bold values indicate interference between viruses in the same interference group.

$^b$Not determined
Table 3.3: Ecotropic vector titers measured on *M. dunni* cells from different sources.

<table>
<thead>
<tr>
<th>LAPSN pseudotype</th>
<th>Group</th>
<th>Strain</th>
<th>Expt.</th>
<th>Titer (FFU/ml) on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dunni-v</td>
</tr>
<tr>
<td>Ecotropic</td>
<td>PE501</td>
<td>1</td>
<td></td>
<td>5 x 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>3 x 10^4</td>
</tr>
<tr>
<td></td>
<td>1387</td>
<td>1</td>
<td></td>
<td>6 x 10^5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>1 x 10^6</td>
</tr>
<tr>
<td></td>
<td>AKR623</td>
<td>1</td>
<td></td>
<td>2 x 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>2 x 10^4</td>
</tr>
<tr>
<td>Amphotropic</td>
<td>PA317</td>
<td>1</td>
<td></td>
<td>2 x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>5 x 10^5</td>
</tr>
</tbody>
</table>

Cells were seeded at 10^5 per 3.5 cm-diameter well of 6 well culture trays one day prior to infection and stained for AP<sup>+</sup> foci as described (40) two days after infection. Each value is the mean of duplicate assays which varied by no more than 27% from the mean.
Table 3.4: Interference of Representatives of Six Murine Leukemia Virus Groups In *M. dunni* Cells$^a$

<table>
<thead>
<tr>
<th>LAPSN pseudotype</th>
<th>Titer [log(FFU/ml)]</th>
<th>Interfering MLV</th>
<th>Ecotropic AKR623</th>
<th>Polytropic 1E</th>
<th>Xenotropic NZB</th>
<th>Amphotropic 4070A</th>
<th>10A1</th>
<th>MDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecotropic</td>
<td>PE501</td>
<td>3.8±0.1</td>
<td>&gt;3.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Polytropic</td>
<td>1E</td>
<td>7.2±0.1</td>
<td>-</td>
<td>&gt;6.4</td>
<td>5.9±0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xenotropic</td>
<td>AKR6</td>
<td>6.7±0.1</td>
<td>-</td>
<td>1.0±0.3</td>
<td>6.0±0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amphototropic</td>
<td>PA317</td>
<td>6.0±0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.6±0.1</td>
<td>5.9±0.3</td>
<td>-</td>
</tr>
<tr>
<td>10A1</td>
<td>PT67</td>
<td>6.3±0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.8±0.0</td>
<td>6.3±0.1</td>
<td>-</td>
</tr>
<tr>
<td>MDEV</td>
<td>MDEV</td>
<td>6.0±0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.5±0.8</td>
</tr>
</tbody>
</table>

$^a$The LAPSN vector with the indicated pseudotypes was used to infect dunni/LN cells or dunni/LN cells infected with the indicated interfering MLVs, and the apparent vector titers in FFU/ml were determined. The vector titer is indicated in the Table as the log$_{10}$ of the titer determined on dunni/LN cells, and interference values are expressed as the log$_{10}$ of the fold interference, or log$_{10}[(\text{LAPSN titer on uninfected dunni/LN cells})/(\text{LAPSN titer on dunni/LN cells infected with the interfering virus})]$. All values are means of the log$_{10}$ values ± SD from at least two independent experiments with duplicate determinations performed in each experiment. Interference values of ±0.3 (±2-fold interference) are indicated by (-) to indicate a lack of interference. These negative values ranged from -0.2 to 0.3 with standard deviations ±0.1.
<table>
<thead>
<tr>
<th>Size markers (kbp)</th>
<th>EcoRI</th>
<th>XhoI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dunni-nv</td>
<td>dunni-nv</td>
</tr>
<tr>
<td></td>
<td>dunni-v</td>
<td>dunni-v</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td></td>
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</tr>
<tr>
<td>4.4</td>
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<tr>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3.1: Southern analysis of DNA from dunni-v and dunni-nv cells shows a similar pattern of fragments that hybridize to MDEV. Cellular DNA was digested with EcoRI or XhoI and 10 μg samples were analyzed using a radiolabeled SalI fragment from pMDEV9 (15) that contains MDEV gag, pol, and env sequences. The positions and sizes of HindIII-digested lambda phage DNA markers are indicted at left.
Chapter IV

SEQUENCE ANALYSIS OF MUS DUNNI ENDOGENOUS VIRUS (MDEV) REVEALS A HYBRID VL30/GALV-LIKE STRUCTURE AND A DISTINCT ENVELOPE

Summary

Mus dunni endogenous virus (MDEV) can be activated from Mus dunni cells by exposing the cells to hydrocortisone or 5-iodo-2’-deoxyuridine. Interference analysis reveals that MDEV uses a different receptor for cell entry than those used by other murine retroviruses. The entire genome has now been sequenced, revealing an LTR-gag-pol-env-LTR structure typical of simple retroviruses of the murine leukemia virus genus, with no additional open reading frames between env and the LTR. The LTRs and other noncoding regions of MDEV are most closely related to those of VL30 elements, while the majority of the coding sequences are most closely related to those of gibbon ape leukemia virus (GALV). MDEV represents the first example of a naturally-occurring, replication-competent virus with sequences closely related to VL30 elements. The U3 region of MDEV contains six near perfect 80 bp repeats and the beginning of a seventh, and the region expected to contain the packaging sequence contains approximately four imperfect 33 bp repeats. The receptor-specificity domains of the envelope are unique among retroviruses, and show no apparent similarity to known proteins.

Introduction

Mus dunni endogenous virus (MDEV) is transcriptionally inactive in cultured Mus dunni cells (dunni cells), but can be activated by treatment of the cells with either
hydrocortisone or 5-ido-2'-deoxyuridine (73). Activation can be easily measured because MDEV can package Moloney murine leukemia virus (Mo-MLV)-based retrovirus vectors. Once activated, MDEV will continue to replicate in dunni cells and can infect many other cell types (15). MDEV is endogenous to Mus dunni wild mice (also known as Mus terricolor) at a proviral copy number of 1-2 per genome, and there is at least one other element in dunni cells that hybridizes to MDEV probes but has a different restriction map (15). MDEV has not been found in the genomes of Asian mice related to Mus dunni or any other species (ref. 4 and unpublished results). It is unknown whether MDEV causes pathology or is ever activated in Mus dunni mice.

MDEV does not interfere with known murine leukemia viruses (MLVs), indicating that it uses a different receptor for cell entry (80). MDEV also does not interfere with some nonmurine retroviruses such as gibbon ape leukemia virus (GALV) (15). The endogenous cat retrovirus RD114 was found to interfere at a low level with MDEV, but this interference was observed in only one cell line (G355 cat glial cells) (15). Furthermore, this interference was weak and variable from one experiment to another, making it unclear whether MDEV and RD114 share a receptor in G355 cells. Other members of the RD114 interference group, such as SNV or MPMV, have not been found to interfere with MDEV (15). The MDEV receptor is widely expressed among species, as indicated by the ability of a retroviral vector pseudotyped by MDEV to transduce cells from many species (15).

Molecular clones of MDEV were obtained to study its genome and receptor usage (15). However, the clones were unable to produce infectious virus after transfection into permissive cells. Here we describe the correction of a clone that renders it infectious, and show that the resulting virus is in the same interference group as the biological isolates. In addition, we have determined the entire sequence of MDEV and describe some unique features of the MDEV genome.
Materials and Methods

Nomenclature. Cells that contain a retroviral vector and/or contain and express a retrovirus are indicated by the cell name followed by a slash and the names of the vectors or viruses, e.g., G355/LAPSN for G355 cells containing the retroviral vector LAPSN or dunni/N2+activated MDEV for Mus durnii cells that contain both the N2 vector and MDEV activated with hydrocortisone. LAPSN(PA317) refers to the viral form of the LAPSN retroviral vector packaged by PA317 cells, which express the amphotropic MLV envelope.

Cell culture. G355 feline embryonic glial cells (33) were grown in McCoy's medium with 15% fetal bovine serum (FBS). D17 dog cells (ATCC CCL 183), 293 human kidney cells (47), and Mus durnii (64) cells were grown in Dulbecco's modified Eagle's medium with 10% FBS. There are two Mus durnii cell strains available which originated from the same mouse, and they can be distinguished by whether medium exposed to the cells becomes viscous (80). Dunni-v cells (those that make the medium viscous, as opposed to dunni-nv cells) can be activated to produce MDEV and were used in these experiments.

Dunni/N2 cells infected with activated MDEV were prepared by exposing dunni/N2 cells to 90 μM hydrocortisone sodium succinate. Dunni/N2 cells infected with virus generated from pMDEV were prepared by transfecting pMDEV into G355/LAPSN cells, passaging the cells in the presence of 4 μg Polybrene per ml for 24 d to allow for viral spread, and transferring limiting dilutions of harvested medium to dunni/N2 cells. After passage, the resulting dunni/N2+MDEV cells were stained for alkaline phosphatase-positive (AP+) foci to confirm the absence of cells transduced by contaminating LAPSN vector. In preparation of dunni/N2 cells infected with virus derived from pMDEV, the cells
were not exposed to hydrocortisone or 5-iodo-2'-deoxyuridine and so were not expressing the endogenous MDEV.

**Retroviral vectors.** LAPSN is an Mo-MLV-based retroviral vector that contains a human placental alkaline phosphatase reporter gene and a neomycin phosphotransferase gene (81), and N2 is a similar vector that contains a neomycin phosphotransferase gene (4).

**MDEV molecular clones.** A circular DNA form of MDEV, with 2 adjacent LTRs, was cut in the pol gene with EcoRI and cloned into the EcoRI site of pBluescriptII KS+ (pBSII KS+) (Stratagene) to generate pMDEV9 (15), the prototype MDEV clone that was sequenced and from which other MDEV plasmids were derived. A frameshift mutation in the env gene of pMDEV9 was corrected by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and primers of the sequence 5'-CAG GGT CAG AAA GGA AAG CTG CAA CAA GAA TG-3' and 5'-CAT TCT TGT TGC AGC TTT CCT TTC TGA CCC TG-3'. The frameshift correction was confirmed by sequencing. Finally, a 13 kb plasmid with an intact (depermuted) MDEV genome was constructed, called pMDEV. MDEV sequences in this pUC19-based plasmid extend from a SalI site in env, through a single 5' LTR, the entire region between the LTRs, and a single 3' LTR, and continue to the SalI site in gag.

**PCR.** To analyze the envelope frameshift mutation, PCR was performed with Taq polymerase (Promega) according to the manufacturer's instructions with the primers 5'-TTGGTGGCCTGTACTCCACACCTG-3' and 5'-CTCTCCTATTTTGCACTACTACCTC-3'. Thermocycling was done with one cycle of 10 min at 95°C; 30 cycles of 1 min at 94 °C, 1 min at 71°C, and 2 min at 72°C; and one cycle of 5 min at 72°C. Templates included genomic DNA from unactivated *Mus dunni* cells and G355/LAPSN+MDEV cells, as well as negative controls such as DNA from uninfected G355 cells and no template. The
PCR products were cloned into pT7Blue (Novagen) according to the manufacturer's protocol. Several clones from separate PCR amplifications of various templates were sequenced.

**Virus assays.** Marker rescue assays were employed to determine whether the MDEV molecular clones were capable of producing infectious virions. G355/LAPSN cells or 293/LAPSN cells were plated at $2 \times 10^5$ cells per 3.5 cm well of 6-well plates on day 1. On day 2, $5 \mu$g of plasmid DNA was transfected into the cells by calcium phosphate precipitation. On day 3, the medium was replaced. On day 4, the medium was harvested, clarified by centrifugation at 4,000 x g for 5 min at 4°C, and stored at -80°C prior to assay for the LAPSN vector.

LAPSN vector titer was determined by plating target cells at $1 \times 10^5$ or $2 \times 10^5$ cells per 3.5 cm diameter well of a 6-well dish on day 1. On day 2, the medium was replaced with medium containing $4 \mu$g of Polybrene per ml, and the virus stock added. On day 4, the cells were stained for AP$^+$ foci as described (40), and virus titers are expressed as AP$^+$ foci per ml of test medium.

**DNA sequencing.** Portions of the MDEV clone pMDEV9 were subcloned into pBSII KS+ and deletions were made with exonuclease III using the method of Clark and Henikoff (25). Sequencing was performed on the nested deletion constructs using dye primer ABI PRISM sequencing kits and analyzed with a 373A DNA sequencer and sequence analysis software (Applied Biosystems, Foster City, CA). Regions of poor sequence were resequenced after making appropriate primers and using the ABI PRISM dye terminator kits. Both strands of MDEV were completely sequenced, and the sequences were assembled into contigs and further analyzed using Sequencher 3.0 (Gene Codes Corporation, Inc., Ann Arbor, MI).
We have numbered the nucleotides of the MDEV genome beginning with the presumptive cap site, the first nucleotide of the R region. This putative cap site was identified by comparison of MDEV to the VL30 element VL3 (accession X03489) in which the cap site has been mapped (108). The complete nucleotide and polyprotein sequences of MDEV have been deposited into GenBank under accession number AF053745.

**Sequence analysis.** To elucidate the nature of the breakpoint regions, alignments were created between MDEV, VL30 BVL-1, and GALV using the MACAW program (113). To infer phylogenetic relationships of the MDEV sequences to other retroelements and retroviruses, Clustal W was used to make sequence alignments, create phylogenetic trees, and to evaluate the trees by bootstrap analysis (123). Clustal W creates alignments using a progressive pairwise method and creates phylogenetic trees with a neighbor-joining method. When sequences were of different lengths due to incomplete sequence data, as for the PERVs in the pol tree, positions with gaps were excluded in the phylogenetic analysis so that only those regions for which all sequences are represented were analyzed. TreeView (95) and Canvas (Deneba Software, Miami, FL) were used to view and print the phylogenetic trees. Some branches were rotated using the Canvas program without changing the branch lengths. This does not alter the information contained in the tree, which is based only on branching order and branch length.

**Results**

**Correction of a mutation in the envelope gene renders an MDEV molecular clone infectious.** Six molecular clones of MDEV were isolated from a library of extrachromosomal DNA from G355 cat cells infected one day earlier with MDEV (15). To prepare the library, extrachromosomal circular DNA was cut with EcoRI and cloned into pBSII KS+. Sequencing demonstrated that the MDEV EcoRI site was in pol.
Different orientations of the virus, the presence of one versus two LTRs, and the presence of extraneous DNA fragments in the pBSII KS+ EcoRI cloning site demonstrated that the clones were independent. A vector-rescue assay was employed to determine whether the molecular clones were competent to provide \textit{gag}, \textit{pol}, and \textit{env} gene products in trans to a vector. The clones were digested with \textit{EcoRI}, religated at low concentration to rejoin the \textit{pol} gene, and transfected into G355/LAPS N cells, but none of the clones were competent to rescue the LAPS N vector. However, three clones were competent to rescue LAPS N when cotransfected with pSX2 (75), a plasmid that expresses the 10A1 MLV envelope protein (data not shown). This indicated that these clones can express functional Gag and Pol proteins after religation of the \textit{pol} gene, but not a functional Env protein. Sequencing of one clone, pMDEV9, demonstrated that there was a +1 frameshift in the envelope gene. Sequence of the corresponding region of the other five clones revealed the same frameshift, indicating that they had originated from a common defective virus.

