An Array of Murine Leukemia Virus-Related Elements Is Transmitted and Expressed in a Primate Recipient of Retroviral Gene Transfer

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Direct RNA-PCR analyses of T-cell lymphomas that developed in rhesus macaques during a gene transfer experiment revealed the presence of several different recombinant murine leukemia viruses (MuLV). Most prominent was the expected MuLV recombinant, designated Mo_{LTR}Ampho_{env}, in which the amphotropic *env* of the helper packaging virus was joined to the long terminal repeat (LTR) of the Moloney MuLV-derived vector. This retrovirus does not exist in nature. An additional copy of the core enhancer acquired from the vector LTR may have augmented the replicative properties of Mo_{LTR}Ampho_{env} MuLV in several different rhesus cell types compared with the prototype amphotropic MuLV_{4070A}. Unexpectedly, at least two types of mink cell focus-forming MuLV elements, arising from endogenous retroviral sequences expressed in the murine packaging cell line, were also transmitted and highly expressed in one of the macaques. Furthermore, murine virus-like VL-30 sequences were detected in the rhesus lymphomas, but these were not transcribed into RNA. The unanticipated presence of an array of MuLV-related structures in a primate gene transfer recipient demands ever-vigilant scrutiny for the existence of transmissible retroviral elements and replication-competent viruses possessing altered tropic or growth properties in packaging cells producing retroviral vectors.

Retroviral vectors derived from murine leukemia virus (MuLV) and containing the amphotropic MuLV_{4070A} 70-kDa surface glycoprotein (gp70SU) as their envelope (22) have been used for the efficient transfer of a variety of genes into primary mammalian cells (4, 34, 36, 49). However, the use of such vectors for human gene therapy has raised concern about the possibility of generating retroviral derivatives possessing novel biological properties in view of the morbidity and mortality associated with retrovirus-induced diseases (55). A long-standing concern has been the possible contamination of amphotropic MuLV vector preparations with replication-competent retroviruses (RCR), capable of establishing an infection in a potential human recipient. This concern was in fact realized when 3 of 10 rhesus macaques developed rapidly fatal CD2⁺ CD8⁺ T-cell lymphomas 6 months after the engraftment of autologous stem cells previously infected with retroviral vector (N2) preparations contaminated with RCR (19).

The N2 vector administered to these primates used the transcriptionally potent Moloney (Mo) MuLV long terminal repeat (LTR) to direct expression of the transduced gene, *neo*^R, and the adjacent *cis*-acting Ψ RNA packaging sequence to encapsidate its RNA (35). The A2 "producer" cell line, derived from a mouse packaging cell line (PA317) that constitutively synthesized amphotropic MuLV_{4070A} proteins (5), released N2-*neo*^R vector particles competent for only a single round of infection of target stem cells (11). However, RCR were also generated in the A2 producer cells, presumably as a consequence of the superinfection procedure designed to elevate the titer of N2 vector (10, 11, 18). It had been predicted

that any RCR emerging in the A2 producer line would be the product of a homologous recombination event that restored the amphotropic MuLV genomic configuration by incorporating the LTR and Ψ elements of the vector (38). The RCR from A2 cells was initially considered clinically inconsequential for rhesus macaques because earlier inoculations with the prototype amphotropic MuLV, MuLV_{4070A}, and other possibly similar amphotropically packaged RCR had been reported to induce a retroviral infection that rapidly cleared (14–16).

The initial characterization of the rhesus tumors by DNA PCR or Southern blot hybridization demonstrated the presence of amphotropic MuLV envelope sequences in DNA samples from lymphoma and peripheral blood mononuclear cells (PBMC) from all three macaques (19). In a more recent study, the RCR present in the serum of one of the affected animals was passaged in mouse NIH 3T3 or mink CCL64 cells and its proviral DNA was subsequently cloned from the Hirt-fractionated DNA of these two rodent cell types (57). Restriction enzyme mapping of full-length viral DNA clones and nucleotide sequence analysis of selected *env* genes indicated that 32 of 33 clones contained amphotropic MuLV *env* sequences and 1 had a mink cell focus-forming (MCF) virus-related *env* sequence.

In the present study, we have utilized the power of reverse transcriptase PCR (RT-PCR) to directly examine all possible MuLV-related RNA elements actually present in two of the RCR-induced lymphomas. Any MuLV-related transcripts detected in macaque tissue by RT-PCR would correspond to retroviral structures that have been transmitted to and expressed in rhesus recipients of gene therapy vectors, because *in vitro* mutagenesis of retroviral genomes has shown that integration into the chromosomal DNA is required for the efficient production of viral mRNAs (48). Using this approach, we have identified a variety of viral mRNAs in two of the rhesus lymphomas, including several species of endogenous MuLV transcripts that were expressed from previously unreported ele-

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ments transferred from the packaging cell line to the monkeys. The unappreciated transmission to, and expression of, a variety of endogenous MuLV transcripts in a rhesus lymphoma is a clear demonstration that unwanted RNA species from murine packaging systems can be transferred, along with the desired transduced gene, to a recipient animal. In addition, we have isolated an RCR directly from the plasma of one of the affected monkeys and have shown that it is far more infectious for several different rhesus macaque cell lines than is the prototype amphotropic MuLV_{4070A}. This altered host range phenotype is a direct consequence of linking the amphotropic envelope to the Mo MuLV LTR, a combination of retroviral sequences that does not exist in nature.

MATERIALS AND METHODS

Characterization of virus from rhesus tissues. Thymic lymphoma tissue (1.2 g) from rhesus macaque 15445 was ground to a fine powder under liquid nitrogen, resuspended in 10 ml of RPMI 1640 medium, and frozen and thawed twice. This tissue homogenate and plasma from rhesus macaque 15445 were clarified by centrifugation at $10,000 \times g$ for 10 min and then passed through a 0.2- μ m-pore-size filter. The tissue or plasma filtrates (approximately 1 ml) were then diluted to 12 ml in phosphate-buffered saline (PBS), and virus particles were pelleted by ultracentrifugation in a Beckman SW-41 rotor at $100,000 \times g$ for 1 h. Virion pellets were resuspended in 50 μ l of PBS, and a 5- μ l aliquot was assayed for RT activity by measuring the incorporation of [32 P]dNTP with an oligo(dT) · poly(rA) template in the presence of 0.6 mM Mn^{2+} or 5 mM Mg^{2+} as the divalent cation (40, 45, 53). FRhL-2 rhesus lung fibroblast cells (CCL160; American Type Culture Collection, Rockville, Md.), growing as monolayers in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and supplemented with 30 ng human epidermal growth factor (Boehringer Mannheim, Indianapolis, Ind.) per ml, were infected with 100 μ l of 15445 plasma filtrate. In control experiments, *Mus dunni* cells were infected with amphotropic MuLV_{4070A} (both supplied by J. Hartley, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) or phytohemagglutinin-activated (1 μ g/ml) rhesus PBMC inoculated with simian immunodeficiency virus (SIV_{MAC239}; supplied by R. Desrosiers, New England Primate Center, Southborough, Mass.). Supernatant medium was harvested at regular intervals and assayed for RT activity in the presence of Mg^{2+} (the rhesus PBMC infection) or Mn^{2+} (the FRhL-2 and *M. dunni* cell infections).

Identification of primers and broad-specificity PCR of MuLV components. Multiple type C murine retroviral sequences, excluding those associated with MuLV retroviral vectors and virally transduced oncogenes, were retrieved from the GenBank and European Molecular Biology Laboratory databases. The CLUSTAL V program (26) was then used to align these MuLV-related sequences into four libraries, approximately 2.5 kb in size, encompassing the LTR-*gag*, *gag-pol*, *pol*, and *env*-LTR regions. On the basis of this alignment, two sets of PCR primers were constructed for amplifying MuLV LTR sequences by using an oligonucleotide synthesizer (Applied Biosystems Inc., Foster City, Calif.). The first primer pair (U5 primer 1+, 5'-TCAGCGGGGCTCTTTCAT TTG-3'; and U3 primer 2-, 5'-ACAGGTGGGGTCTTTCATTC-3'), predicted to hybridize to the termini of most MuLV LTRs, was capable of amplifying from the untranslated 5' (U5) to the untranslated 3' (U3) region of envelope-encoding mRNAs from a wide variety of MuLV. The second primer pair, used to deduce the structure of the U3 regions of MuLV-related LTRs, consisted of a forward primer annealing to U3 regions of most MuLVs (universal U3+, 5'-CAAGCTAGCTTAAGTACGCCA-3') and a reverse primer annealing to the R region shared by most MuLVs (universal R-, 5'-AGGGTTATT GGATACACGGGA-3'). Some mouse endogenous proviruses have minor sequence differences located in the center of the universal R- primer but can still be amplified under the 55°C, 1-min annealing conditions used.

RT-PCR for retroviral mRNA species. Total cellular RNA was harvested by extraction with RNazol (TelTest Inc., Friendswood, Tex.), and poly(A)⁺ RNA was selected by the Micro-Fast Track oligo(dT) cellulose method (Invitrogen, San Diego, Calif.). For first-strand cDNA synthesis, poly(A)⁺ RNA was denatured at 94°C for 2 min in 15 mM methyl mercuric hydroxide in the presence of 130 mM 2-mercaptoethanol and 0.5 μ g of random hexamers or oligo(dT) and then subjected to two 1-h cycles of reverse transcription with avian myeloblastosis virus RT, using a cDNA cycle kit (Invitrogen). Full-length *env* mRNAs were amplified with the U5 primer 1+ forward and U3 primer 2- reverse primers for 30 cycles (94°C for 1.5 min, 57°C for 1.5 min, and 72°C for 3 min). Thirty cycles of PCR (94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min) with the universal U3+ and R region primers were performed to elucidate the U3 structure of discrete MuLV *env* mRNA species; the amplified product was analyzed on a 2% agarose gel. Purified PCR bands were cloned into the pCRII vector (Invitrogen), and cDNA inserts were sequenced (46) and compared with the GenBank and European Molecular Biology Laboratory libraries by using the FASTA program (44).

To delineate the *env* and LTR structures of discrete mRNAs, nested PCR was

performed on diluted products from RT-PCR initially with U5 primer 1+ (above) as the forward primer and universal R- as reverse primer and then with MuLV *env* specific forward primers (i) Amphi+ (5'-ACGGGATGCTCTAAAG TTGCCTGT-3'), (ii) AmphiΔ+ (5'-GATGCTCCTTCTGTGGCCCCCTG-3'), (iii) MCF+ (5'-AGGGCCCCGTGTTATGATTCTTCAGT-3'), or (iv) Mo+ (5'-CTGTCCAGGTATAAAGATAATAAGTGG-3') *env* genes with reverse LTR primers for either Universal R (above), Mo U3- (5'-TGCTGAGGGCTGGAC CGCA-3'), or Amphi and MCF U3- (5'-GAAACTGCTGAAGGTTGGGCC AT-3') sequences.

To amplify VL30 sequences, two VL30 PCR primers targeted to regions of VL30 with low sequence homology to the MuLV or simian sarcoma virus (2), were employed: 5'-TGTAAGAAAATGAGCACTGGGTGAG-3' (VL30 +2178) and 5'-CAAGTATGCCACAGGTCTTTTCAGG-3' (VL30 -2620). DNA or cDNA from the lymphoma of rhesus macaque 15445 was amplified for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

RESULTS

Infectivity of retroviruses from rhesus macaque tissues. In a previous analysis of three rhesus macaques that developed lymphomas after engraftment with CD34⁺ bone marrow cells that were infected with a MuLV-derived vector carrying a neomycin resistance gene, replication-competent virus was detected in the serum of two of the animals by the S⁺L⁻ assay (19). The presence of virus in the serum of one of the macaques (no. 15445) was also inferred in a more recent study in which MuLV-related molecular clones were obtained from mouse NIH 3T3 fibroblasts or mink CCL64 cells following incubation with the serum of the affected animal (57). In an attempt to directly isolate and characterize potentially infectious retroviruses present in rhesus macaque 15445, we prepared $100,000 \times g$ virion pellets from the plasma or 0.2- μ m-pore-size filtrates of tumor homogenate from animal 15445; Mn^{2+} -dependent (~20,000 cpm/ml) but not Mg^{2+} -dependent RT activity (40, 45, 53) was detected in both samples. This result provided a direct demonstration that macaque 15445 was indeed viremic. It also eliminated several simian retroviruses (simian immunodeficiency virus, simian T-cell leukemia virus, Mason-Pfizer monkey virus, and simian D-type retrovirus) with an Mg^{2+} cation requirement for RT activity as possible causative agents of the observed lymphomas (17, 20, 27, 30, 33).