To determine the exact nature of the frameshift mutation, a portion of the MDEV envelope was PCR-amplified from DNA of unactivated \textit{Mus dunni} cells and of G355/LAPS N cells that had been infected with activated MDEV using primers flanking the frameshift region, as described in Materials and Methods (data not shown). Amplification reactions performed on DNA from uninfected G355 cells or performed with no DNA did not yield detectable product. Sequence analysis of cloned PCR products derived from both unactivated \textit{Mus dunni} cell DNA and G355/LAPS N+MDEV DNA demonstrated that pMDEV9 had an extra A residue at position 6168 within the first variable region (VRA) of \textit{env}, generating the +1 frameshift. Due to the presence of several differences peripheral to the frameshift mutation that may have resulted from errors by the \textit{Taq} polymerase, we corrected the envelope frameshift by site-directed mutagenesis rather than by subcloning a PCR-generated envelope fragment into the MDEV plasmids. The corrected plasmid was
then used to construct a plasmid containing an intact (depermuted) copy of MDEV, called pMDEV.

The clone pMDEV was then transfected into 293/LAPSN cells to assess the ability of the clones to rescue the LAPSN vector (Table 4.1). Transfection of pBSII KS+ did not result in rescue of the LAPSN vector. Transfection of pMDEV did result in packaging of the LAPSN vector, although at a lower titer than by transfection of pAMS, which encodes a replication-competent amphotropic MLV (AM-MLV). It is unclear whether the LAPSN titers resulting from transfection of pMDEV were low because pMDEV represents a nonoptimal copy of MDEV (transfections had to be optimized before successful LAPSN rescue could be demonstrated) or whether this simply reflects the biology of the virus. Indeed, the biological isolates of MDEV also package LAPSN at a much lower titer than does AM-MLV (about 100-fold lower).

We next tested the ability of MDEV to rescue LAPSN following transfection of pMDEV into G355/LAPSN cells, which are permissive for MDEV replication. Again, transfection of pBSII KS+ did not yield detectable LAPSN production whereas transfection of pMDEV produced a low titer (300 FFU/ml) of LAPSN. In similar experiments, we have found that transfection of pAMS into G355/LAPSN cells yields a titer of >10^5 after 2 days. We passaged the transfected cells in the presence of Polybrene to determine whether the LAPSN titers would climb to those observed when packaged by wild-type MDEV. We assessed the LAPSN titers at 24 days, and found that the LAPSN was being produced at levels equal to those achieved by the biological isolates (9 x 10^4 FFU/ml).

Packaging of a vector requires only that trans-acting sequences of a retrovirus be intact. These include gag, pol, and env, and sequences necessary to express them. Functional cis-acting sequences are also required for a retrovirus to be replication-competent; these sequences include those necessary for packaging, reverse-transcription,
and integration. Spread of the MDEV genome, requiring functional cis-acting sequences, presumably occurred during the passage of the transfected G355/LAPSN cells as assayed by the increased packaging of LAPSN. To be certain that the cis sequences were intact, MDEV virus derived from the molecular clone was titrated on naive G355/LAPSN cells. G355/LAPSN cells that received at least 0.1 μl of MDEV virus became positive for LAPSN production (2 x 10^4 to 2 x 10^5 FFU/ml) after 16 days, demonstrating that the cis-acting sequences were intact and that the MDEV genome-containing virions were present at an infectious titer of approximately 10^4 per ml.

**Virus derived from the molecular clone uses the same receptor as the MDEV biological isolates.** Northern and Southern blot analysis indicates that the MDEV molecular clones represent the activated MDEV virus (15). Additional evidence would be provided by interference analysis to demonstrate that virus derived from the molecular clone uses the same receptor as MDEV that had been activated by hydrocortisone. Viral interference experiments rely on the observation that cells that have been productively infected with a replication-competent retrovirus are resistant to superinfection by a virus or vector that uses the same receptor for cell entry. As seen in Table 4.2, the entry of LAPSN pseudotyped by MDEV from either source was strongly impeded by the presence of MDEV from either source. The LAPSN(PA317) control shows an equivalent titer on all cells, showing that the block is not due to a general resistance to retroviral infection. Similar experiments have been done using chimeric virus containing the cloned MDEV envelope and Mo-MLV gag and pol, demonstrating that the block is due to env sequences rather than gag or pol sequences (data not shown). This interference demonstrates that the virus derived from the MDEV molecular clone uses the same receptor as that used by hydrocortisone-activated MDEV. Because the MDEV receptor is not known to be used by any other retrovirus, these experiments strongly
indicate that the molecular clone represents MDEV. The reciprocal nature of the interference also shows that the virus derived from activation of dunni cells with hydrocortisone does not contain a detectable mixture of viruses.

**Genomic structure and open reading frames of MDEV.** MDEV has the genomic structure of a simple retrovirus of the murine leukemia virus (MLV) group, with 3 major open reading frames (ORFs), corresponding to *gag*, *pol*, and *env* and no significant ORFs between the envelope and 3' LTR (Fig. 4.2). MDEV has a UAG (amber) stop codon separating the *gag* and *pol* genes, and 2 stop codons each at the end of the *pol* and *env* genes. The MDEV provirus is predicted to be 9,480 base pairs after the loss of 4 bp upon integration. The genomic and spliced envelope RNAs are predicted to be 8,655 and 3,383 bases long, respectively, not including the polyA tails. These predicted sizes are consistent with the results of previous Northern and Southern blot analyses (ref. 15 and data not shown).

In addition to the Gag protein that initiates at an ATG in the gag ORF, many MLVs synthesize a glycosylated Gag protein encoded by sequences upstream of the gag ATG start codon. Translation of this protein in Mo-MLV has been shown to initiate at an upstream CTG start codon (98). The *gag* ORF of MDEV also extends upstream of the ATG start codon and contains a CTG embedded in a sequence identical to that of Mo-MLV (ACC CTG GGA GAC GTC CCA GG, position 419-438), indicating that MDEV may make a similar glycosylated Gag protein. This high level of sequence identity between these two distantly related retroviruses (there is 44% amino acid identity between the two in the gag ORF starting at the putative CTG start codon) suggests an important role for this region.

There is an additional ORF within the *gag* gene, in the +1 reading frame with respect to *gag*. This ORF has at its beginning an ATG codon, making it potentially capable of encoding a protein of 163 amino acids, but the sequence surrounding this codon
(CCGATGC, position 1059-1065) does not fit the consensus for efficient translation (62). GALV also has a similar ORF potentially capable of encoding a protein of 117 amino acids, and a stretch of 66 amino acids shows similarity to that encoded by the MDEV ORF. However, the similar regions of these ORFs coincides with a highly conserved region of gag; thus, their similarity is likely to be due to sequence constraints on the gag gene. The potential MDEV ORF protein shares no similarity with those predicted from sequences in the GenBank database. We have not determined whether this ORF is translated into a protein.

Several areas of repeats exist in the MDEV genome (Fig. 4.3). The first is a series of more than six near perfect tandem 80-bp repeats in the beginning of the U3 (repeat A at positions 7,838-7,917; 7,918-7,997; 7,998-8,077; 8,078-8,157; 8,158-8,237; 8,238-8,317, and a partial repeat at 8,318-8,329). The second consists of two identical but separate copies of a 16 bp repeat (repeat B at 170-185 and 304-319), and the third consists of more than three tandem imperfect 33 bp repeats (repeat C at positions 485-517, 518-550, 551-583, and a partial repeat at 584-611) located between the LTR and the start of gag. The repeats will be discussed below.

The MDEV putative splice donor (AG●GTAAG, positions 233-239) is located between the primer binding site (PBS) and gag gene, and the putative splice acceptor sequence (CCCTCCTCTTGCTTATTCATTAAAG●G, position 5,480-5,507) is located in the pol gene.

MDEV appears to be a chimera between a VL30 and a virus similar to GALV. Sequence analysis demonstrated that the LTRs of MDEV are most similar to VL30 sequences, while the majority of the coding sequences are most similar to those of GALV (Figs. 4.1, 4.2). To model a recombination event between a VL30 element and a
virus similar to GALV that may have generated MDEV, we used the MACAW program to find similarity among the genomes of MDEV, the VL30 element BVL-1, and GALV. BVL-1 was chosen to represent the VL30 because it is the VL30 element most similar to MDEV for which the complete sequence is available. The MDEV genome shares similarity with the VL30 genome from the start of the genome through a conserved region in the gag gene. In that region of the gag gene between nucleotide 919 and 944 (corresponding to the region between amino acids 66 and 75 of the nonglycosylated Gag, shown as the upper shaded sequence block in Fig. 4.2), MDEV is highly similar to both the VL30 and GALV. Beyond that region, MDEV is similar to GALV. Therefore, the 5’ recombination event appears to have been a homologous recombination in a region of the gag gene that is conserved between VL30s and MLV-type viruses. While the amino terminus of Gag appears to be derived from the VL30 element (90% identity with VL30 BVL-1 in the first ~70 amino acids of nonglycosylated Gag), this region is also highly similar at the protein level to the Gag of GALV (70% identity).

The 3’ recombination appears to have occurred in a region of conservation encompassing the env termination codon and the polypurine tract between nucleotides 7,770 and 7,813 (shown as the shaded box near the bottom of Fig. 4.2). Unlike the 5’ recombination breakpoint, however, the sequences immediately surrounding the putative homologous recombination region are not similar among any of the three genomes. Only when the sequences are examined further from the conserved region is it apparent that the sequences towards the env gene are most similar to GALV sequences, and the sequences in the LTR most similar to those of VL30s.

The MDEV LTR defines a new VL30 class. The 928 bp LTR is composed of U3, R, and U5 regions with predicted lengths of 750, 99, and 79 bp, respectively. The beginning of the U3 was identified by the consensus att site AATGAA (position 7,807-
7,812) which is immediately downstream of the polypurine tract AAGAAAAAGGGGGG (position 7,792-7,806; see Fig. 4.2). The U3/R junction was identified by comparison to the VL30 element VL3 (accession number X03489) in which the cap site has been mapped (108). The R/U5 boundary of the LTR was identified by alignment to several VL30s that were sequenced from cDNA clones, where the beginning of the polyA tail indicates the end of the R region (87). The end of the U5 was identified by the presence of the inverted att site TTCATT (position 173-178) which matches the U3 att site and is adjacent to the primer binding site (see repeat B of Fig. 4.3).

The U3 is the most divergent region among VL30 LTRs, and has accordingly been used to define 4 families of VL30s (87). To assess the relationship of MDEV to other VL30s, we aligned the MDEV U3 with those of other VL30s using the ClustalW program. An alignment was created using a progressive pairwise method, an unrooted tree was produced with the neighbor-joining method, and bootstrap analysis performed to assess the validity of the branch points. Inspection of the tree shows that MDEV defines a fifth VL30 family (Fig. 4.4).

The MDEV U3 region contains five perfect 80 bp repeats, a repeat with a single base pair mismatch, and the beginning of a seventh perfect repeat. Because the U3 region is that which contains the promoter and enhancers, we examined the U3 repeat for the presence of consensus enhancer elements. At the beginning of each repeat is a consensus VL30 retinoic acid receptor binding domain of the sequence TGAACCTTCTTTCACCC, where the underlining identifies the imperfect direct repeats that are characteristic of such binding sites. This sequence matches consensus sequences shown to be active in other VL30 LTRs (55). Other potential binding sites in the MDEV U3 but downstream of the repeats are those for NF-1 (position 8,461-8,474) and NF-kB (position 8,489-8,499) (87). A putative TATA box with the sequence TATATAA is present (position 8,528-8,534), but
a CAAT box is not apparent. A putative polyadenylation signal of the sequence ATTTAAA is located in each R region (positions 83-88 and 8,639-8,644). Although MDEV can be activated by hydrocortisone, there are no apparent consensus glucocorticoid response elements in the U3.

**The MDEV UTR is similar to those of VL30 elements.** The untranslated region (UTR) between the LTR and *gag* is most similar to the UTRs of VL30 elements. This region contains the primer binding site (PBS), repeats B, the putative splice donor, and the putative packaging signal, which may extend into *gag*.

The 18 nucleotide PBS (position 179-196) is similar to the 3' end of the glycine tRNA, which is characteristic of VL30 elements. However, the MDEV PBS has a single base mismatch (TGGTGCGTTGGCCGGGAA, with the mismatch underlined), while those sequenced from other VL30s are a perfect match for the 3' end of murine tRNA\textsuperscript{Gly} (TGGTGCAATTGGCCGGGAA).

Repeats B are located at the end of the LTR and upstream of the presumed glycosylated Gag CTG start codon. The first copy (TTCTTCATTGGTGCCG, with the beginning of the PBS underlined, position 170-185) overlaps the U5-PBS border and the second occurs downstream of the putative splice donor (S.D. at position 233-239 and 2nd repeat at position 304-319). The final nucleotide of the first repeat is that which does not match the VL30 PBS sequence. The VL30 BVL1 aligns well with MDEV throughout this region, and the regions that correspond to the MDEV repeats can be readily identified in the VL30s. Although the two MDEV repeats are identical, the VL30 repeats share only 60% identity.

The MDEV putative splice donor (AG•GTAAG, position 233-239) is located in the UTR in a region highly similar to those of VL30s, and the putative splice acceptor sequence
(CCCTCCTCTTTGTATCTTTAAG•G, position 5,480-5,507) is located in the pol gene in sequence similar to that of GALV.

The Majority of MDEV coding regions are most similar to GALV. The beginning of the gag gene is most similar to gag sequence of VL30 elements, while the majority of the gag gene and the pol gene are most similar to those of GALV. The env gene, while not closely related to that of any known retrovirus, is most similar to that of GALV and the porcine endogenous retroviruses (PERVs) (65).

GAG: MDEV appears to encode both a nonglycosylated Gag protein with an ATG translational start codon, and an extended, glycosylated Gag protein with a CTG translational start codon. The amino-terminus of the MDEV Gag, corresponding to approximately 70 amino acids of nonglycosylated Gag and 170 amino acids of glycosylated Gag, is encoded by sequence derived from a VL30. This region of a VL30 gag is one of the few regions of VL30s that show strong sequence similarity to retroviruses (50% identity among VL30 BVL-1, MDEV, GALV, and Mo-MLV in the first 70 amino acids of nonglycosylated Gag). This portion of the gag gene of VL30 BVL-1 is one of the longest open reading frames found in VL30s, which tend to have the gag and pol genes obliterated by numerous stop codons and frameshift mutations.

The MDEV Gag polyprotein is predicted to be 522 amino acids and 59 kD, and the glycosylated Gag polyprotein is predicted to be 622 amino acids and 69 kD prior to glycosylation. The nucleocapsid portion of Gag has a single Cys-His box that matches the consensus spacing of CX₂CX₄HX₄C. Between the first two cysteines is usually found an aromatic amino acid; while this is tyrosine in most MLVs and GALV, it is phenylalanine in MDEV.

MDEV has approximately four imperfect ~33 bp repeats (repeats C) between the CTG start codon of the glycosylated Gag and the ATG start codon of the nonglycosylated
Gag (Fig. 4.3). The repeats contain 33 bp and so would not disrupt the reading frame of gag. BVL-1 and NVL-3, two VL30s for which the entire sequence is known, have 5 and 6 imperfect repeats, respectively, of about the same size in the same region. These repeats, while similar between the two VL30s, are generally of a different sequence than the MDEV repeat. A common motif is present of the sequence GAATCC^C/gACCCC in MDEV and GAGTCCCAACCTC in the two VL30s.