To avoid a possible artifact related to the propagation and selection of the recovered virus in rodent tissue culture systems, a variety of rhesus cell types were infected with filtered (pore size, 0.2 μ m) plasma or virus prepared from the pelleted plasma of rhesus 15445. Fig. 1A shows that the RCR isolated from the pelleted (or unpelleted) rhesus plasma readily infected the rhesus lung fibroblast cell line, FRhL-2. The same virus could also be propagated in rhesus kidney (FMk), rhesus bronchial fibroblast (4MBr-5), HeLa, and the broadly permissive (for MuLV) *M. dunni* cell lines (32) but not in rhesus PBMC activated with PHA (data not shown). The virus progeny released from FRhL-2 cells exhibited robust infection kinetics during a second cycle of infection of the same cells, whereas an equivalent amount (based on RT activity) of the prototype amphotropic MuLV_{4070A} produced only relatively low levels of infectious virus (Fig. 1B). In contrast, both the rhesus plasma virus and amphotropic MuLV_{4070A} replicated to high levels in *M. dunni* cells (Fig. 1C).

Cloning and sequencing of the abundantly expressed *env* RNAs. To avoid the possibility of not detecting novel retroviral recombinants as a consequence of their elimination during the isolation procedure, the thymic lymphoma of rhesus 15445 (the animal with the best-preserved tissue samples) was directly examined by using PCR primers and amplification strategies capable of detecting the mRNAs of most MuLVs and their derivatives. Alignment of several exogenous and endogenous MuLV sequences identified a set of oligonucleotides, mapping

FIG. 1. Growth characteristics of the retroviruses isolated from rhesus macaque 15445. (A) Infection of the rhesus lung fibroblast cell line, FRhL-2, with an inoculum of 100 μ l of plasma from macaque 15445 ($\blacksquare$) containing 5,500 cpm of (Mn^{2+} -dependent) RT activity, virions pelleted by ultracentrifugation from 500 μ l of macaque 15445 plasma ($\blacksquare$) containing 20,000 cpm of RT activity, or a medium control ($\times$). The days of cell passage are marked with an arrow. (B) In a second round of infection with equivalent amounts (100,000 cpm of RT activity) of the macaque 15445 plasma virus and amphotropic MuLV_{4070A} ($\triangle$), both produced in FRhL-2 cells, the 15445 plasma virus had significantly greater infectivity for FRhL-2 cells than did the amphotropic MuLV_{4070A}. (C) However, both of these viruses exhibited similar infectivity in *M. dunni* fibroblasts, which are highly permissive for most MuLV strains (32).

to the inner boundaries of the U5 and U3 termini, that could prime RT-PCR for any C-type MuLV retrovirus-derived mRNA, including those containing unique *env* genes or recently captured *v-onc* elements (52, 54). RT-PCR amplification of mRNA extracted from the 15445 lymphoma yielded a single product of 2.4 kb (Fig. 2A), the size expected for a correctly spliced MuLV *env* mRNA. This 2.4-kb band was also the major PCR product present in rhesus FRhL-2 fibroblasts infected with the 15445 plasma virus isolate (Fig. 2B).

Twelve independent *env* cDNA clones were obtained following RT-PCR, and their nucleotide sequences were determined.

FIG. 2. Lymphoma from rhesus macaque 15445 and FRhL-2 fibroblasts infected with virus from macaque 15445 plasma express full-length *env* mRNA related to murine C-type retroviruses. RT-PCR for MuLV *env* with mRNA from FRhL-2 cells (lanes 1 and 4), thymic lymphoma tissue from rhesus 15445 (lanes 2 and 3), and FRhL-2 cells infected with plasma from macaque 15445 after 9 (lane 5) or 36 (lane 6) days in culture was performed. A 1-kb size ladder is shown at the left of each panel. Template cDNA was primed with either random hexamers (lanes 1, 2, 5, and 6) or oligo(dT) (lanes 3 and 4), and PCR was performed with primers complementary to the terminal U5 and U3 regions of the LTR of wide variety of C-type retroviruses (26).

Nine corresponded to the prominent 2.4-kb band amplified from the macaque 15445 tumor, and three corresponded to the same-sized band amplified from FRhL-2 fibroblasts productively infected with the virus recovered from rhesus macaque plasma (Fig. 3). The 5'- and 3'-terminal regions of all of the clones were readily aligned (44) with the Mo MuLV LTR, known to be present in the N2 vector. However, two different types of MuLV *env* sequence were identified between the 5' splice junction site and a common 3' *Cla*I site located 100 nucleotides (nt) upstream from the *env* translation termination signal (Fig. 3). Of the 12 clones, 9 contained the gp70SU *env* sequence of amphotropic MuLV_{4070A}, which is the *env* gene of the helper provirus in the A2 packaging cell line. These clones were identical to the previously reported V/H proviral DNA clone, recovered from rodent cells exposed to the serum of macaque 15445 (57). We designated these *env* mRNAs Mo_{LTR}Ampho_{env} because they contained the Mo MuLV LTR linked to amphotropic MuLV *env* sequences (see below). Two of these nine clones, one from each source, contained a 6-bp deletion within the *env* coding region and were designated Mo_{LTR}Ampho_{env} Δ . This deletion results in minor changes to the second variable receptor-binding domain (see Fig. 5B), which is known to affect virus tropism (6, 7, 37, 41), and had also been previously identified during the cloning of proviral DNA from infected rodent cells (57).

A second group of RNA-derived clones (3 of 12), all from the thymoma tissue, contained sequence for the p15 transmembrane protein (p15TM) of the amphotropic MuLV_{4070A} helper linked to the sequence for gp70SU that best aligns with Friend MCF MuLV (FrNx strain [1]) except for a short segment upstream of the gp70SU/p15TM processing site, which shares only 92% homology with MCF MuLV_{FrNx} (Fig. 3, positions 1510 to 1635). This *env* RNA was designated Mo_{LTR}Ampho/MCF_{env} and contained sequences identical to those present in one of the 33 proviral DNA clones previously characterized (and designated AMP/MCF [GenBank S70177]) (57). It is of interest that two previously reported endogenous polytropic *env* segments (GenBank M17326 and M17327) (51) are very closely related (97.6%) to Mo_{LTR}Ampho/MCF_{env} through this region. However, the homology of these two endogenous polytropic sequences through the upstream gp70SU coding region is slightly lower than that observed with the

FIG. 3. The lymphoma from rhesus macaque 15445 contains *env* mRNA for three types of MuLV. The consensus sequences from the three types of *env* cDNA clones obtained from macaque 15445 tumor tissue were aligned, with the top (reference) line of each group of six, designated Mo_{LTR}Ampho_{env} (GenBank accession number U36602), representing the most common (five of nine) type of clone obtained. The second line is a variant of Mo_{LTR}Ampho_{env} containing a 6-bp deletion (Δ) at the site indicated by the bullets above the reference sequence (●●●●) and designated Mo_{LTR}Ampho_{env} Δ (GenBank accession number U36800); the third sequence is the second most prevalent type of *env* cDNA clone (three of nine) and is designated Mo_{LTR}Ampho/MCF_{env} (GenBank accession number U36991). The bottom three lines in this alignment show the *env* mRNA sequences of prototype MuLV: amphotropic 4070A virus, the FrNx strain of MCF virus, and Mo ecotropic virus. A dash (—) indicates a break in the sequence, and a period (.) indicates that the nucleotide at that position is identical to the reference sequence. RNA and protein features, including the primer-binding site for reverse transcription (PBS) and the *Cla*I restriction site, are indicated. The identity of the primers used for the PCR are also shown at the distal ends of the sequence.

Friend MCF MuLV_{FrNx} (GenBank K01384) (97.1% compared with 99.0%).

Transcriptionally activated U3 promoters in the rhesus macaque lymphoma. The structure of the LTRs directing the synthesis of viral mRNA in the macaque lymphomas was also determined by RT-PCR with U3/repeat (R) region primer pairs capable of amplifying the 3' untranslated regions of a variety of MuLV transcripts (Fig. 4A). Four discrete U3 region products were directly amplified from both the 15445 and 88049 lymphomas, as well as from *M. dunni* cells infected with plasma-derived virus from animal 15445 (Fig. 4B, lanes 1, 2,

and 3). Although significantly less abundant than the faster-migrating bands, the faint, most slowly migrating band was repeatedly detected in lymphomas from rhesus macaques 15445 and 88049. Sequencing of 30 of these cDNA clones, amplified from both lymphomas, revealed that the four different RT-PCR products contained one to four copies of a core enhancer element (1, 3, 31, 43, 50) between nt 117 and 191 (Fig. 4A). In contrast, the predominant MuLV transcript in the vector-producing A2 line contained a U3 element with two core enhancer motifs (Fig. 4B, lane 5), although small amounts of mRNAs with one or three copies of the enhancer were also

FIG. 4. Retroviruses from lymphoma tissue of rhesus macaques 15445 and 88049 have multiple copies of the transcriptional core enhancer element from Mo MuLV U3. (A) A sequence alignment showing that the U3 and R regions of 1 of the 30 independent PCR clones amplified from macaque 15445 was identical to the sequence of this region of Mo MuLV but not of amphotropic MuLV_{4070A}. Other PCR clones of this region differed from Mo MuLV by deleting or duplicating the core enhancer region (boxed from positions 117 to 191). The binding regions of murine transcription factors (LVa, NF1, Lvb, AP-3, LVC, NF1, and GRE), the TATA and CAT boxes (50), and the locations of the oligonucleotide primers for PCR are shown above the alignment. The rhesus virus sequence at the extremities, shown in lowercase type, was obtained from other experiments. (B) The structure of enhancer elements in the U3 region of MuLV-related RNA was examined by RT-PCR analysis of mRNA from thymic lymphoma tissue of macaque 15445 (lane 1) or macaque 88049 (lane 2), from *M. dunni* cells infected with plasma from macaque 15445 after 36 days in culture (lane 3) or amphotropic MuLV_{4070A} (lane 4), from A2 producer cells for the N2 vector (lane 6), from uninfected rhesus FRhL-2 fibroblasts (lane 6), or from uninfected *M. dunni* fibroblasts (lane 7). PCR was performed with the universal U3 forward primer and the universal R reverse primer to regions of MuLV shared by many MuLVs. PCR products were analyzed on a 2% agarose gel, with a 100-bp ladder at the left as a size marker.

FIG. 5. Envelope mRNAs from rhesus macaque 15445 and 88049 tumors have joined amphotropic MuLV *env* to the Mo MuLV LTR or MCF *env* to Mo MuLV LTR. Some MCF *env* remained joined to MCF LTR in the tumor of macaque 15445. The first step of nested RT-PCR was performed with the U5 primer 1 (Fig. 3) as the forward primer and universal R (Fig. 4) as the reverse primer on RNA from lymphoma tissue from macaque 15445 (lanes 1, 7, and 13), macaque 88049 (lanes 2, 8, and 14), *M. dunni* fibroblasts infected with macaque 15445 plasma (lanes 3, 9, and 15) or MuLV_{4070A} amphotropic virus (lanes 4, 10, and 16), uninfected FRhL-2 rhesus fibroblasts (lanes 5, 11, and 17), or uninfected *M. dunni* cells (lanes 6, 12, and 18). Several different primer pair combinations were used in the second step of the nested PCR, including (i) MuLV *env* forward primers specific for AmphiΔ (A, lanes 1 to 6), Amphi (A, lanes 7 to 12), MCF (A, lanes 13 to 18), and Mo (B, lanes 1 to 6) with the reverse complement of the universal U3 forward primer (Fig. 4); (ii) MuLV *env* forward primers specific for AmphiΔ (B, lanes 7 to 12), Amphi (B, lanes 13 to 18), or MCF (C, lanes 1 to 6) with the reverse primer specific for Mo U3 (Fig. 4); and (iii) MuLV *env* forward primers specific for Amphi (C, lanes 7 to 12) or MCF (C, lanes 13 to 18) with the reverse primer specific for Amphi/MCF U3 (Fig. 4).

present. U3 elements containing four core enhancers were not detected in the A2 producer cells. This U3 structure is clearly different from that present in the LTR of the amphotropic MuLV_{4070A}, which contains a solitary core enhancer element (Fig. 4B, lane 4). A similar PCR analysis of the LTR present in the original N2 plasmid (35), using the same primer pair, generated the expected product: a U3 region containing two copies of the core enhancer (data not shown).

Retroviral envelope and LTR sequence combinations in mRNAs from rhesus macaque lymphomas. RT-PCR was next used to evaluate the envelope and LTR composition of discrete MuLV mRNA species present in the two rhesus lymphomas or in infected *M. dunni* fibroblasts. The strategy was to use combinations of four different upstream MuLV envelope primers (Mo ecotropic, amphotropic, "deleted" amphotropic, and MCF [see Materials and Methods]) and three different downstream U3 LTR reverse primers (the reverse complement of the universal U3, the Mo ecotropic, and the amphotropic/Friend MCF [Fig. 4A]) for mRNA amplification. The Amphi U3 reverse primer shown in Fig. 4A is identical to 22 of 23 nucleotides from this region of the Friend MCF MuLV_{FrNx} and is very closely related to comparable segments of other MCF MuLV U3 regions. Therefore, it has been designated the Amphi/MCF U3 primer. This primer is capable of amplifying some sequences associated with recombinant MCF *env* mRNAs, such as Friend MCF MuLV_{FrNx}. However, some MCF *env* transcripts expressed from endogenous polytropic MuLV sequences share less nucleotide sequence homology

with the Amphi/MCF U3 primer (51). As a consequence, such endogenous MCF-related *env* transcripts may not efficiently amplify. In fact, increasing the PCR annealing temperature markedly reduces the number of endogenous MCF-related *env* bands amplified by the Amphi/MCF primer (data not shown).