**POL:** The molecular MDEV clones were originally obtained by cloning EcoRI-cut extrachromosomal, circular DNA into pBSII KS+. Sequence analysis showed that EcoRI had cut MDEV within the pol gene. To be sure that a portion of the pol gene was not lost due to two EcoRI sites in close proximity, we aligned the MDEV protein sequence that spans the EcoRI site with the corresponding sequence of GALV. No gaps were observed in the MDEV sequence. This evidence, coupled with the data that demonstrate that the molecular clones encode functional Pol proteins, indicate that none of the MDEV pol gene was lost during cloning.

Pol proteins of MLV-type viruses are translated by misreading of the stop codon UAG as CAG to create a GagPol fusion protein with glutamine occupying the position of the stop codon. The resulting MDEV Gag-glutamine-Pol fusion protein would be expected to be 1726 amino acids with a molecular weight of 192 kD.

The readthrough needed to express pol is theorized to be due to effects of RNA secondary structure on translation rather than editing of the RNA (127,128). The RNA has been predicted to form a pseudoknot just downstream of the UAG for MLV-type retroviruses, and support for hairpins in Mo-MLV has been obtained through complementary mutation analysis (39,127,128). A pseudoknot structure for GALV has been proposed, but mutational analysis has not been conducted on the predicted hairpins (128). A corresponding structure can be drawn with the related but different MDEV
sequence, so that the sequences can be compared to find evidence for the existence of the hairpin (Fig. 4.5). Comparison of the two hypothetical pseudoknots shows that differences in one strand of stem 1 are accompanied by changes in the other strand of the stem, consistent with the existence of stem 1 in GALV and MDEV. The residues U6, C7, G35, G36, U38, A39, A40, U44, U50 have previously been found to be absolutely conserved in MLV-type retroviruses and again are conserved in MDEV.

An unrooted tree has been generated based on the PR-RT region of Pol protein sequences (Fig. 4.6). The MDEV sequence is distinct but most closely resembles that of GALV. The PERVs, which have also recently been reported to resemble GALV, have been included in the phylogenetic analysis. It is apparent that MDEV is more closely related to GALV than are the PERVs. A tree based on Gag proteins has a similar structure (data not shown), but the PERVs could not be included because their Gag sequences have not been reported.

**ENV:** The env ORF overlaps the pol ORF by 146 nucleotides (~49 amino acids), which is very similar to the overlap in GALV, PERV-A, and PERV-B (~46, ~41, and ~42 amino acids, respectively). The immature MDEV envelope is predicted to be a total of 673 amino acids prior to protease cleavage. The signal sequence of the envelope is not sufficiently similar to other retroviruses to predict a cleavage site, but the regions surrounding the other presumed cleavage sites can be aligned to other retroviruses. Based on such comparisons, we predict that cleavage between the surface domain (SU) and transmembrane domain (TM) occurs immediately downstream of the sequence RWKR, and cleavage of the R peptide from the cytoplasmic tail occurs approximately 16 amino acids from the carboxy terminus. Such cleavage would produce an SU domain of 479 amino acids (including the signal peptide) and a TM domain of 178 amino acids (lacking the R
peptide). The envelope is predicted to have a molecular weight of approximately 75 kDa prior to glycosylation.

The MDEV envelope has variable regions that correspond in position to the variable receptor-specificity domains identified in other MLVs, with alignable scaffold regions surrounding the variable regions (Fig. 4.7) (ref. 7). The envelope of RD114, which may share a receptor with MDEV in G355 cells, was not included in Fig. 4.7 because it could not be aligned well to the other MLVs, making the identification of the variable regions problematic. Variable region A (VRA) of MDEV is similar to that of GALV in length (47 versus 53 amino acids, respectively), but is considerably longer than that of PERV (17 amino acids). Variable region B (VRB) of MDEV is intermediate in length between those of GALV and PERV (34, 20, and 41 amino acids, respectively), and variable region C (VRC) is approximately the same length among the three (7-10 amino acids). In all cases, no similarities in sequence were observed among the variable regions, and no significant similarity was observed between the MDEV variable regions and the RD114 envelope. The predicted protein sequences encoded by the MDEV env variable regions were not similar to those potentially encoded by DNA sequences in GenBank.

There are 6 putative N-linked glycosylation sites of the structure N-X-S/T in the MDEV SU (Fig. 4.7). All but the first, which is in the variable region B (VRB), are conserved well among MLV-type viruses.

The MDEV envelope has 19 cysteines in SU, 16 of which are conserved among various MLVs. Disulfide bridges involving the corresponding conserved cysteines of Friend murine leukemia virus have been mapped, a subset of which was later confirmed by X-ray crystallography (36,67). By comparing the MDEV envelope to the Friend MLV envelope, we predict disulfide bridges between cysteines 77 and 114; 131 and 152; 144 and 157; 211 and 216; 345 and 348; 374 and 429; 393 and 405; and 437 and 449. This
leaves 3 remaining cysteines in the MDEV SU. The first of these (Cys22) is likely lost upon removal of the signal sequence, leaving one in VRA (Cys103) and one in VRB (Cys199). The structure of the Friend MLV envelope shows that VRA and VRB lie next to one another in 3 dimensional space, making it possible that the two variable regions of MDEV are linked by a disulfide bond.

The TM region of the MDEV envelope aligns well with other MLVs. The hydrophobic fusion domain, the membrane-spanning domain, and the regions corresponding to the alpha helices of Mo-MLV TM are all highly conserved (37). The cytoplasmic tail is approximately the same length as that in most MLV-type viruses.

To evaluate the relationship of the MDEV envelope to those of other retroviruses, an alignment was created with envelopes of MLV-type viruses (and MPMV, which shares a receptor with several MLV-type viruses) using the ClustalW program. Fig. 4.8 shows an unrooted tree based on the alignment, with viruses that share receptors grouped in ovals. In this diagram, bootstrap analysis indicated confidence values of greater than 97% for all branch points except for those indicated, which had confidence values of 46% and 93%. The MDEV envelope is not very closely related to any particular envelope, though it appears to be closest to those of GALV and the PERVs. This is consistent with trees based on Gag and Pol alignments, in which the closest relative to MDEV is GALV (except at the amino terminus of Gag, in which the closest relative to MDEV is a VL30). Because some experiments indicate overlap in receptor usage between MDEV and RD114, it is useful to note that the envelopes of MDEV and RD114 are not closely related.
Discussion

VL30 elements are retroelements found in mice, rats, and potentially other species, that are very similar to retroviruses in their structure and replication (42). They have LTRs that are structurally similar to retroviruses but not similar in sequence. VL30s have *gag* and *pol* genes that have domains of recognizable similarity to retroviral *gag* and *pol* genes, but of the VL30s characterized thus far, none would be expected to encode functional proteins due to the presence of numerous stop codons and frameshift mutations (Fig. 4.1, ref. 3,53). Furthermore, no VL30 has been characterized that has an *env* gene. Indeed, the elements were originally defined as having genomic RNAs of 30 Svedbergs, a size that would not be predicted to have an appreciable *env* gene. VL30 elements have been called retrotransposons because their genome indicates an intracellular replication cycle.

Many cis-acting sequences of VL30 elements have been found to be intact, including promoters, sequences necessary for reverse-transcription, and sequences necessary for integration. Despite the lack of sequence similarity between the packaging signals of VL30s and MLVs, VL30 genomes can be efficiently packaged by MLVs and spread from cell to cell (51). Copackaging of retroelements is thought to facilitate their recombination.

MDEV appears to have been generated by such recombination events between a VL30(s) and a virus similar to GALV, yielding a structure containing VL30 LTRs and interior sequences similar to those of GALV. Inspection of the putative recombination breakpoint regions indicates that it was homologous recombination events that generated MDEV. This may have occurred during first strand synthesis of DNA by jumping of the elongating chain to the corresponding region of the other copackaged RNA template. GALV is a primate virus, but belongs to the MLV group of retroviruses due to its sequence, and is thought to have originated from a xenotropic mouse retrovirus (xenotropic...
here referring to host range, not interference group) (18,66). It is therefore likely that the recombination event that generated MDEV occurred in a mouse.

Examples of MLV-VL30 recombinants exist, but apparently none have been replication-competent. Itin and Keshet successfully selected clones from a BALB/c genomic library that hybridized to both a VL30 clone and an Mo-MLV clone. Hybridization analysis demonstrated that the clone VM-1 has VL30 LTRs and gag and pol sequences similar to those of Mo-MLV (56). This clone did not hybridize to the Mo-MLV env, however, and its size indicates that it could not contain a full complement of genes. Harvey, Kirsten, and Rasheed murine sarcoma viruses, isolated from rats after inoculation with MLVs, have the inverse structure as compared to MDEV. They have MLV LTRs and some interior sequences related to rat VL30s, which incidentally do not have sequences closely similar to murine VL30s. In this case, it has been proposed that recombination with rat VL30s facilitates acquisition of the ras oncogene (71). VL30-MLV recombinants have also been selected using an integration-defective MLV, in which recombination with VL30 sequences provided intact att sites (86). MDEV differs from these examples in that it is naturally-occurring and replication-competent.

The U3 region is the least conserved region of VL30 LTRs, and has been used to divide VL30 elements among 4 families, each with multiple representatives (Fig. 4.3) (87). The MDEV LTR represents a fifth family, and is about twice as long as a standard VL30 LTR due to the presence of multiple near-perfect 80-bp repeats (repeats A) in the beginning of the U3 region. Because the U3 region contains the promoter and enhancers, we examined the repeat for the presence of consensus enhancer elements. At the beginning of each repeat is a consensus VL30 retinoic acid receptor binding domain, shown to be active in other VL30 LTRs (55). Retinoic acid receptor binding domains consist of short direct repeats separated by a spacer, and the MDEV sequence differs from the VL30 sequences in
that it has a 5 nucleotide spacer instead of a 2 nucleotide spacer. In other systems, however, a spacing of 5 shows a more vigorous response to retinoic acid than does 2 (97). We have attempted, unsuccessfully, to activate MDEV from dunni cells with retinoic acid, but these experiments may have been complicated by the lack of appropriate retinoic acid receptor expression in the dunni cells. Other consensus binding sites in the MDEV U3 are those for NF-1 and NF-κB (87). MDEV can be activated by exposing dunni cells to hydrocortisone, but no glucocorticoid response elements were observed in the U3.

The MDEV primer binding site (PBS) is for the glycine tRNA, which is characteristic of VL30 elements; other MLVs use either tRNA\textsuperscript{Pro} or tRNA\textsuperscript{Gln}. However, the MDEV PBS has a single base mismatch to the 3' end of murine tRNA\textsuperscript{Gly}, while those sequenced from other VL30s are a perfect match. It is unknown whether this mismatch is a mutation in our molecular clone, whether MDEV is tolerant for polymorphism at this site, or whether the molecularly cloned PBS matches the 3' end of the cat tRNA\textsuperscript{Gly} perfectly. The latter is possible since MDEV was last replicated in G355 cat cells during the cloning process; the 3' end of the tRNA is copied during reverse transcription and so the PBS should match the tRNA of the cells in which it was last propagated.

MDEV has two repeat areas in the 5' end of the genome. The first copy of repeat B overlaps the U5-PBS border and the second occurs downstream of the putative splice donor. The VL30 BVL1 aligns well with MDEV throughout this region, and it is noteworthy that while the MDEV repeats are identical, the VL30 repeats have only 60% identity; without alignment to MDEV, it is not apparent that the VL30 sequences represent repeats. This indicates that either the VL30 repeats have degenerated since an original duplication event or that a second MDEV repeat was generated in an area of similar sequence. In either case, the original MDEV repeat is likely the one that overlaps the U5/PBS boundary, since this is a critical region that would be sensitive to mutation. MDEV
also has four imperfect 33 bp repeats (repeats C) between the CTG and ATG start codons of glycosylated and nonglycosylated Gag. We inspected the two full-length VL30-sequences in the GenBank database, BVL-1 and NVL-3, for a similar repeated sequence. These VL30s have 5 and 6 imperfect repeats, respectively, of about the same size in the same region. Unlike the MDEV repeats, the length of the VL30 repeats is not a perfect multiple of 3, and would therefore create frameshifts. This has dubious significance, as VL30 Gag proteins have lost selective pressure as evidenced by the numerous frameshift mutations elsewhere in characterized VL30 gags. These repeats, while similar between the two VL30s, are generally of a different sequence than the MDEV repeat. A common motif can be found of the sequence GAATCC$^5_g$ACCCC in MDEV and GAGTCCCACCTC in the two VL30s. That the sequences of these repeats are well conserved within an element, but differ between MDEV and the VL30s, suggests that repeats of that sequence region were selected for independently. Alternatively, a process such as gene conversion could have homogenized repeats that had previously evolved. The significance of these repeats and putative motifs remains unknown, but they may play an important role at the nucleotide level in the packaging signal or at the protein level in the extended glycosylated Gag.

MDEV generates a spliced envelope message in infected cells, consistent with the biology of other retroviruses (15). The putative splice donor of MDEV is similar to VL30 sequences, while the putative splice acceptor is similar to GALV sequences. The consensus splice donor sequence exactly matches those of VL30s BVL-1 and NVL-3, the presence of which has previously been noted (88). However, no VL30 has been identified that has an envelope gene, leaving the requirement of a splice donor questionable. Although it is possible that a short consensus sequence could coincidentally be present at the proper location in a VL30, it is also possible that VL30s were originally derived from infectious retroviruses that had envelopes and therefore a splicing requirement. Perhaps a
VL30 with an envelope will eventually be identified. Indeed, an intracisternal A-type particle (IAP), a member of a class that had also been previously considered to be void of envelopes, has been cloned that appears to have an envelope sequence (105).

Retroviral interference experiments have shown that MDEV uses a novel receptor among murine retroviruses, but may share a receptor with the endogenous cat virus RD114 in G355 cat glial cells. Fig. 4.8 shows that the MDEV and RD114 envelopes are not closely related, and no similarity exists in the variable regions that are responsible for receptor specificity. This does not rule out the possibility of common receptor usage; while retroviruses that use common receptors tend to have closely-related envelopes, there are exceptions. For example, receptor interference can be observed among 10A1, GALV and FeLV-B, which also do not have envelopes that are closely related by sequence. While the MDEV envelope is not closely related to that of any other retrovirus, it is perhaps the most similar to the envelopes of GALV and the PERVs. Again, there is no similarity in the receptor-binding regions. While common receptor usage between MDEV and PERVs has not yet been evaluated, MDEV and GALV have been found to use distinct receptors in all cells tested thus far.

The mechanisms by which retroviruses evolve to use different receptors are poorly understood. One possibility is that a virus can dramatically change receptor usage by recombination with cellular sequences that encode a protein that binds to the new receptor. This type of event could produce a virus capable of infecting new cells, giving it a replicative advantage. Similar acquisition of cellular sequences by retroviruses is well-documented in the case of oncogenes. A prediction of this envelope cassette-swapping hypothesis is that the variable regions of retroviral envelopes resemble cellular proteins. Therefore, the amino acid sequences of the variable regions of MDEV and other MLV-type retroviruses were used to search the databases for similar sequences, but none of statistical
significance were found. Perhaps the rapid mutation rate of retroviruses obliterates the primary sequence while the envelope is fine-tuned to mediate efficient entry via the new receptor, while the general tertiary structure of that sequence is maintained.