RCRs containing either the full-length or 6-bp-deleted amphotropic MuLV envelope linked to MuLV U3 sequences (Fig. 5A, lanes 1 to 3) and more specifically to a Mo U3 LTR (Fig. 5B, lanes 7 to 9 and 13 to 15) were present in the two lymphomas and *M. dunni* cells, infected with the 15445 plasma virus inoculum. The two bands amplified in the macaque 15445 lymphoma specimen with the Mo reverse U3 primer (Fig. 5B, lanes 7 and 13) represent *env* transcripts joined to U3 segments presumably containing one or two core enhancer elements, respectively. As expected, neither lymphoma contained a prototype amphotropic MuLV *env* transcript, readily detected in MuLV_{4070A}-infected *M. dunni* cells (Fig. 5C, lanes 7 to 10) or the ecotropic Mo MuLV *env* RNAs (Fig. 5B, lanes 1 and 2).

As noted above, three of nine *env* cDNA clones, amplified directly from macaque 15445 lymphoma, contained sequences very similar to that found in Friend MCF MuLV (13, 23, 51, 57). PCR analysis of the LTRs linked to these MCF MuLV *env* transcripts revealed the presence of at least two RNA subsets. The first (Mo_{LTR}-Amphi/MCF_{env}) contained MCF *env* sequences joined to Mo MuLV U3 regions containing multiple core enhancers (on the basis of several LTR amplification experiments similar to those shown in Fig. 4B). This type of MCF *env* RNA was present in the lymphoma from animal 15445 but not from animal 88049 (Fig. 5C, lanes 1 and 2) and is consistent with previous DNA analyses of these macaque tumors (57). An RCR containing this constellation of sequences was not detected in *M. dunni* cells infected with 15445 plasma virus (Fig. 5C, lane 3). Although this identical MCF *env* sequence had previously been identified in an amplified proviral DNA sample in animal 15445, it was not associated with an authentic Mo MuLV LTR. Instead, it was linked to an LTR containing a 22-bp insertion that disrupted one of the core enhancer elements (57). The latter type of LTR alteration was never detected in either lymphoma by RT-PCR.

A second unusual class of MCF *env* mRNAs, containing the Amphi/MCF U3 configuration rather than the Mo MuLV U3, was also detected in lymphoma 15445 (Fig. 5C, lane 13). Multiple species of these MCF *env* transcripts were detected, one of which was significantly larger (by approximately 200 nt) than the MCF *env* RNA linked to Mo MuLV LTR sequences (compare with Fig. 5C, lane 1). Determination of the precise structure of these unique MCF-related transcripts will require the sequencing of this class of RT-PCR clones, but their presence suggests that endogenous MuLV-related sequences in the mouse packaging line were incorporated into progeny particles, transmitted to the rhesus macaques, and subsequently expressed in the lymphoma that developed in the primate recipient. We have also conducted side-by-side RT-PCR analyses of endogenous MuLV RNAs in the A2 producer cell line and the macaque 15445 lymphoma by using the Amphi/MCF

FIG. 6. Endogenous retrovirus-like VL30 elements were transmitted from the murine producer cells into the DNA of the rhesus macaque lymphomas but were not expressed as RNA in rhesus macaques. PCR for the VL30 *pol* region was performed by using as template either DNA (A) or cDNA from RNA (B) of the A2 producer clone (lanes 1), rhesus 15445 tumor (lanes 2), macaque 88049 tumor (lanes 3), uninfected rhesus PBMC (lanes 4), or uninfected FRhL-2 rhesus fibroblasts (lanes 5). A 100-bp size ladder is shown at the left of each panel (lanes M).

A.

Structure	RNA element	Packaging efficiency	Documented transmission to recipient cells
	recombinant viral vector	high	+
	helper virus (MuLV _{4070A} derivative)	very low	-
	endogenous retroviral elements	high	+
	virus-like VL-30	high	+
	mouse cell mRNAs	very low	rarely

B.

Structure	env RNA Designation	env type	U3 type	Infectious for :		
				Mus dunni	FRhL-2	
1a.		Mo _{LTR} - Ampho _{env}	4070A ampho	Moloney	yes	yes
1b.		Mo _{LTR} - AmphoΔ _{env}	4070A ampho - 6bp	Moloney	yes	yes
2a.		Mo _{LTR} - Ampho/MCF _{env}	MCF gp70SU + ampho p15TM	Moloney	no	no
2b.		MCF (endogenous)	MCF - like	MCF	no	no

U3 primer and the following four upstream *env* region primers: (i) Ampho/MCF common *env* (Fig. 3, nt 945 to 964), (ii) MCF *env* (Fig. 3, nt 851 to 896), (iii) 5.3-5A *env* (57) (Fig. 3, nt 885 to 905), and (iv) 5.3-5B *env* (57) (Fig. 3, nt 1292 to 1315). In each case, a comigrating full-length *env*/LTR amplified band was detected (data not shown).

PCR was also performed to assess whether murine retrovirus-like VL30 (2) sequences were transmitted to the rhesus macaques (Fig. 6). A VL30-specific 469-bp PCR product, amplified from the DNA of the murine A2 producer clone (Fig. 6A, lane 1), was verified by sequence analysis (data not shown). Comigrating PCR products were also obtained from lymphoma DNA samples from both rhesus macaques 15445 and 88049 but not from the DNA of uninfected rhesus PBMC or FRhL-2 fibroblasts. The VL30 elements present in the rhesus lymphoma DNA were not transcribed as RNA, because the VL30-specific 469-bp RT-PCR product from A2 RNA was not amplified from either of the two rhesus lymphomas (Fig. 6B). This result indicates that VL30 RNA, transcribed in the A2 cells producing the N2 vector, was packaged into retrovirus particles, transferred to rhesus stem cells, and transmitted to rhesus monkeys subsequent to engraftment.

DISCUSSION

Most analyses of the potential risks associated with retrovirus-mediated gene therapy have focused on replication-competent amphotropic MuLVs generated from viral or cellular elements present in producer cell lines (Fig. 7A). In contrast to other classes of murine retroviruses, the pathogenic potential of amphotropic MuLVs, including the well-studied strain 4070A, has been judged to be extremely low to nonexistent (14–16). The reported rapid clearance of virus from the blood and the absence of any untoward effects following inoculation of macaques with amphotropic MuLV_{4070A} are consistent with this assessment (4). However, the direct RT-PCR analyses of two rhesus lymphomas described in this report clearly demonstrate that the RCR detected in both tumors was not a prototype amphotropic RCR (Fig. 6B) but, rather, was a MuLV recombinant consisting of an amphotropic *env* gene joined to a Mo MuLV LTR (Fig. 7B, lines 1a and 1b), a combination of murine retroviral motifs not present in previously reported virus isolates. A virus with the same constellation of elements was directly isolated from the serum of one of the affected macaques and, in contrast to the amphotropic MuLV_{4070A}, grew to high levels in cultured primate cells. In this regard and, with the possible exception of the class of elongated MCF *env* mRNAs (Fig. 5C, lane 13), none of the four MuLV *env* mRNAs expressed in the macaque 15445 lymphoma is a transcript associated with a known murine retrovirus (Fig. 5B).

The presence of the unique Mo_{LTR}Ampho_{env} RCR in both lymphomas, coupled with its enhanced infectivity for rhesus fibroblast cells compared with that of amphotropic MuLV_{4070A} (Fig. 1), strongly implicates it as the causative agent of the two lymphomas examined. The extended host range of the recov-

ered Mo_{LTR}Ampho_{env} RCR most probably reflects the acquisition of an additional core enhancer element, the principal genetic difference between it and amphotropic MuLV_{4070A}, which contains only a single copy of the core enhancer (Fig. 5A). Multimerization of enhancer motifs has previously been reported to increase the transcriptional activity of viral promoters. For example, a simian virus 40 mutant containing an inactivated core enhancer element regained wild-type growth properties by spontaneously duplicating a 15-bp enhancer motif during long-term passage in tissue culture (24, 25). Similarly, the suppressed infection kinetics and reduced leukemogenicity of a Mo MuLV derivative, containing an inserted polyomavirus regulatory sequence in its LTR, were abrogated by a reversion event that triplicated the 75-bp Mo MuLV core enhancer element (8, 12).

In the present study, Mo_{LTR}Ampho_{env} RCRs containing two or more copies of the enhancer element were prominent in samples of the two lymphomas. Because analogous *env* transcripts with two core enhancer predominated in the packaging line, it is possible that the triplicated and quadruplicated enhancer elements were selectively expanded during or after infection/engraftment of the rhesus stem cells. Exposure of an individual or, in this case, rhesus macaques to an RCR possessing a more active promoter and augmented replicative potential very probably led to multiple cycles of spreading infection, particularly in the context of bone marrow ablation and immunosuppression. The multiple integration events attending each cycle of infection also increased the likelihood of activating a cellular oncogene in three of the inoculated macaques (57).

One of the more surprising findings of this work was detection of a variety of MCF *env* transcripts in one of the two macaque lymphomas (Fig. 5C, lane 13). The inadvertent transfer of such sequences from a mouse packaging cell line to human cells during tissue culture infections with a retroviral vector has been reported previously (47). Our results clearly demonstrate the transmission of multiple endogenous MCF elements from murine producer cells to an animal engrafted with stem cells infected with a retroviral vector. These endogenous elements contributed to the formation of provirus encoding Mo_{LTR}Ampho/MCF_{env} transcripts, which were quite abundant in macaque 15445 lymphoma, as judged by the frequency (33%) of the RT PCR cDNA clones bearing MCF *env* sequences (six of the nine clones contained amphotropic sequences, and three contained MCF *env* sequences [Fig. 7B, line 2a]). Furthermore, some of these endogenous elements served as recombination partners in the A2 producer line, generating the Mo_{LTR}Ampho/MCF_{env} MuLV. The acquisition of the Mo MuLV LTR could explain the abundance of the Mo_{LTR}Ampho/MCF_{env} mRNA transcripts in macaque 15445 lymphoma. Although we were unable to isolate an RCR containing this combination of MuLV segments in long-term *M. dunni* and rhesus cell cultures, an RCR related to Mo_{LTR}Ampho/MCF_{env} MuLV was nonetheless identified following a 14-day infection of CCL64 mink cells (57).

FIG. 7. Retrovirus-related *env* RNAs present in the lymphoma tissue of macaque 15445 are derived from transmissible elements in the murine packaging cells. (A) Several elements in murine producer or packaging cells, apart from the vector, possess a packaging signal (Ψ) which directs efficient encapsidation and transmission to recipient mammalian cells. The Ψ site has been removed (X) from the packaging sequences. Endogenous retroviral elements and viruslike elements mostly contain defects (X) that disrupt *gag*, *pol*, and *env* genes, but recombination events may generate an RCR, as indicated by dashed lines. (B) The predominant *env* RNAs identified in the lymphomas from rhesus macaques 15445 and 88049 were recombinants that joined the amphotropic *env* (1a) or an altered amphotropic *env* (affecting the variable receptor-binding [VRB] region) (1b) to the U3-terminal region of Mo MuLV from the N2 vector, except that duplications of the core enhancer element (E) had occurred. In addition, the tumor from macaque 15445 but not 88049 contained an *env* RNA in which a segment of *env* from MCF was substituted within the Mo_{LTR}Ampho_{env} *env* RNA (2a). A unique species of MCF *env* RNA, approximately 200 nt longer than any of the other MuLV *env* transcripts, was also detected. This elongated MCF transcript, diagrammatically shown in line 2b, may contain U3 sequences derived from endogenous MuLV-related sequences expressed in the packaging cell line.

At present, we do not know the precise structure of the elongated species of MCF *env* mRNA amplified with the Amphi/MCF U3 primer. This transcript was clearly transmitted, integrated, and expressed in the lymphoma of one rhesus macaque, yet no virus of this type was ever recovered from the plasma or lymphoma of animal 15445. It is tempting to speculate that this large species of MCF *env* RNA may contain endogenous MuLV-related elements (3, 31, 43, 51) expressed in the A2 packaging cell line. Nucleotide sequencing of the elongated species of MCF *env* transcripts will be required to delineate their structure and origin. It is also of interest that the macaque 15445 and 88049 lymphomas contained unique virus-like VL30 sequences (9, 56) when analyzed by DNA PCR with primer pairs for mouse VL30 (56) (Fig. 6A). However, no murine VL30 transcripts were detected by RT-PCR analysis of RNA (Fig. 6B), and no transcripts for any other "captured" mouse sequences (52) (Fig. 7A) were detected with primer pairs capable of amplifying transcripts bounded by highly conserved MuLV U5 and U3 terminal domains (data not shown).