This work has demonstrated that virus derived from an MDEV molecular clone uses the same receptor as a biological isolate. Sequence analysis has demonstrated that MDEV is a chimera between a VL30 element(s) and a virus similar to GALV, making MDEV the first example of a naturally-occurring, replication-competent retrovirus with major functional sequences similar to VL30 elements. Current work is directed at creating packaging cells that express the unique MDEV envelope for gene transfer applications, evaluating the recombinant nature of the native provirus in Mus dunni cells, and exploring the significance of the LTR repeats.
Table 4.1: Titer of LAPSN rescued by transfection of pMDEV into 293/LAPSN cells

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>LAPSN titer (FFU/ml) assayed on D17 cells(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBSII KS+</td>
<td>&lt;5</td>
</tr>
<tr>
<td>pMDEV</td>
<td>$2 \times 10^2 \pm 20$</td>
</tr>
<tr>
<td>pAMS</td>
<td>$3 \times 10^5 \pm 7 \times 10^4$</td>
</tr>
</tbody>
</table>

\(^a\) Results are averages of 4 experiments ± S.D.
Table 4.2: MDEV derived from the molecular clone displays reciprocal interference with a biological isolate.

<table>
<thead>
<tr>
<th>LAPSN pseudotype</th>
<th>Dunni/N2</th>
<th>Dunni/N2 + activated MDEV</th>
<th>Dunni/N2 + cloned MDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA317</td>
<td>1 x 10^6</td>
<td>1 x 10^6</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>Activated MDEV</td>
<td>8 x 10^4</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Cloned MDEV</td>
<td>4 x 10^4</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Uninfected dunni/N2 cells and dunni/N2 cells that had been previously infected with either MDEV activated from dunni cells or MDEV derived from the pMDEV molecular clone were plated at 2 x 10^5 cells per 3.5 cm well on day 1. The cells were exposed to LAPSN with the indicated pseudotypes on day 2, and were stained for AP^+ foci on day 4. Values are expressed as the mean of duplicates which varied by no more than 20% from the mean. The experiment was repeated by plating 1 x 10^5 cells per 3.5 cm well, with nearly identical results.
MDEV

promoter and enhancers (expression)
Ψ (packaging)
PBS (reverse-transcription)
att site (integration)

polypurine tract (reverse-transcription)
att site (integration)
polyA signal (expression)

9.5 kb

VL30 (Virus-like element of 30 Svedbergs)

CTG ATG

~5 kb

Similarity to VL30 elements
Similarity to GALV (gibbon ape leukemia virus)

Fig. 4.1: MDEV has derived its cis-acting sequences from a VL30, and its trans-acting sequences from a virus similar to GALV.
Fig. 4.2: Genomic structure of the proviral form of MDEV. Genomes of a VL30, MDEV, and GALV were aligned using the MACAW program; the alignment is shown at the putative breakpoint regions. The recombinant junctions are presumed to have occurred in the homologous regions shown in the shaded boxes. S.D. indicates the putative splice donor, S.A. indicates the putative splice acceptor, CTG indicates the start codon of the extended glycosylated Gag, and ATG indicates the start codon of the nonglycosylated Gag.
Fig. 4.3: Repeated regions of MDEV. Repeats A are nearly perfect, so only one copy is shown; the nucleotide indicated in bold is a C in the sixth repeat. Repeat A has a consensus retinoic acid binding site DR5, consisting of the underlined imperfect direct repeats separated by 5 spaces. Repeats B are perfect repeats, so only one copy is shown. The first copy of repeat B overlaps the U5/PBS boundary.

Repeat C is imperfect, so all 4 copies are shown with differences in bold; these 33 bp repeats are within the coding region of the extended glycosylated Gag. Repeat C has an underlined sequence similar to a sequence found in corresponding repeats of VL30 elements. CTG indicates the start codon of the glycosylated Gag, and ATG indicates the start codon of the nonglycosylated Gag.
Fig. 4.4. The MDEV U3 region defines a fifth VL30 group. The phylogenetic tree is based on the nucleotide sequence of the U3 regions of VL30s and MDEV. The sequences are indicated by GenBank locus name. The VL30 BVL-1, which was compared to MDEV in Fig. 1, is indicated here as MMBVL1 in group III. The four VL30 groups defined by Nilsson and Bohm are indicated, with some additional VL30 sequences added, and the MDEV U3 defines a fifth group. Five of the perfect 80 bp repeats of the MDEV U3 were eliminated prior to making the alignment to make the U3 regions approximately the same size. The alignment, phylogenetic tree, and bootstrap analysis were performed with the ClustalW program, and the tree was drawn with the TreeView program. All major branchpoints between VL30 groups have confidence values of >98% except for the branchpoint that indicates that MDEV is most closely related to group 1, which was observed in 638 out of 1,000 trials.
Fig. 4.5: Differences between the MDEV and GALV sequences are consistent with a proposed model of a GALV pseudoknot. The sequences start at the pol stop codon. In this figure and in the text referring to this figure, position 1 is defined as the nucleotide immediately downstream of the stop codon. Hydrogen bonding is indicated by dots and sequence differences are shown in bold, with boxes drawn around differences in the proposed hairpins. The GALV pseudoknot is redrawn from Wills et al., 1994, and the MDEV pseudoknot has been drawn by analogy.
Fig. 4.6: The MDEV Pol is most closely related to that of GALV. The protein alignment, a phylogenetic tree, and bootstrap analysis were performed using the ClustalW program and the tree was viewed using the TreeView program. Only those positions of the alignment with no gaps were used to create the phylogenetic tree. All major branchpoints had confidence values of >98% as measured by bootstrap analysis except for branchpoints among the closely-related members of the mouse virus group at the left.
Fig. 4.7: The variable regions of MDEV show no similarity to those of GALV or PERV. The variable regions shaded, and the putative glycosylation sites of the MDEV envelope are indicated by branched lines.
Fig. 4.8: Phylogenetic tree based on the envelope protein sequences of selected MLV-type viruses, SRV-1, and MPMV. Viruses observed to share receptors in at least one cell type are grouped together in ovals, and the receptor is indicated in the cases where it is known. Note that FeLV-B belongs to the phosphate transporter group but cannot be included in that oval in a two-dimensional tree. MDEV and RD114 were not grouped due to the inconsistent nature of the interference observed in G355 cells. The alignment, tree, and bootstrap analysis were performed with the ClustalW program. All branch points have a confidence value of >97% as measured by bootstrap analysis except for those indicated: the MDEV branchpoint was observed in 459 out of 1,000 trials, and the Friend/Mo-MLV branch point was observed in 930 out of 1,000 trials. A phylogenetic tree created by excluding positions with a gap for any sequence was nearly identical.
Chapter V

*MUS DUNNI ENDOGENOUS VIRUS (MDEV) EXHIBITS LTR EXPANSION*

**Summary**

*Mus durni* endogenous virus (MDEV) normally lies transcriptionally silent in *Mus durni* cells, but can be activated by treatment of the cells with hydrocortisone. Here we provide evidence for the following model of MDEV activation and replication: The MDEV provirus endogenous to *Mus durni* cells has a chimeric structure similar to that of the molecular clone, but instead of multiple 80 bp repeats in the U3, has only two 12 bp mini-repeats separated by 68 bp of intervening sequence. Hydrocortisone directly stimulates the LTR, allowing a low level of replication. The replication offers opportunity for generation of multiple 80 repeats in a process we call LTR expansion. The progeny with expanded LTRs are positively selected due to increased promoter strength, and this selection is likely balanced by natural instability of retroviral repeats and constraints imposed by packaging limits. The result is that an infectious MDEV population is widely heterogeneous, carrying from 3.15 to 11.15 repeats in the LTR.

**Introduction**

*Mus durni* endogenous virus (MDEV) normally lies transcriptionally inactive in durni cells, but can be activated by treating the cells with 5-ido-2'-deoxyuridine or hydrocortisone 21-succinate (73). Once activated, MDEV can replicate in durni cells and can be passaged to many other cells. Interference analysis demonstrates that MDEV uses a
novel receptor among murine retroviruses (80). Retroviral vectors pseudotyped by MDEV can transduce cells from many species, demonstrating that the MDEV receptor is commonly expressed (15).

Molecular clones of MDEV were obtained to further study its envelope and receptor usage (15). Sequence analysis revealed many interesting features in addition to a distinct envelope (130). MDEV has a hybrid structure, with the majority of the coding regions derived from a virus similar to gibbon ape leukemia virus (GALV) and long terminal repeats (LTRs) derived from Virus-Like 30 elements (VL30s). VL30s are retroelements that have a similar structure and replication cycle to retroviruses, but have important differences (42). The cis-acting sequences of characterized VL30s have been found to be intact, making VL30s capable of expression, incorporation within a virion, reverse transcription, and integration. However, the trans-acting sequences have been disrupted; the *gag* and *pol* genes have been found to contain numerous nonsense and frameshift mutations, and no VL30s have been identified with an *env* gene. VL30s can be propogated, however, by parasitizing murine leukemia viruses (MLVs) by being packaged within virions of a superinfecting MLV. MDEV was likely generated by recombination during reverse transcription of copackaged genomes of a VL30 and a virus similar to GALV. MDEV is first example of a replication-competent retrovirus with sequences derived from VL30 elements.

The U3 region of the MDEV LTR, which contains the promoter and enhancers, is unusual in two respects. First, the sequence of the MDEV U3 defines a novel, fifth VL30 family. Second, the MDEV U3 region contains more than six 80 bp repeats, which is likely the highest U3 repeat number observed in a retrovirus. Except for a single nucleotide in the sixth repeat, all 6.15 repeats are identical. This means that only a single mutation had occurred in the ~500 bp region since the repeats were generated. Because the error rate of
retroviral reverse transcriptase is high (\(10^{-4}\) mutations per nucleotide per round of replication), we hypothesized that the repeats in the molecular clone were of recent origin, and were generated during or after the activation of the MDEV. Here, we provide evidence that the native MDEV provirus has only 1.15 repeats (two 12 bp minirepeats separated by 68 bp of intervening sequence), that hydrocortisone directly activates the provirus, that LTR expansion (generation of multiple repeats) is a common event occurring during activation or propagation of the virus, and that the LTR expansion provides MDEV a replicative advantage.

**Materials and Methods**

**Nomenclature.** Cells that contain a retroviral vector and/or contain and express a retrovirus are indicated by the cell name followed by a slash and the names of the vectors or viruses, e.g., G355/LAPSN for G355 cells containing the retroviral vector LAPSN or G355/LAPSN+MDEV for G355 cells containing the LAPSN vector and productively infected with MDEV. LAPSN(PA317) refers to the viral form of the LAPSN retroviral vector packaged by PA317 cells, which express the amphotropic MLV envelope. LAPSN is a retroviral vector that encodes a heat-stable human placental alkaline phosphatase (AP) and neomycin phosphotransferase (81).

**Plasmids.** pMDEV9 and pMDEV have been described previously (130). pSEAP contains a human placental alkaline phosphatase gene that has a stop codon engineered to prevent translation of the transmembrane tail (secreted alkaline phosphatase, seap, ref. 11) followed by a human growth hormone polyadenylation sequence (2), but no promoter. pM1SEAP and pM6SEAP are derived from pSEAP and have an MDEV LTR with 1.15 repeats and 6.15 repeats, respectively, inserted in the proper orientation upstream of the seap cDNA. pMoSEAP has a truncated Moloney murine leukemia virus (MoMLV) LTR
upstream of the *seap* gene. The LTR has been truncated on the 5' end up to the *NheI* site and truncated on the 3' end to the *BanI* site in the R region, which leave the enhancers and promoter intact. pEQ176 is a plasmid that contains the CMV promoter driving the *lacZ* gene encoding β-galactosidase.

**PCR and Southern analysis.** To amplify the breakpoint region, PCR was performed with the high fidelity *Pwo* polymerase (Boehringer Mannheim) according to the manufacturer's instructions. The primers 5'-GCCCACCCTGTGGCCACCACATGAAAGAAAACCCACGAGACAAC-3' (with the bold residues complementary to MDEV) and 5'-CATCTTGAGGTGTGTTCCAGG-3' correspond to the beginning of the MDEV envelope and a region within the U3 downstream of the multiple 80 bp repeats, respectively. The samples were initially heated to 94°C for 2 min., followed by 10 cycles of 15 s at 94°C, 30 s at 58°C, and 110 s at 72°C; 20 cycles of 15 s at 94°C, 30 s at 58°C, and 110 s (plus a cumulative 20 s extension for each cycle) at 72°C; and finished with 7 min. at 72°C.

Southern blotting was performed according to standard procedures (72). Probes were radiolabelled by random priming (Boehringer Mannheim) and were hybridized to blots in a sodium phosphate solution (24).

**Cell culture.** G355 feline embryonic glial cells were grown in McCoy's medium with 15% fetal bovine serum (FBS). D17 dog cells (ATCC CCL 183) and *Mus dunni* tail fibroblasts (64) were grown in Dulbecco's modified Eagle's medium with 10% FBS. There are two *Mus dunni* cell strains available which originated from the same mouse, and they can be distinguished by whether medium conditioned by the cells becomes viscous (80). Dunni-v cells (those that make the medium viscous, as opposed to dunni-nv cells) can be activated to produce MDEV and were used in these experiments. Activation of MDEV was performed as previously described (73).
**Virus production and assay.** Virus-containing medium was harvested and debris removed by centrifugation at 4000g for 5 min at 4°C. Aliquots were stored at -80°C until titration.

Titers of LAPSN vector stocks were determined by plating the target cells at $1 \times 10^5$ or $2 \times 10^5$ cells per 3.5 cm-diameter well of a 6-well plate on day 1, replacing the medium with fresh medium containing 4 μg of Polybrene (Sigma) per ml and various dilutions of the vector stock in a final volume of 2 ml on day 2, and staining the cells for AP$^+$ foci (40) on day 4.

**Secreted human alkaline placental phosphatase (SEAP) and β-galactosidase (β-gal) assays.** SEAP assays were begun by plating G355 or dunni cells at $2 \times 10^5$ cells per 3.5 cm diameter well of 6 well plates on day 1. On day 2, the medium was replaced and 4 μg of test plasmid was cotransfected with 1 μg of pEQ176 by calcium phosphate precipitation into G355 cells or by using the Lipofectamine reagent and the manufacturer's protocol (Gibco) for dunni cells. The medium was replaced on day 3. On day 4, the medium and cellular lysates were harvested. The medium was removed from the cells, was heated to 68°C for 30 min to inactivate the background alkaline phosphatase activity, and was centrifuged at 4000 g for 5 min at 4°C, after which the supernatants were aliquoted and stored at -80°C until assayed for SEAP activity. SEAP assays were performed by mixing 100 μl test medium, 100 μl 2X SEAP buffer (2 M diethanolamine, 1 mM MgCl$_2$, 10 mM L-homoarginine), and 5 μl MUP solution (11.4 mg 4-methylumbelliferyl phosphate (Sigma) per ml dimethyl sulfoxide [DMSO]) in wells of a 96 well plate. The plate was incubated at 37°C, and 10 μl aliquots were periodically removed and mixed with 100 μl stop solution (100 mM glycine pH 10) in wells of a black 96 well plate. The fluorescence was then read with a Microfluor microfluorometer (Dynatech Industries, Inc.) To harvest the cellular lysates, cells were rinsed in phosphate buffered
saline (PBS), rinsed in PE (10 mM NaPO₄ pH 7.5, 1 mM EDTA), and exposed to 1 ml of PE+1% nonidet P-40 (Sigma). The lysed cells were then scraped with a rubber policeman into 1.5 ml microcentrifuge tubes and centrifuged at 12000 rpm for 10 min. Aliquots of the supernatants were frozen at -80°C until assayed for β-gal activity. For the β-gal assay (12), 100 μl of test lysate was mixed with 100 μl Z-buffer (60 mM Na₂HPO₄•7H₂O, 40 mM NaH₂PO₄•7H₂O, 10 mM KCl, 1 mM MgSO₄•7H₂O, 50 mM 2-mercaptoethanol, 1 mg/ml BSA) and 1 μl MUG solution (15 mg 4-methylumbelliferyl β-D-galactoside, Sigma, per ml DMSO) in wells of a black 96 well plate. The plate was incubated at 37°C and periodically read in a Microfluor microfluorometer (Dynatech Industries, Inc.). For both the SEAP and β-gal assays, the fluorescence units were plotted against the time, and the activity was taken as the slope (fluorescence units/min). SEAP and β-gal assays were also performed with 1/10 the amount of substrate or test sample, and a corresponding 10-fold decrease in activity was observed, with relative activity ratios among the samples remaining approximately the same.