Although the N2 vector used for the rhesus gene transfer experiment has now been replaced by a new generation of vectors possessing smaller regions of homology with the helper virus, breakouts of RCR in vector production lots still occur at low frequencies, even with regions of homology as short as 8 to 10 nt (42). Because the new packaging cell lines that accompany the latest vectors are still derivatives of murine NIH 3T3 fibroblasts, the regeneration of such RCRs might also be abetted by the copackaging of endogenous MCF MuLV RNAs generated in mouse producer cell lines, as suggested by our results. Such endogenous MuLV elements contain encapsidation signals and could function as viable recombination partners with the vector or a different copackaged endogenous retroviral mRNA (shown diagrammatically in Fig. 7A) during the reverse transcription step of the subsequent cycle of infection (28, 29, 58). For these reasons, great vigilance should be exercised to rigorously exclude unanticipated RCRs from reagents to be administered to immune system ablated or deficient patients. Additional cell types (e.g., mink, primate) could be used for the detection and isolation of RCRs with unusual tropic and replicative properties (4). Alternatively, the development of retrovirus packaging systems (e.g., quail, canine, or human diploid cells) that generate either no MuLV-related transcripts or low levels of retrovirus RNAs that are incapable of recombining with existing MuLV-based vectors would greatly reduce production of potential RCRs. The use of *ex vivo* packaging systems such as liposomes (39) or procedures that directly deliver a desired DNA segment, such as gene-gun technology (21), might eventually prove safer.

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REFERENCES

- Adachi, A., K. Sakai, N. Kitamura, S. Nakanishi, O. Niwa, O. Matsuyama, and A. Ishimoto. 1984. Characterization of the *env* gene and long terminal repeat of molecularly cloned Friend mink cell focus-inducing virus DNA. *J. Virol.* **50**:813–821.
- Adams, S. E., P. D. Rathjen, C. A. Stanway, S. M. Fulton, M. H. Malim, W. Wilson, J. Ogden, L. King, S. M. Kingsman, and A. J. Kingsman. 1988. Complete nucleotide sequence of a mouse VL30 retro-element. *Mol. Cell. Biol.* **8**:2989–2998.
- Amanuma, H., F. Laigret, M. Nishi, Y. Ikawa, and A. S. Khan. 1988. Identification of putative endogenous proviral templates for progenitor mink cell focus-forming (MCF) MuLV-related RNAs. *Virology* **164**:556–561.
- Anderson, W. F., G. J. McGarrity, and R. C. Moen. 1993. Report to the NIH recombinant DNA advisory committee on murine replication-competent retrovirus (RCR) assays. *Hum. Gene Ther.* **4**:311–321.
- Armentan, D., S. F. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa. 1987. Effect of internal viral sequences on the utility of retroviral vectors. *J. Virol.* **61**:1647–1650.
- Battini, J.-L., O. Danos, and J. M. Heard. 1995. Receptor-binding domain of murine leukemia virus envelope glycoproteins. *J. Virol.* **69**:713–719.
- Battini, J.-L., J. M. Heard, and O. Danos. 1992. Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukemia viruses. *J. Virol.* **66**:1468–1475.
- Belli, B., A. Patel, and H. Fan. 1995. Recombinant mink cell focus-inducing virus and long terminal repeat alterations accompany the increased leukemogenicity of the Mo+PyF101 variant of Moloney murine leukemia virus after intraperitoneal inoculation. *J. Virol.* **69**:1037–1043.
- Besmer, P., U. Olshevsky, D. Baltimore, D. Dolberg, and H. Fan. 1979. Virus-like 30S RNA in mouse cells. *J. Virol.* **29**:1168–1176.
- Bestwick, R. K., S. L. Kozak, and D. Kabat. 1988. Overcoming interference to retroviral superinfection results in amplified expression and transmission of cloned genes. *Proc. Natl. Acad. Sci. USA* **85**:5404–5408.
- Bodine, D. M., K. T. McDonagh, S. J. Brandt, P. A. Ney, B. Agricola, E. Byrne, and A. W. Neinhuis. 1990. Development of a high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **87**:3738–3742.
- Brightman, B. K., C. Farmer, and H. Fan. 1993. Escape from *in vivo* Moloney mink cell focus-inducing viruses driven by the Mo+PyF101 long terminal repeat (LTR) by LTR alterations. *J. Virol.* **67**:7140–7148.
- Cloyd, M. W., J. W. Hartley, and W. P. Rowe. 1980. Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. *J. Exp. Med.* **151**:542–552.
- Cornetta, K., R. C. Moen, K. Culver, R. A. Morgan, J. R. McLachlin, S. Sturm, J. Selegue, W. London, R. M. Blaese, and W. F. Anderson. 1990. Amphotropic murine leukemia retrovirus is not an acute pathogen for primates. *Hum. Gene Ther.* **1**:15–30.
- Cornetta, K., R. A. Morgan, and W. F. Anderson. 1991. Safety issues related to retroviral-mediated gene transfer in humans. *Hum. Gene Ther.* **2**:5–14.
- Cornetta, K., R. A. Morgan, A. Gillio, S. Sturm, L. Baltrucki, R. O'Reilly, and W. F. Anderson. 1991. No retroviremia or pathology in long-term follow-up of monkeys exposed to a murine amphotropic retrovirus. *Hum. Gene Ther.* **2**:215–219.
- Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers. 1985. Isolation of T-cell tropic HTLV-III like retrovirus from macaques. *Science* **228**:1202–1204.
- Danos, O., and R. C. Mulligan. 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. USA* **85**:6460–6464.
- Donahue, R. E., S. W. Kessler, D. Bodine, K. McDonagh, C. Dunbar, S. Goodman, B. Agricola, E. Byrne, M. Raffeld, R. Moen, J. Bacher, K. M. Zsebo, and A. W. Neinhuis. 1992. Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *J. Exp. Med.* **176**:1125–1135.
- Fine, D. L., and G. Schochetman. 1978. Type D primate retroviruses: a review. *Cancer Res.* **38**:2123–2139.
- Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **90**:11478–11482.
- Hartley, J. W., and W. P. Rowe. 1976. Naturally occurring murine leukemia viruses in wild mice: characterization of a new amphotropic class. *J. Virol.* **19**:19–25.
- Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. USA* **74**:789–792.
- Herr, W., and J. Clarke. 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. *Cell* **45**:461–470.
- Herr, W., and Y. Gluzman. 1985. Duplications of a mutated simian virus 40 enhancer restore its activity. *Nature (London)* **313**:711–714.
- Higgins, D. G., A. J. Bleasby, and R. Fuchs. 1992. CLUSTAL V: improved software for multiple sequence alignment. *Comput. Appl. Biosci.* **8**:189–191.
- Homma, T., P. J. Kanki, N. W. King, Jr., R. D. Hunt, M. J. O'Connell, N. C. Letvin, M. D. Daniel, R. C. Desrosiers, C. S. Yang, and M. Essex. 1984. Lymphoma in macaques: association with virus of human T-lymphotropic family. *Science* **225**:716–718.
- Hu, W.-S., and H. M. Temin. 1990. Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. *Proc. Natl. Acad. Sci. USA* **87**:1556–1560.
- Jones, J. S., R. W. Allan, B. Seufzer, and H. M. Temin. 1994. One retroviral RNA is sufficient for synthesis of viral DNA. *J. Virol.* **68**:4097–4103.
- Kanki, P. J., M. F. McLane, N. W. King, N. L. Letvin, R. D. Hunt, P. Sehgal, M. D. Daniel, R. C. Desrosiers, and M. Essex. 1985. Serologic identification and characterization of a macaque T-lymphotropic retrovirus closely related to HTLV-III. *Science* **228**:1199–1201.