**Sequence analysis.** Sequencing reactions were performed using M13 dye primer ABI PRISM sequencing kits or by using appropriate primers with the ABI PRISM dye terminator kits. Sequences were analyzed with a 373A DNA sequencer and sequence analysis software (Applied Biosystems, Foster City, CA) The sequences were assembled into contigs and further analyzed using Sequencher 3.0 (Gene Codes Corporation, Inc., Ann Arbor, MI).

**Results**

**Elements exist in unactivated dunni cells that have the same recombinant structure as MDEV.** We have used the polymerase chain reaction
(PCR) to determine whether an element(s) exists in the *Mus dunni* genome that has the VL30/GALV-like chimeric structure of the molecular clones. A region encompassing the 3' putative recombination breakpoint was amplified from *Mus dunni* cells (both dunni-v and dunni-nv cells) and from G355 cat cells infected with MDEV using primers that flank the recombination breakpoint (Fig. 5.1). The 5' primer corresponds to the beginning of the envelope lying within sequence similar to that of GALV, whereas the 3' primer is embedded in VL30 sequence in the LTR downstream of the repeat region. Unambiguous amplification products were obtained when DNA from *Mus dunni* cells or MDEV-infected cells was used as a template, while no specific amplification products were obtained when DNA from uninfected G355 cells was used as a template. No differences were observed between the dunni-v or dunni-nv samples, consistent with the cells being derived from the same mouse (80). Southern blots were performed to ascertain that the amplification products specifically hybridized to an MDEV envelope probe (Fig. 5.1). The dunni cells used in this experiment had not been activated with hydrocortisone nor iododeoxyuridine, and so were not expressing MDEV. Thus, the dunni PCR products were most likely amplified from the native provirus(es), demonstrating that an element(s) exists in the *Mus dunni* genome with a chimeric VL30/GALV-like structure.

Close inspection of the ethidium-stained gel showed two closely-spaced dominant bands present in the dunni lanes, consistent with previous Southern blotting evidence that demonstrates additional elements related to MDEV in the *Mus dunni* genome (15). Inspection of the ethidium-stained gel also revealed multiple, evenly spaced bands in the G355/LAPSN+MDEV lanes, but only a single band in the pMDEV9 lane. Additionally, the products derived from the dunni DNA appear to be about 400 bp smaller than those derived from the infected cells or pMDEV9 (Fig. 5.1). Both the multiple bands in the
G355/LAPSN+MDEV lanes and the size discrepancies can be explained by differences in U3 repeat numbers, described in detail below.

To molecularly clone a 3' breakpoint region, PCR products amplified from dunni DNA were digested with SalI (located in env) and XbaI (located in the LTR) and separated on an agarose gel. The band corresponding to that containing the breakpoint region (~400 bp) was isolated and ligated into the appropriate restriction sites of pBSIIKS+. Sequence analysis of clones demonstrated that some of these clones had a sequence 99% identical to that of the molecular clone pMDEV9. These clones likely represent the native MDEV provirus; the differences may be due to errors by Pwo polymerase during PCR amplification or due to errors by reverse-transcriptase during passage of MDEV prior to molecular cloning.

**An element consistent with an MDEV provirus containing 1.15 U3 repeats, but not 6.15 repeats, exists in Mus dunni cells.** We hypothesize that the native MDEV provirus contains 1.15 repeats rather than 6.25 repeats as present in the molecular clone. A prediction of this hypothesis is that the native proviral SalI-XhoI env-LTR fragment is 679 bp, rather than the 1079 bp of the molecular clone. To examine this possibility, we performed southern blots using DNA from dunni, G355/LAPSN+MDEV, and G355/LAPSN cells (Fig. 5.2). Supporting our hypothesis, a strongly-hybridizing fragment in dunni DNA digested with SalI-XhoI has a size consistent with 679 bp, and there is no fragment consistent with 1079 bp. Fragments of higher molecular weight also hybridized with the probe, consistent with previous evidence that there are additional elements in the Mus dunni genome that are related to MDEV. The band seen in the G355/LAPSN+MDEV lane is smeared, but is centered around the 1079 bp fragment observed with the plasmid. (There are two bands in the plasmid lane because pMDEV9 has two adjacent LTRs; the relevant SalI-XhoI env-LTR fragment is 1079 bp, and a structurally
irrelevant XhoI-XhoI fragment is 928 bp.) The smearing observed in the G355/LAPSN+MDEV lane prompted additional experiments to determine whether it represented a range of fragment sizes.

An MDEV population has a variable number of repeats. For greater resolution in examining the MDEV U3 repeats, additional blots were conducted after digesting genomic DNA with AluI (Fig. 5.3). G355/LAPSN cells were infected with 100 μl, 10 μl, 1 μl, 0.1 μl, 0.01 μl, or 0 μl of MDEV-containing medium. After passaging the cells for 2 weeks, the medium was harvested and the genomic DNA obtained from the cells. Titration of the LAPSN vector in the harvested medium demonstrated that the cultures that had received at least 0.1 μl of MDEV became positive for LAPSN production (with each positive culture producing a titer of approximately 5x10^4 AP^+ FFU per ml), indicating a productive MDEV infection. Genomic DNA from each positive culture from passage 1 is shown under the heading “log dilutions”. The Southern analysis shows a ladder with an 80 bp increment in each case, indicating that the MDEV population contains viruses with different numbers of 80 bp repeats. The probe should also hybridize to the 5' LTR of each integrated provirus, but because the next AluI site in the cellular genomic DNA would vary according to the integration site, this hybridization should only produce a low level background smear. No hybridization was observed in a lane containing DNA from uninfected G355/LAPSN cells. For reference, the 768 bp AluI fragment derived from pMDEV on the right side of the blot contains 6.15 repeats. Assuming each successively higher band has one additional repeat, there are viruses within the MDEV population that have as many as 11.15 repeats.

Additional serial passages (passages 2 and 3) were performed as described above. The DNA from each culture that received limiting dilutions of MDEV is shown under the heading “serial limiting dilutions”. Each of these cultures presumably received 1-10
infectious units, yet each culture displays a full complement of repeat numbers. That the full array of repeats is present in each limiting dilution culture indicates that the repeat number can be easily changed during the viral life cycle.

The last two lanes marked “molecularly-derived MDEV” contain DNA from G355/LAPSN cells that were transfected with the molecular clone pMDEV and passaged for 24 days to allow for viral spread. Although the source of MDEV in this case had 6.15 of repeats, the repeat array is again observed, demonstrating that variants with different repeat numbers can be derived from a defined number in a short history of passage. The predominant band corresponds to the 6.15 repeats of the transfected plasmid, but variants of up to 10.15 repeats are present.

The blot in Fig. 5.3 and similar blots provided evidence that MDEV viruses can have variable numbers of repeats. This prompted us to re-examine the molecular clones that we had previously isolated from a library of extrachromosomal DNA from infected G355 cells. Six molecular clones were obtained during the cloning of MDEV (15), and the prototype clone pMDEV9 was originally sequenced and found to have 6.15 bp repeats (accession number AF053745). Sequence analysis of the other clones demonstrated that we had isolated clones containing 3.15 and 4.15 repeats as well (data not shown). No U3 alterations were observed other than the number of repeats, providing additional evidence that the ladders observed are only due to variation of the repeat number.

**Independent activants of MDEV are not identical with respect to repeat number.** Despite different passage histories, all of the MDEV samples shown in Fig. 5.3 had originally been derived from the same MDEV producer cell line G355/LAPSN+MDEV clone 16. Additional samples were required to determine whether the expanded LTR with variable numbers of repeats is a general feature of activated MDEV or is specific to this activant. Cultures of dunni/LAPSN cells were independently exposed
to hydrocortisone or 5-ido-2'-deoxyuridine as described previously to activate the endogenous MDEV (73). The activation of MDEV was ascertained by measuring the packaging of the LAPS(N) vector, and the MDEV was passaged to naive G355/LAPS(N) cells. After passage of these cells for 2 weeks, the medium and genomic DNA were harvested. Shown in Fig. 5.4 is a Southern blot of the resulting genomic DNAs that had been digested with AluI and probed as part of the same blot as that in Fig. 5.3. The first lane represents MDEV activated by hydrocortisone, similar to the samples in Fig. 5.3, and the other two lanes represent MDEV activated by 5-ido-2'-deoxyuridine. In two cases, an 80 bp ladder is observed, although containing fewer repeats on average than the samples shown in Fig. 5.3. The middle sample contains only one band. We have not further characterized the LTR structure of this MDEV sample. In all cases, the bands are larger than what can be explained by an MDEV LTR with 1.15 repeats, which would be 368 bp. All three cultures were producing similar titers of LAPS(N)(MDEV) at the time of DNA harvest, indicated below the blot, and these titers were similar to those produced by the cultures shown in Fig. 5.3.

**Expanded MDEV LTRs exhibit greater promoter activity in G355 and dunni cells.** We hypothesized that expanded LTRs are selected due to promoter strength. To test this, a series of constructs were made with different promoters driving the expression of a secreted human placental alkaline phosphatase (SEAP) (Fig. 5.6). Each plasmid was cotransfected with pEQ176 (a plasmid that expresses β-galactosidase) into G355 cells. Two days after transfection, both the medium containing the SEAP and the cells containing β-gal were harvested separately. Fluorometric assays were performed on the medium samples using the substrate 4-methylumbelliferyl phosphate (MUP), which fluoresces after cleavage of the phosphate by SEAP, and were performed on the cell lysates with the substrate 4-methylumbelliferyl β-D-galactoside (MUG), which fluoresces
after cleavage of the galactoside by β-galactosidase. SEAP and β-gal assays were also performed with 1/10 the amount of substrate or test sample, and a corresponding 10-fold decrease in activity observed, with relative activity ratios among the samples remaining approximately the same. No differences in SEAP activity were detected among the plasmids pBSIIKS+, which doesn’t contain a seap gene; pSEAP, which contains a seap gene but no promoter; and pM1SEAP, which contains the MDEV LTR with 1.15 repeats as a promoter to drive expression of the seap gene (Fig. 5.6). However, transfection of the constructs pM6SEAP, which contains the MDEV LTR with 6.15 repeats driving the seap gene, and pMoSEAP, which contains a MoMLV promoter and enhancers driving the seap gene, did result in detectable SEAP. Equivalent levels of β-galactosidase activity in all cellular lysates indicated that the differences in SEAP activity were not due to differences in transfection efficiency. Sequence analysis of the promoter region of pM1SEAP that had been digested with restriction enzymes and ligated demonstrated that no errors had occurred during the plasmid construction. Two additional clones of pM1SEAP were also tested to rule out bacterial mutation of the seap gene, and again yielded no detectable activity. Statistical analysis showed that the MDEV LTR with 6.15 repeats had at least 4.5 fold greater activity than did the MDEV LTR containing 1.15 repeats. The truncated MoMLV LTR had at least 65-fold greater activity than did the MDEV LTR with 6.15 repeats. Results consistent with these were obtained by transient transfection into dunni cells (Fig. 5.5).

The MDEV LTR is responsive to hydrocortisone in dunni cells. Stable transfectants of the SEAP constructs in dunni cells were obtained in two separate experiments. The relative strengths of the the various constructs were inconsistent between the two sets of stable transfectants, but Southern blot analysis demonstrated that the
inconsistencies were most likely due to dramatically different copy numbers of the seap constructs introduced into the cells, which varied by more than 10 fold.

Despite the inconsistent nature of stable plasmid introduction (and relative expression) into dunni cells, a strong induction of activity was observed by hydrocortisone on all 4 cell lines containing MDEV constructs, including pM1SEAP, but no induction was observed for any of the cell lines containing other constructs (Fig. 5.6). Hydrocortisone has no effect on the SEAP assay (data not shown), but it did slow the growth of the cells, so that there were approximately 2.5 fewer cells in the treated dishes than in the untreated dishes at the time of medium harvest. The decrease in cells could explain the approximately 3-fold drop in activity observed for the pMoSEAP stables in experiment 2. Thus, after normalizing to cell number, we observe an induction of up to 47-fold for an unexpanded MDEV LTR (47-fold for pM1SEAP in experiment 2) that we hypothesize to be present in the native MDEV provirus.

Discussion

We propose the following model of *Mus dunni* endogenous virus (MDEV) activation: The MDEV provirus native to the *Mus dunni* genome has a chimeric VL30/GALV-like structure but contains only 1.15 80 bp repeats (two 12 bp minirepeats separated by 68 bp of intervening sequence) in the U3 region, rather than the 6.15 repeats contained by the molecular clone. It normally lies transcriptionally quiescent in *Mus dunni* cells, but treatment with hydrocortisone induces a low level of replication by activating the LTR. The initial replication offers the opportunity for generation of multiple repeats, a process we refer to as LTR expansion. Viruses with expanded LTRs are then selected due to stronger promoters. The repeat number of a replicating population is dynamic, with
constant selection for expanded LTRs likely being balanced by deletions and packaging constraints.

This model can be divided into four key hypotheses: 1) the native MDEV provirus has a chimeric structure but only 1.15 repeats, 2) the MDEV LTR is responsive to hydrocortisone, 3) expanded MDEV LTRs containing multiple repeats exist in a replicating population, and 4) the expanded LTRs offer a selective advantage due to stronger promoters. We present evidence for these hypotheses in this paper.

The first hypothesis of the model grew from our inspection of the MDEV sequence. The LTRs of the MDEV molecular clone contain multiple 80 bp repeats in the U3, and their near-perfect nature lead us to hypothesize that they were of recent origin, and not present in the native MDEV provirus. Southern analysis, PCR analysis, restriction analysis of bulk PCR products, and sequence analysis of clones generated by PCR all support the hypothesis that the native MDEV provirus has a chimeric VL30/GALV-like structure with 1.15 repeats, and provide no evidence of a provirus containing 6.15 repeats (Figs. 5.1 and 5.2 and data not shown).

The second hypothesis of the model is that the MDEV LTR is responsive to hydrocortisone, an agent that activates MDEV. To address this, we have created Mus dunni cells that have been stably transfected with constructs containing the MDEV LTR promoting a gene encoding a secreted form of human placental alkaline phosphatase (SEAP). Fluorometric assays demonstrated that the MDEV LTR shows up to 47 times greater activity in the presence of concentrations of hydrocortisone that activate MDEV. Thus, MDEV is likely activated by the effects of hydrocortisone on the promoter activity.

The third hypothesis of the model is that expanded LTRs exist in a replicating population. The expanded LTRs could be created by a simple mechanism: because the repeat number is not integral, two 12 bp minirepeats separated by 68 bp would be present
in an MDEV containing 1.15 repeats. These minirepeats could facilitate LTR expansion by allowing homologous misannealing during reverse transcription. Any number of repeats could be generated by such a mechanism, and indeed retroviruses have been observed to generate tandem repeats from sequences surrounded by small repeats (115). The Southern blots demonstrate that an MDEV population contains viruses with widely different numbers of 80 bp repeats, from 3.15 to 11.15. These blots prompted us to sequence the corresponding region of additional molecular clones, which has demonstrated clones containing 3.15, 4.15, and 6.15 repeats. No differences other than repeat number were observed among the clones, indicating that the laddering effect seen in Figs. 5.3 and 5.4 is simply due to different numbers of repeats rather than due to higher order changes in the LTR. We have put MDEV through population bottlenecks, either by infecting cells with limiting doses of MDEV or by deriving virus from a molecular clone that contains 6.15 repeats. After two weeks of passage, the full array of repeats was observed, indicating that the repeat number is dynamic through replication cycles. Independently-activated populations of MDEV also display expanded LTRs with variation in repeat number. Thus, PCR evidence (Fig. 5.1), Southern blots (Figs. 5.3 and 5.4), and sequence analysis of molecular clones showing differences in repeat number offer profound evidence that expanded LTRs exist in a replicating population.

The fourth hypothesis is that the expanded LTRs offer a selective advantage through promoter strength. That the expanded LTRs are selected is supported by several observations. First, repeats in retroviruses are unstable (106) and would not be expected to be maintained in the absence of selection. Second, the MDEV populations with multiple U3 repeats are likely derived from a provirus that has 1.15 repeats, and we have observed no evidence of an actively-replicating MDEV containing only 1.15 repeats. Because the U3 region is that which contains the promoter and enhancers, the selective advantage of LTR
expansion is likely due to promoter strength. To test this, we compared the activities of an MDEV LTR containing 1.15 repeats (the hypothetical, unexpanded LTR of the native provirus) versus 6.15 repeats (the LTR of the prototype MDEV molecular clone) in transient transfection SEAP assays. The assays could not detect any promoter activity from the 1.15 repeat-containing LTR, but could detect at least 4.5 fold greater activity from the expanded LTR (Fig. 5.5). Viruses containing the stronger, expanded LTRs would likely be selected during passage. Perhaps variants with excessive numbers of repeats are actively selected against due to packaging constraints or passively due to natural instability of repeats in retroviruses, creating a distribution centered around an optimal number of repeats.

Other investigators have observed synergistic cooperation among three U3 repeats of other VL30 LTRs, both at the level of binding factors in nuclear extracts (99) and at a functional level (13). Although these repeats are of different sequence than the MDEV repeats, the MDEV repeats may work in a cooperative manner as well. We have inspected the MDEV repeats for the presence of consensus enhancer elements, and have only observed a potential retinoic acid receptor binding site; it is likely, however, that each repeat contains multiple binding sites for nuclear factors.

The promoter assays demonstrate that even the expanded MDEV LTR has 65 fold lower activity than the MoMLV promoter in G355 cells. This is consistent with relative titers observed, in which the titer of MDEV is generally about 100 fold lower than that of MoMLV. Since VL30 LTRs show tissue-specific activity (87), it is possible that the MDEV LTR does not require a high degree of expansion to be effective in some cell types. Expansion could be detrimental for LTRs that function very well in a cell type, such as the MoMLV LTR in G355 cells, because an overload of viral transcripts could be toxic to the cell, thereby eliminating the selective advantage.
Inspection of the MDEV genome has lead us to propose a model of MDEV activation and replication. Experimental results are consistent with this model, and indicate that MDEV may display unique behavior among retroviruses.
Fig. 5.1. Elements with a chimeric VL30/GALV-like structure exist in the *Mus dunni* genome. Amplification products from PCR performed with primers that flank the MDEV 3′ recombination breakpoint were electrophoresed on an agarose gel and Southern blotted. The blot was then probed with the *MfeI-BsaBI env* fragment of pMDEV9. At the bottom is a proviral map of the molecular clone, with the *gag* gene shown starting at the glycosylated Gag CTG start codon. The LTRs are identical, but the 3′ LTR is shown with the repeats to illustrate that the primers span the repeat region.
Fig. 5.2. An element consistent with an MDEV provirus containing 1.15 repeats exists in the *Mus dunni* genome. Genomic DNA (10μg) or pMDEV9 plasmid DNA (10pg left pMDEV9 lane, 100 pg right pMDEV9 lane) were digested with *XhoI* or *XhoI* plus *SalI* as indicated below the blot, run on an agarose gel, blotted, and probed with a radiolabelled *SalI*-*XbaI* env-containing fragment of pMDEV9. The ethidium bromide stain of the gel prior to blotting indicated approximately equal loading of genomic DNA samples (data not shown). The two panels are from the same gel and blot, with irrelevant plasmid-containing central lanes removed.
Fig. 5.3. A population of MDEV has a variable number of repeats. Except for the each end lane, all lanes contain DNA from G355/LAPSN cells that had been infected with MDEV as indicated and described in Results. Genomic DNA (10μg) or pMDEV DNA (10pg) were digested with AluI, electrophoresed, blotted, and hybridized to a radiolabelled 80 bp XbaI fragment corresponding to one repeat. The ethidium bromide stain of the gel prior to blotting indicated approximately equal loading of genomic DNA samples (data not shown). The two panels are from the same gel and blot, with intervening lanes (shown in Fig. 5.4) removed. Below the blot is a map of the 3' half of an MDEV provirus. The LTR has been divided into U3, R, and U5 regions, with the XhoI site overlapping the U3/R boundary.
Fig. 5.4. Independently activated populations of MDEV are not identical. G355/LAPSN cells were plated at $10^6$ cells per 10 cm diameter dish, infected with 1 ml of independently-activated MDEV samples in the presence of 4 μg polybrene per ml, and passaged for 2 weeks, at which time the media and genomic DNAs were harvested. This panel is from the same gel and blot as shown in Fig. 5.3; refer to the legend for further experimental details. (A two-fold longer exposure was required for good visualization of these bands.) The LAPSN titer produced by each culture at the time of genomic DNA harvest is indicated below the blot.
Fig. 5.5. The Expanded LTR has greater promoter strength in G355 and dunni cells. The promoter constructs are shown at the bottom of the figure. Thick boxes indicate LTR sequences (with the cap site indicated by the bent arrow), the SEAP coding region, and a human growth hormone 3' untranslated region containing a polyadenylation sequence (AATAAA indicated by pA). The top graph shows results from transfection experiments with G355 cells (left half) and dunni cells (right half). For the G355 transfections, three separate calcium phosphate precipitates were prepared for each plasmid, which was cotransfected with a plasmid encoding β-galactosidase to control for transfection efficiencies. This experiment was repeated several times, and also with different substrate and medium concentrations, with consistent results. For the dunni transfections, three separate Lipofectamine (Gibco) liposome preparations were prepared for each plasmid, and the SEAP assay was repeated with nearly identical results. The data is shown in each case as the mean with error bars representing the standard deviation.
Fig. 5.6. The MDEV LTR is responsive to hydrocortisone in *Mus dunni* cells. Stable transfectants were obtained by cotransfecting each SEAP construct with the selectable plasmid pSV2neo (a gift from Paul Berg at Stanford University) with at a ratio of 20:1 by electroporation with the BioRad Gene Pulser according to the manufacturer's instructions. In each case, selection in 0.7 mg active G418 (Gibco) per ml resulted in at least 16 clones which were pooled into a polyclonal population. On day 1, the pooled cells were plated at 2x10^5 cells per 3.5 cm-diameter well of 6 well dishes, 6 wells per cell lines. On day 2, the medium was changed, and 3 wells of each cell line were exposed to 10^-4 M hydrocortisone 21-hemisuccinate (Sigma). On day 3, the medium in each well was replaced with the same as on day 2. On day 4, the medium samples were harvested and treated as described above for the assay. Data are the means of 3 values, and the error bars represent the standard deviations. “Experiment 1” and “Experiment 2” refer to 2 sets of stable transfectant cell lines. Each experiment has been repeated with similar results. The stable line obtained with pMoSEAP in experiment 1 was not included because it had no activity above pSEAP and Southern analysis was unable to demonstrate the presence of the seap gene.
Activity (Fluorescent units / min, normalized to copy number)

Plasmid:

- pSEAP
- pMISEAP
- pM6SEAP
- pSEAP
- pMISEAP
- pM6SEAP
- pM6SEAP

Experiment 1

- Experiment 2

- no hydrocortisone
- $10^{-4}$ M hydrocortisone
Chapter VI

RETROVIRUS PACKAGING CELLS BASED ON *MUS DUNNI*
ENDOGENOUS VIRUS FACILITATE TRANSDUCTION OF CHO AND
PRIMARY HEMATOPOIETIC CELLS

Summary

*Mus dunni* endogenous virus (MDEV) infects a wide variety of cell types from many different species. To take advantage of this broad host range, we have constructed packaging cells (PD223) that produce virions bearing the MDEV envelope. PD223 cells are able to package Moloney murine leukemia virus-based vectors at titers exceeding $4 \times 10^5$ infectious units per ml in the absence of contaminating replication-competent retrovirus. Vectors packaged by PD223 cells are able to transduce CHO cells, which are resistant to transduction by many retroviruses, at ≥ 25-fold higher efficiency than do vectors having other pseudotypes. A vector packaged by PD223 was found to be among the most efficient for transducing primary baboon and human CD34+ cells.

Introduction

Retroviral vectors have become important tools for studying biology and will increasingly be used to transfer genes for therapeutic purposes. A crucial step in vector production is the packaging of vectors within retroviral virions. This can be achieved by a replication competent retrovirus which copackages the vector along with its genome. However, the virions containing the viral genomes cannot be separated from the virions containing the vector genomes, leading to transmission of the replication-competent virus to
the target cells. This can pose many problems, such as further spread of the vector, interference with subsequent vectors that carry the same envelope protein, and infection of other tissues if transduced cells are introduced into an organism. Alternatively, packaging cells can provide the fundamental retroviral gene products Gag, Pol, and Env in trans to the vector in the absence of replication-competent viruses.

A crucial step in retrovirus transduction is the binding of the virus envelope protein (Env) to a specific cellular receptor. If a cell does not express a receptor that is recognized by the retroviral Env, it will not be efficiently transduced. Because cells vary in their expression of specific retroviral receptors, it is important to have available a variety of packaging cells that express different envelopes that recognize different receptors. Viral interference experiments show that the recently-described Mus dunni endogenous virus (MDEV) uses a novel receptor among murine retroviruses (80). Retroviral vectors pseudotyped by wild-type MDEV can infect different cell types from a variety of species including mouse, rat, hamster, cat, dog, quail, and human (15). We have therefore constructed packaging cells that express the MDEV envelope to take advantage of its novel receptor usage, and have evaluated the potential of vectors packaged by PD223 for use in hematopoietic gene therapy.

**Materials and Methods**

**Nomenclature.** Cells that contain a virus or vector are indicated by the cell name followed by the vector or virus, e.g. NIH 3T3/MDEV or PD223/LAPSN. A vector in viral form is indicated by the name of the vector followed, in parentheses, by the name of the virus or packaging cells that have pseudotyped the vector, e.g. LAPSN(MDEV) or LAPSN(PD223). The pseudotype refers to the envelope protein used to package a retroviral vector regardless of what Gag and Pol proteins were used. Transduction is
defined as the transfer and expression of a gene by a retroviral vector. LAPSNo expresses a human placental alkaline phosphatase (AP) (81), and LNCGo expresses a mutated, humanized green fluorescent protein (GFP).

**PCR, site-directed mutagenesis, and plasmid construction.** MDEV Env expression plasmids were required for construction of the packaging cells. The original MDEV molecular clones contain a frameshift mutation in the envelope gene, so an intact MDEV envelope was generated either by site-directed mutagenesis to correct the frameshift or by amplifying the entire Env by the polymerase chain reaction (PCR). The intact MDEV envelopes were cloned into the 10A1 MLV Env expression plasmid pSX2 to generate MDEV Env expression plasmids.

The Env expression plasmid pMDEV9ex was generated with an envelope that was corrected by site-directed mutagenesis. The elucidation of the frameshift mutation and subsequent correction by site-directed mutagenesis has been described previously (Wolgamot MDEV seq. paper, submitted). pMDEV9ex was created by subcloning a 2165 bp *BamHI- XmnI* fragment containing the corrected MDEV *env* into the 3948 bp fragment of the 10A1 murine leukemia virus Env expression plasmid pSX2 prepared by digestion with *BsaBI* and partial digestion with *BamHI*.

The pMEX series of plasmids contain the MDEV envelope amplified with primers that overlap the MDEV *env* start and stop codons. The PCR was performed with *Pwo* polymerase (Boehringer Mannheim, IN) according to the manufacturer’s instructions using the primers 5’-GCCCAACCTGTGCCCACCATGAAGAAACCACCAAGACAAC-3’ and 5’-GCCTTTAACATAGGCTCTAATCCTAGAGCGAG-3’. Thermocycling was performed by heating to 94°C for 2 min, followed by 10 cycles of 94°C for 15 s, 62°C for 30 s, and 72°C for 80 s; 20 cycles of 94°C for 15 s, 62°C for 30 s, and 72°C for 80 s plus an additional cumulative 20 s per cycle; with a final extension at 72°C for 7 min. Templates included plasmid DNA containing an MDEV *env* that had been corrected by site-directed
mutagenesis, DNA from unactivated dunni cells, G355/LAPSN + MDEV cells, uninfected G355 cells, and no DNA. PCR products resulting from amplification from plasmid DNA, dunni DNA, or G355/LAPSN + MDEV DNA were cloned into the 3835 bp DraIII-BsaBI fragment of pSX2. pMEV\textsuperscript{dunni}, upon which the packaging cells are based, contains an envelope amplified from DNA of unactivated dunni cells.

**Cell culture.** D17 dog cells (ATCC CCL 183), NIH 3T3 TK\textsuperscript{-} murine cells (74), LPGS cells (76), and all packaging cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) (Hyclone, UT). The packaging cells used were PA317 (74), PG13 (76), PT67 (75), LLENZB, PM571 (82), PE501 (78), FLYRD (29), and PD223. G355 cells (33) were grown in McCoy’s modified Eagle’s medium with 15% FBS, and CHO cells (ATCC CCL 61) were grown in a-MEM with 5% FBS. Primary human CD34+ cells were cultured in RPMI+10% FBS.

**Virus production and assay.** Virus-containing medium was harvested, centrifuged at 4000g for 5 min at 4°C to remove cells and debris, and medium samples were stored at -80°C until titration.

Titers of LAPSN vector stocks were determined by plating target cells on day 1 at 1x10^5 or 2x10^5 cells per 3.5 cm well of a 6 well plate, replacing the medium with fresh medium containing Polybrene (4 mg/ml; Sigma) and a dilution of the vector stock in a final volume of 2ml on day 2, and staining the cells for AP\textsuperscript{+} foci (40) on day 4.

Titers of LNCG vector stocks were determined by plating and infecting the target cells as described above, followed by counting GFP\textsuperscript{+} foci using a Leica MZ8 dissection microscope equipped with GFP Plus filters.