31. Khan, A. S., and M. A. Martin. 1983. Endogenous murine leukemia proviral long terminal repeats contain a unique 190-base-pair insert. *Proc. Natl. Acad. Sci. USA* **80**:2699–2703.
32. Lander, M. R., and S. K. Chattopadhyay. 1984. A *Mus dunni* cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses. *J. Virol.* **52**:695–698.
33. Marx, P. A., D. H. Maul, K. G. Osborn, N. W. Lerche, P. Moody, L. J. Lowenstein, R. V. Henrickson, L. O. Arthur, R. V. Gilden, M. Gravell, W. T. London, J. L. Sever, J. A. Levy, R. J. Mumm, and M. B. Gardner. 1984. Simian AIDS: isolation of a type D retrovirus and transmission of the disease. *Science* **223**:1083–1086.
34. McLachlin, J. R., K. Cornetta, M. A. Eglitis, and W. F. Anderson. 1990. Retroviral-mediated gene transfer. *Prog. Nucleic Acid Res. Mol. Biol.* **38**: 91–135.
35. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895–2902.
36. Miller, A. D., D. R. Trauber, and C. Buttimore. 1986. Factors involved in production of helper-free retrovirus vectors. *Somatic Cell Genet.* **12**:175–183.
37. Morgan, R. A., O. Nussbaum, D. D. Muenchau, L. Shu, L. Couture, and W. F. Anderson. 1993. Analysis of the functional and host range-determining regions of the murine ecotropic and amphotropic retrovirus envelope proteins. *J. Virol.* **67**:4712–4721.
38. Muenchau, D. D., S. M. Freeman, K. Cornetta, J. A. Zwiebel, and W. F. Anderson. 1990. Analysis of retroviral packaging lines for generation of replication-competent virus. *Virology* **176**:262–265.
39. Nabel, G. J., E. G. Nabel, Z. Y. Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, and A. E. Chang. 1993. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA* **90**:11307–11311.
40. Oroszlan, S., M. Barbacid, T. D. Copeland, S. A. Aaronson, and R. V. Gilden. 1981. Chemical and immunological characterization of the major structural protein p28 of MMC-1, a rhesus monkey endogenous type C virus: homology with the major structural protein of avian reticuloendotheliosis virus. *J. Virol.* **39**:845–854.
41. Ott, D., R. Friedrich, and A. Rein. 1990. Sequence analysis of amphotropic and 10A1 murine leukemia viruses: close relationship to mink cell focus-inducing viruses. *J. Virol.* **64**:757–766.
42. Otto, E., A. Jones-Trower, E. F. Vanin, K. Stambaugh, S. N. Mueller, W. F. Anderson, and G. J. McGarrity. 1994. Characterization of a replication-competent retrovirus resulting from recombination of packaging and vector sequences. *Hum. Gene Ther.* **5**:567–575.
43. Ou, C.-Y., L. R. Boone, and W. K. Yang. 1983. A novel sequence segment and other nucleotide structural features in the long terminal repeat of a BALB/c mouse genomic leukemia. *Nucleic Acids Res.* **11**:5603–5620.
44. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
45. Rabin, H., C. V. Benton, M. A. Tainsky, N. R. Rice, and R. V. Gilden. 1979. Isolation and characterization of an endogenous type C virus of rhesus monkey. *Science* **204**:841–842.
46. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
47. Scadden, D. T., B. Fuller, and J. M. Cunningham. 1990. Human cells infected with retrovirus vectors acquire an endogenous murine provirus. *J. Virol.* **64**:424–427.
48. Schwartzberg, P., J. Colicelli, and S. Goff. 1984. Construction and analysis of deletion mutations in the pol gene of Moloney murine leukemia virus: a new viral function required for productive infection. *Cell* **37**:1043–1052.
49. Sorge, J., D. Wright, V. D. Erdman, and A. E. Cutting. 1984. Amphotropic retrovirus vector system for human cell gene transfer. *Mol. Cell. Biol.* **4**:1730–1737.
50. Speck, N. A., and D. Baltimore. 1987. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney murine leukemia virus enhancer. *Mol. Cell. Biol.* **7**:1101–1110.
51. Stoye, J. P., and J. M. Coffin. 1987. The four classes of endogenous murine leukemia virus: structural relationships and potential for recombination. *J. Virol.* **61**:2659–2669.
52. Swain, A., and J. M. Coffin. 1992. Mechanism of transduction by retroviruses. *Science* **255**:841–845.
53. Tainsky, M. A. 1981. Analysis of the virogenes related to the rhesus monkey endogenous type C retrovirus in monkeys and apes. *Virology* **37**:922–930.
54. Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy. 1984. Pathogenesis of retrovirus-induced disease, p. 785–998. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
55. Temin, H. M. 1990. Safety considerations in somatic gene therapy of human disease with retrovirus vectors. *Hum. Gene Ther.* **1**:111–123.
56. Torrent, C., C. Gabus, and J.-L. Darlix. 1994. A small and efficient dimerization/packaging signal of rat VL30 RNA and its use in murine leukemia virus-VL30-derived vectors for gene transfer. *J. Virol.* **68**:661–667.
57. Vanin, E. F., M. Kaloss, C. M. Broscius, and A. W. Nienhuis. 1994. Characterization of replication-competent retroviruses from nonhuman primates with virus-induced T-cell lymphomas and observations regarding the mechanism of oncogenesis. *J. Virol.* **68**:4241–4250.
58. Zhang, J., and H. M. Temin. 1993. Rate and mechanism of nonhomologous recombination during a single cycle of retroviral replication. *Science* **259**: 234–238.