**Flow immunocytometry and fluorescence-activated cell sorting (FACS).** To select the highest virus-producing clone of PD223/LAPSN, a FACS-enrichment approach was used. On day 1, PD223 cells were plated at 10^5 cells per 3.5 cm
diameter well of 6-well dishes. On day 2, the medium was replaced with fresh medium containing Polybrene (4 mg/ml) and either 200 ml (MOI~1) or 20 ml (MOI~0.1) of LAPSN(PA317)-containing medium, previously titered at 1x10^6 AP⁺ FFU/ml on NIH 3T3 cells. On day 4, the cells were harvested, resuspended in 2.4G2 conditioned medium to block FcgII receptors and labelled using anti-human placental alkaline phosphatase (clone 8B6, Dako, CA) which had been conjugated with biotin. After washing, the cells were stained using streptavidin phycoerythrin (PE; Dako) and further washed. Prior to sorting, cells were resuspended in phosphate buffered saline containing 2% FBS, DNAseI (10 mg/ml) and propidium iodide (1mg/ml; Molecular Probes, Eugene, OR). Analysis and sorting was performed on a FACSVantage II (Becton Dickinson, CA) using CellQuest II software. Dead cells and debris were excluded by gating on forward and high angle light scatter and absence of propidium iodide staining. Doublets were minimized using forward light width and area parameters. After gating, fluorescent cells were sorted into wells of a 96 well plate using an automated cell deposition unit. For the primary screen of the PD223/LAPSN clones, D17 cells were plated at 5x10^4 per well of 24-well plates on day 1. On day 2, the medium was replaced with fresh medium containing Polybrene (4 mg/ml), and exposed to 5 ml of test medium from each clone. On day 4, the cells were prepared for flow cytometry as described above using conjugated mouse anti-human placental AP antibody followed by staining with FITC-conjugated anti-mouse antibody (PharMingen). A secondary screen was performed by directly titering the LAPSN vector in medium conditioned on selected clones.

To select the best clone of PD223/LNGC, a similar approach to that described above was used except that either 1 ml (MOI~1) or 100 ul (MOI~0.1) of LNGC(PT67)-containing medium was added to the PD223 cells on day 2. The LNGC(PT67) stock had previously been found to have a titer of 2x10^5 GFP⁺ FFU/ml. Cells were gated as
described above and single GFP-positive cells were sorted into wells of 96-well plates. For the primary screen, D17 cells were infected as described for the primary screen above. A secondary screen was performed by directly titering the LNCG vector in medium conditioned on selected clones.

To evaluate the transduction of human CD34\(^+\) cells by LAPS(N)(MDEV), fresh normal bone marrow cells were selected for the presence of the CD34\(^+\) antigen using an immunomagnetic column protocol. On day 1, the cells were prestimulated with flt-3 ligand (100 ng/ml), IL-6 (100 ng/ml), stem cell factor (100 ng/ml), and megakaryocyte growth and development factor (50 ng/ml). On day 2, cells were plated at 50,000 cells per well in a 24-well Pronectin F culture plate (Protein Polymer Technologies) in 500 ml of medium and transduced with 500 ml of LAPS(N)(PD223), LAPS(N)(PG13) or LAPS(N)(FLYRD) in the presence of Polybrene (4 mg/ml). The transduction was repeated on day 3, and the percentage of alkaline phosphatase-expressing cells was evaluated by flow immunocytometry on day 5 as described above. Human cells were obtained using protocols approved by the institutional review board.

**Sequence analysis.** Sequencing was performed on the nested deletion constructs using M13 dye primer ABI PRISM sequencing kits and analyzed with a 373A DNA sequencer and sequence analysis software (Applied Biosystems, Foster City, CA). The reverse strand of pMEX\(^{dnnr}\) was sequenced by introducing deletions with exonuclease III using the method of Clark and Henikoff (25). Regions of poor sequence data, and the positive strand in the region where the pMEX\(^{dnnr}\) sequence differed from the sequence of pMDEV9, were resequenced after designing appropriate primers and using the ABI PRISM dye terminator kits. The sequences were assembled into contigs and further analyzed using Sequencher 3.0 (Gene Codes Corporation, Inc., Ann Arbor, MI).
Results

Generation and sequence analysis of MDEV Env expression

**Plasmids.** The original molecular clones of MDEV contained a frameshift mutation in the envelope gene that was corrected using two approaches, described below. Once obtained, the intact MDEV envelopes were cloned into the 10A1 MLV envelope expression plasmid pSX2 to generate MDEV Env expression plasmids. pSX2 was chosen because it was found to express the highest levels of 10A1 Env out of a series of constructions (75).

pMDEV9ex contains an MDEV env that was corrected by site-directed mutagenesis. To characterize the exact nature of the MDEV env frameshift, a region of the MDEV envelope was amplified by PCR from DNA of unactivated dunni cells and G355/LAPSN+MDEV cells. Amplification performed with control templates including G355/LAPSN DNA and no DNA did not yield detectable product. Sequence analysis of cloned PCR products from both dunni cell and G355/LAPSN+MDEV DNA templates revealed that the frameshift mutation had been created by the addition of an extra nucleotide at position 6168 of the MDEV genome, which was removed by site-directed mutagenesis.

The pMEX series of plasmids contain the entire envelope of MDEV amplified using the high fidelity *Pwo* polymerase with various templates. Templates included plasmid DNA containing the same envelope sequence as pMDEV9ex, DNA from unactivated dunni cells, and DNA from G355/LAPSN + MDEV cells. Amplification products were cloned into pSX2, and 20 clones were selected for analysis. pMEX\textsuperscript{dunni} and pMEX\textsuperscript{plasmid}, which are are shown in Table 1, respectively contain an envelope sequence amplified from DNA of unactivated dunni cells and amplified from plasmid DNA containing the same envelope used for construction of pMDEV9ex.

pMEX\textsuperscript{dunni} was used in the construction of the PD223 packaging cells. Sequence analysis showed that it differs from pMDEV9ex at only 1 nucleotide in the env coding
region. The position is at nucleotide 6186 located just downstream of the first variable region (VRA) in the MDEV Env. The nucleotide is an A in pMDEV9ex and the reported MDEV sequence (accession number AF053745), which corresponds to an Arg residue, and a G in pMEXdun, which corresponds to a Gly residue. Sequence analysis of cloned PCR products amplified from DNA from either dunni cells or G355/LAPSN+MDEV cells demonstrated a G residue at position 6186, indicating that pMEXdun contains the envelope sequence of the native MDEV provirus in dunni cells.

**Evaluation of MDEV Env expression plasmids.** The MDEV Env expression plasmids were evaluated by transfecting them into LGPS/LAPSN or by cotransfecting them with the plasmid pLGPS (which expresses the Moloney MLV Gag and Pol) into G355/LAPSN cells and measuring the packaged LAPSN vector 2 days after transfection. An experiment used to screen the 20 pMEX clones showed that all 5 clones containing the MDEV env amplified from plasmid DNA were functional, 5 out of 6 clones amplified from G355/LAPSN + MDEV DNA were functional, but only 2 out of 9 clones amplified from dunni cell DNA were functional. We have shown that elements related to MDEV exist in the *Mus dunni* genome (15), and some of these nonfunctional pMEX plasmids may carry related but defective envelopes. We have not sequenced any of the nonfunctional plasmids.

The transfection experiments indicate that the various functional MDEV env expression plasmids express similar levels of the MDEV Env, as illustrated by a representative experiment shown in Table 6.1. While transfection of pBSIIKS+ did not result in detectable packaging of LAPSN, transfection of any of the Env expression plasmids did result in packaging of LAPSN. No differences were observed among the MDEV Env expression plasmids, including those containing an MDEV env amplified from infected G355 cells (data not shown). The similar function of MDEV Env plasmids containing envelopes amplified from various sources indicates that they do not contain
significant mutations. In each case, however, the LAPSN titers resulting from transfection of the MDEV env expression plasmids were lower than those achieved by transfection of pSX2. This may reflect the biology of the viruses, for 10A1 MLV typically achieves titers that are 100 fold higher than does MDEV.

Viral interference experiments were conducted that demonstrated that the LAPSN vector packaged by the MDEV Env in these transfection experiments used the same receptor as that used by wild-type MDEV (data not shown).

**Creation of the MDEV packaging cells and selection of clones.**

To generate the MDEV packaging cells, pMEX\textsuperscript{dunni} was stably introduced into LGPS cells (76), which are NIH 3T3 cells engineered to express the Moloney murine leukemia virus Gag and Pol, by cotransfection with the selectable plasmid pSV2Δ13-hyg (a gift from Paul Berg at Stanford University) at a ratio of 20:1. The plasmid pMEX\textsuperscript{dunni} was chosen because it functions as well as any of the MDEV Env expression plasmids, it likely contains the env sequence identical to that in the native Mus dunni provirus, and it has less sequence than pMDEV9ex upstream of env that could potentially recombine with the plasmid pLGPS to generate a recombinant replication-competent retrovirus (Discussion and Fig 6.2). The cells were selected in 0.4 mg hygromycin per ml, and 38 clones were isolated by incubating trypsin-soaked Whatman paper disks on the colonies and transferring the disks into individual wells of a 48-well plate. The clones were designated “PD”, for packaging cells based on the Mus dunni endogenous virus envelope. Two screens were employed to determine which clone possessed the best ability to package a retroviral vector (Table 6.2). A primary screen demonstrated that 32 of the 38 clones had the ability to package LAPSN, from which the nine best clones were chosen for a second screen. The second screen was similar to the first, except that the clones were selected in G418 after infection by LAPSN(PE501) to be certain that all cells carried the LAPSN
vector. PD clone 223 (PD223) was found to produce the highest titer of LAPSN in both the primary and secondary screens.

Upon determining that PD223 packaged LAPSN at the highest titer, we cloned PD223/LAPSN cells to obtain a high titer producer line of LAPSN(PD223) which consistently produces a titer of $4 \times 10^4$ AP* FFU/ml. PD223/LNCG cells were also cloned to obtain a high titer producer line of LNCG(PD223) that packages LNCG at levels of $2 \times 10^4$ GFP* FFU/ml.

**Vectors packaged by PD223 cells use the same receptor as MDEV.** Viral interference experiments were conducted to determine whether a vector packaged by the PD223 cells uses the same receptor as MDEV (Table 6.3). Viral interference experiments rely on the observation that a cell that is producing a retroviral Env is resistant to infection by a retrovirus that uses the same receptor as the expressed Env. The entry of LAPSN(PD223) was dramatically impeded by the presence of MDEV that had been activated by hydrocortisone ($>2 \times 10^4$ fold inhibition), while the entry of LAPSN(PA317) was equivalent on both cell types. LAPSN(PD223) and LAPSN(PA317) contain the same Gag and Pol proteins, indicating that the inhibition is Env-specific and at the level of viral entry. A reciprocal experiment has also been conducted, in which entry of LAPSN pseudotyped by wild-type MDEV or PD223 cells was impeded on PD223 cells as compared to NIH 3T3 cells (data not shown). PD223 cells are NIH 3T3 cells that have been engineered to express the MoMLV Gag and Pol and the MDEV Env, so again the blockage was presumably due to expression of the MDEV Env.

**PD223 cells package vectors without contaminating helper virus.** A packaging cell line should package retroviral vectors without producing contaminating replication-competent retroviruses (RCR), or helper virus. S*L* assays are often used to detect helper virus, but these were not used here because previous experiments have shown that wild-type MDEV does not score on S*L* assays utilizing PG4 cells, CCC-81 cells
overlaid with NRK cells, Mv1Lu cells, or SC-1 cells overlaid with Mv1Lu cells (73). The two assays used here consisted of placing test medium harvested from PD223/LAPSN cells on G355/LAPSN or durnni/LAPSN cells, passaging the cells for more than two weeks, and then transferring the medium to G355 or durnni cells. If the test medium contains RCR that can replicate on the cells, that virus should package the LAPSN vector, allowing its detection on the final indicator cells. The same type of cells were used for the final indicator cells as the LAPSN-containing amplification cells to ensure that any amplified virus could infect the indicator cells. Two assays using different cell types were used to enhance the probability of detecting RCR. Table 6.4 shows that assays using either the G355 and durnni cells were able to detect wild-type MDEV, and either assays can also efficiently detect amphotropic MLV (data not shown). Neither assay, however, revealed any RCR in the medium harvested from PD223/LAPSN cells.

**PD223 cells are useful for transduction of CHO cells.** CHO cells provide an important tool for genetic analysis due to their functionally haploid nature (116), and are widely used in biotechnology for production of glycosylated therapeutic proteins. Unfortunately, CHO cells are relatively resistant to transduction by a variety of retroviral vectors, with a block at the level of viral entry (82). We have previously demonstrated that a vector pseudotyped by wild-type MDEV could efficiently transduce CHO cells, so we compared the LAPSN vector packaged by PD223 cells to LAPSN packaged by seven other packaging cells that express different envelopes for the ability to transduce CHO cells (Table 6.3). The titers of LAPSN packaged by the various cell lines were generally low on CHO cells, as predicted. However, the titer on permissive cells (D17 or NIH 3T3) was high for each stock, demonstrating that there was functional virus present. The most efficient entry into CHO cells was achieved by LAPSN(PD223), which transduced CHO at least 25-fold more efficiently than vectors packaged by any of the seven other packaging cells.
Vectors packaged by PD223 cells transduce primary human CD34+ cells. Hematopoietic stem cells are an attractive target for gene therapy. Cells selected for the CD34 surface antigen contain hematopoietic stem cells, and have been used here as a model for hematopoietic stem and progenitor cell transduction. Primary baboon and human CD34+ cells were transduced with LAPSN pseudotyped by PD223 and PG13 (76) packaging cells. PG13 is a packaging cell line that is preferred for transduction of baboon and human CD34+ cells. Additionally, the FLYRD packaging cell line was included in the human cell experiment because it shows promise for transduction of human cells (29); however, it does not infect baboon cells (data not shown). Flow cytometry using an anti-human placental AP antibody revealed two distinct populations of cells, allowing the determination of the percent transduced. In the baboon experiment, the LAPSN packaged by PD223 transduced the CD34+ cells with about 2-fold lower efficiency than did LAPSN packaged by PG13. In the human experiment, the LAPSN packaged by PD223 transduced the cells as efficiently as did LAPSN packaged by PG13, and more efficiently than did LAPSN packaged by FLYRD (Fig. 6.3). In both experiments, mock-transduced CD34+ cells showed ≤ 0.05% transduction. Because CD34+ cells are a heterogeneous population, it is possible that the vectors with different pseudotypes transduced different subpopulations of cells. The only true assay of a stem cell is the measurement of its ability to reconstitute hematopoiesis in a myeloablated animal, so further experiments will be required to further assess the utility of PD223 cells for use in hematopoietic gene therapy.

Discussion

Mus dunni endogenous virus (MDEV) uses a receptor distinct from those used by other murine retroviruses, and this receptor is broadly expressed among mammalian species and cell types. Because presence of the appropriate receptor is often limiting in transduction of
cells by retroviral vectors, we have created packaging cells expressing the MDEV envelope protein to take advantage of its broad host range.

The packaging cells (PD223) contain an MDEV env that was amplified by PCR using the high-fidelity Pwo polymerase. Sequence analysis demonstrated that this envelope differs from the previously-reported MDEV env by a single nucleotide that induces a nonconservative change in the protein sequence at the end of VRA. However, comparison of various MDEV Env expression plasmids indicates that this amino acid change does not significantly affect the function of the Env (Table 6.1). Sequencing of clones derived from separate PCR amplifications indicated that the PD223 cells express an MDEV Env identical in sequence to that encoded by the native MDEV provirus in dunni cells and Mus dunni mice. Consistent with this, interference analysis demonstrated that the vectors packaged by PD223 cells use the same receptor for cell entry as does wild-type MDEV.

An important feature of packaging cells is that they exhibit a low propensity to generate replication-competent retrovirus (RCR). RCR causes problems by inducing further vector spread, making transduced cells resistant to superinfection by another vector that uses the same receptor, or by infecting other tissues if transduced cells are introduced into organisms for gene marking or gene therapy trials. Indeed, lymphomas have been induced in monkeys by contaminating RCR produced by recombination between a vector and sequences in a packaging cell line (31). In this case, there was considerable overlap between vector sequences and the sequences engineered into the packaging cells.

Three recombination events would be required to produce a genome with a potential RCR from the LN series of vectors and the engineered sequences in PD223 cells (Fig. 6.2). Sequence overlap that would facilitate the 5′ recombination event is almost unavoidable with a vector containing the extended MLV packaging signal (ψ'), because it extends into the gag gene (9). The central recombination event would be between MoMLV
sequences at the end of pol in pLGPS and corresponding 10A1 sequences that are upstream of the MDEV Env in pMEX\textsuperscript{dunni}; these 10A1 sequences were included because they have been found to enhance expression in pSX2, the plasmid upon which pMEX\textsuperscript{dunni} is based (75). However, recombination in this region of homology would result in the substitution of 49 conserved amino acids of the end of Pol with 6 irrelevant amino acids encoded by the beginning of the pMEX construct. The final 3’ recombination event would be unlikely because there is no significant similarity between the vector and pMEX\textsuperscript{dunni}.

Recombination leading to RCR has only rarely been observed with the extensively-used PA317 cells and the LN series of vectors (94), which requires only two recombination events. It is therefore reasonable to predict that generation of an RCR would be even more rare in PD223 cells, also considering that the three recombination events described above would only yield an incomplete retrovirus with a deletion in the pol. However, endogenous retroviral sequences could also be involved in recombination, necessitating direct testing for RCR. For this, two vector-rescue helper assays have been employed. Both assays, which are able to detect wild-type MDEV and amphotropic MLV, were unable to detect contaminating RCR from PD223/LAPSN cells (Table 6.4).

CHO cells have become an important tool for genetic analysis due to their functionally haploid nature (116), but are unfortunately relatively resistant to transduction by retroviral vectors. Because this resistance is at the level of entry (82), we have compared the transduction efficiencies of the LAPSN vector packaged by eight different packaging cell lines, each expressing a different envelope. We have found that vectors packaged by PD223 cells transduce CHO cells more than 10 times as efficiently as vectors packaged by the seven other packaging cells (Table 6.5).

Hematopoietic stem cells are an attractive target for gene therapy due to 1) the tremendous cellular amplification as they give rise to all blood cells, and 2) the accessibility and increasing understanding of bone marrow transplantation. Cells selected for the CD34
surface antigen contain hematopoietic stem cells, and have been used here as a model for hematopoietic stem cell/progenitor transduction. We have found that vectors packaged by PD223 transduce human CD34+ cells as efficiently as do vectors packaged by PG13 cells, which are considered to be the most useful for transduction of such cells. Because CD34+ cells are a heterogenous population, it is possible that the vectors transduced different subpopulations of cells. The only true assay of a stem cell is to measure its ability to repopulate the marrow of a myeloablated animal, so further experiments would be required to further assess the potential of PD223 cells to be useful in hematopoietic gene therapy.

In summary, we have taken advantage of the broad host range of MDEV by constructing packaging cells based on the MDEV envelope. Vectors packaged by PD223 cells can transduce a wide variety of cell types, including CHO cells, which are resistant to transduction by vectors packaged by many other packaging cells, and human CD34+ cells, which are considered an important target for human gene therapy. PD223 cells should be useful for general gene transfer applications, studies of retroviral entry, and the cloning of the MDEV receptor, which will enhance our understanding of retroviral evolution.
Table 6.1. Comparison of MDEV Env expression plasmids

<table>
<thead>
<tr>
<th>plasmid</th>
<th>LAPSN titer (AP⁺ FFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBSIIKS⁺</td>
<td>&lt;5</td>
</tr>
<tr>
<td>pSX2</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>pMDEV9ex</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>pMEX&lt;sub&gt;plasmid&lt;/sub&gt;</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>pMEX&lt;sub&gt;dunnii&lt;/sub&gt;</td>
<td>$2 \times 10^3$</td>
</tr>
</tbody>
</table>

*To evaluate the MDEV Env expression plasmids, LGPS/LAPSN cells were plated at $5 \times 10^5$ cells per 6cm diameter dish on day 1. On day 2, 10 μg of each plasmid DNA was transfected in duplicate dishes by calcium phosphate precipitation. The medium was replaced on day 3 and harvested on day 4, as described in Materials and Methods. The titers are expressed as the mean of the values obtained from the duplicate transfections.
Table 6.2. Primary and secondary screens identified the best packaging clone

<table>
<thead>
<tr>
<th>Clone</th>
<th>1° screen LAPS N Titera</th>
<th>2° screen LAPS N Titerb</th>
</tr>
</thead>
<tbody>
<tr>
<td>208</td>
<td>4.9x10³</td>
<td>6x10²</td>
</tr>
<tr>
<td>209</td>
<td>7.5x10³</td>
<td>4x10³</td>
</tr>
<tr>
<td>217</td>
<td>1.4x10³</td>
<td>4x10⁴</td>
</tr>
<tr>
<td>223</td>
<td>1.3x10⁴</td>
<td>1x10⁵</td>
</tr>
<tr>
<td>229</td>
<td>1.2x10³</td>
<td>2x10⁴</td>
</tr>
<tr>
<td>230</td>
<td>1.4x10³</td>
<td>2x10⁴</td>
</tr>
<tr>
<td>235</td>
<td>1.4x10³</td>
<td>100</td>
</tr>
<tr>
<td>260</td>
<td>6.5x10³</td>
<td>3x10⁴</td>
</tr>
<tr>
<td>276</td>
<td>8.2x10³</td>
<td>1x10⁴</td>
</tr>
</tbody>
</table>

a The primary screen was initialized by plating aliquots of the clones at 5x10⁴ cells per 3.5 cm diameter well of a 6 well dish on day 1. On day 2, the medium was replaced with fresh medium containing 4 μg Polybrene per ml, and 200 μl of LAPS(N(PE501) added. The LAPS(N(PE501) stocks had previously found to have a titer of 5x10⁵ AP⁺ FFU/ml on NIH 3T3 TK⁻ cells. On day 3, the medium was replaced with medium containing no polybrene. On day 4, the virus-containing medium was harvested and the titer determined.

b The secondary screen was initialized by plating aliquots of the clones at 5x10⁴ cells per 3.5 cm diameter well of a 6 well dish on day 1. Two wells were plated for each clone. On day 2, the medium was replaced with fresh medium containing 4 μg Polybrene per ml, and 400 μl of LAPS(N(PE501) added to one well. On day 4, the medium was replaced in both wells with medium containing 0.5 mg active G418 per ml. The cells were split 1:5 as they became confluent. Once all of the non-transduced cells had died in the presence of G418, the transduced clones were released from G418 selection. To evaluate LAPS(N production by the selected clones, each was plated at 2.5x10⁵ per 3.5 cm diameter well on day 1. The medium was replaced on day 2, harvested on day 3, and the titer was determined as described in Materials and Methods. LAPS(N titers are expressed as the mean of duplicate assays in which each value varied from the mean by no more than 17%. 
Table 6.3. Vectors packaged by PD223 cells use the same receptor as MDEV.

<table>
<thead>
<tr>
<th>LAPSN pseudotype</th>
<th>LAPSN Titer (FFU/ml)(^a) on:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>duni/N2</td>
<td>duni/N2+MDEV</td>
</tr>
<tr>
<td>PD223</td>
<td>6x10(^4)</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>PA317</td>
<td>1x10(^6)</td>
<td>1x10(^6)</td>
</tr>
</tbody>
</table>

\(^a\)The titer is expressed as the mean of duplicate assays in which each value varied by no more than 6% of the mean. The experiment was repeated with nearly identical results.

\(^b\)The LAPSN(PD223) stocks had a titer of 3x10\(^5\) AP\(^+\) FFU/ml on D17 cells. PA317 refers to a packaging cell line that pseudotypes vectors in the amphotropic MLV envelope.
Table 6.4: PD223 cells do not produce detectable replication-competent retrovirus

<table>
<thead>
<tr>
<th>Source of test medium</th>
<th>Volume added</th>
<th>G355 Assay Titer</th>
<th>dunni Assay Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD223/LAPSN</td>
<td>1 ml</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>PD223/LAPSN</td>
<td>1 ml</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>G355/LAPSN+MDEV</td>
<td>0.1 µl</td>
<td>4x10^4</td>
<td>2x10^4</td>
</tr>
<tr>
<td>G355/LAPSN+MDEV</td>
<td>0.01 µl</td>
<td>6x10^3</td>
<td>&lt;2</td>
</tr>
<tr>
<td>no virus</td>
<td>0 µl</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

\(^a\)G355/LAPSN or dunni/LAPSN cells were plated on day 1 at 2x10^5 cells per 6 cm diameter dish in 3 ml of medium. On day 2, the the test stock was added in the presence of fresh medium containing 4 µg of Polybrene per ml. The cells were passaged in the presence of Polybrene to facilitate viral spread, during which time they were split 1:10 every several days. On day 17, the medium was harvested and titered on G355 cells for the G355 assay or dunni cells for the dunni assay. In all cases, the dunni cells had not been exposed to agents that activate the endogenous MDEV and so were not expressing MDEV.

\(^b\)The volume of test medium that was added to G355/LAPSN cells or dunni/LAPSN cells.

\(^c\)LAPSN titer (AP* FFU/ml) is expressed as the mean of those determined by duplicate assays in which each value varied from the mean by no more than 20% of the mean for the G355 assay and 4% of the mean for the dunni assay.
Table 6.5: Vectors packaged by PD223 efficiently transduce CHO cells.

<table>
<thead>
<tr>
<th>Packaging cells</th>
<th>Envelope</th>
<th>CHO</th>
<th>D17</th>
<th>NIH 3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA317</td>
<td>amphotropic</td>
<td>&lt;20</td>
<td>1x10^6</td>
<td>N.D.</td>
</tr>
<tr>
<td>PG13</td>
<td>GALV</td>
<td>600</td>
<td>3x10^6</td>
<td>N.D.</td>
</tr>
<tr>
<td>PT67</td>
<td>10A1</td>
<td>800</td>
<td>3x10^5</td>
<td>N.D.</td>
</tr>
<tr>
<td>LLENZB</td>
<td>xenotropic</td>
<td>&lt;20</td>
<td>3x10^5</td>
<td>N.D.</td>
</tr>
<tr>
<td>PM571</td>
<td>MCF/ polytropic</td>
<td>&lt;20</td>
<td>N.D.</td>
<td>1x10^5</td>
</tr>
<tr>
<td>PE501</td>
<td>ecotropic</td>
<td>&lt;20</td>
<td>N.D.</td>
<td>1x10^6</td>
</tr>
<tr>
<td>FLYRD</td>
<td>RD114</td>
<td>&lt;20</td>
<td>4x10^6</td>
<td>N.D.</td>
</tr>
<tr>
<td>PD223</td>
<td>MDEV</td>
<td>2x10^4</td>
<td>2x10^5</td>
<td>N.D.</td>
</tr>
<tr>
<td>wild-type MDEV</td>
<td>MDEV</td>
<td>8x10^4</td>
<td>7x10^4</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Cells were plated at 1x10^5 cells per 3.5 cm diameter well of a 6 well dish on day 1, infected on day 2, and stained for AP* foci on day 4, as described in Materials and Methods.

*Titers are expressed as the mean of those determined by duplicate assays, in which each value varied from the mean by no more than 18%. The experiment was repeated with similar results.
Fig. 6.1. Envelope expression constructs. The constructs are drawn to scale, and the plasmid sequences derived from pML-1 are not shown. Each plasmid has the MoMLV LTR promoter, truncated at the 5' end to the Sau3AI site upstream of the enhancers, the MoMLV splice donor, the 10A1 MLV splice acceptor, the envelope coding sequence, and the early simian virus 40 (SV40) polyadenylation sequence. pMDEX represents 20 plasmids in which the MDEV env was amplified from various sources.
Fig. 6.2. Homologous recombination among the engineered sequences could not generate a replication-competent retrovirus. The vector LXSN has been included in the diagram as an example of a vector that may be used with the PD223 cells. Recombination between pLGPS and pMEX\textsuperscript{danna} would result in the deletion of a portion of \textit{pol}, and there is no similarity between pMEX\textsuperscript{danna} and an LN-series vector. Areas of similarity are indicated by shaded boxes bridging two constructs. The LTRs and coding regions are shown as boxes. \textit{npt} indicates the neomycin phosphotransferase gene, arrows indicate the cap sites of promoters, pA indicate polyadenylation sites, ΔLTR indicates the MoMLV LTR truncated to the \textit{Sau3AI} site upstream of the enhancers, SV indicates simian virus 40 sequences, Ψ- indicates the deletion of the retroviral packaging signal, and TAG indicates the conversion of the Gag ATG start codon to a stop codon in LXSN.
Fig. 6.3. The LAPSNN vector packaged by PD223 efficiently transduces baboon and human CD34+ cells. CD34+ were prestimulated with cytokines and transduced with LAPSNN that had been packaged by the indicated packaging cells. Each value represents the average of three separate transductions, with the standard deviations indicated.
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(Contains material from Chapter I)


(Contains material from Chapter II)


(Contains material from Chapter III)


(Contains material from Chapter IV)


(Contains material from Chapter V)


(Contains material from Chapter VI)
VITA

Greg was born in El Paso, Texas, to Thomas Craig Wolgamot and Bonnie Harding Wolgamot on November 9, 1969. Shortly thereafter, the family moved to Great Falls, Montana. Throughout his childhood, his parents encouraged him to learn about nature and the world, and he became interested in science.

A great believer in education, his mother attended the College of Great Falls to complete a degree in teaching. During this time, she experienced numbness in her legs and problems with balance, and was diagnosed by her husband as having multiple sclerosis. Although she experienced increasing difficulties, she maintained an enthusiastic attitude and completed her education. She was never able to realize a career in teaching, but nonetheless taught her family and peers many important lessons. Partly by observing the dramatic impact that health has on peoples’ lives, Greg became interested in medicine.

Greg graduated from C. M. Russell High School in 1988 and attended Oregon State University, where he studied biochemistry and biophysics. It was there that he began doing research, in the laboratory of George F. Rohrman. Along with Christian Gross, they studied baculovirus structural proteins. They identified a previously unknown capsid protein as well as a protein that forms crystal-like inclusion bodies in infected cells. Greg graduated at the top of his class from Oregon State University in 1992.

The Medical Scientist Training Program at the University of Washington was a perfect place to learn the science of medicine. Greg began the program in 1992, and after briefly studying X-ray crystallography in the laboratory of Ronald Stenkamp, joined the laboratory of A. Dusty Miller to study viruses and their application to treatment of disease.

His thesis project was initiated by the accidental discovery of a previously unrecognized virus that was thought to be a novel human virus. His thesis concerns the demonstration that the novel virus is a mouse virus, the cloning of the virus, characterization of the virus, and the building of packaging cells based on the virus that