JOURNAL OF VIROLOGY, July 1994, p. 4241–4250
0022-538X/94/$04.00+0

Vol. 68, No. 7

Characterization of Replication-Competent Retroviruses from Nonhuman Primates with Virus-Induced T-Cell Lymphomas and Observations Regarding the Mechanism of Oncogenesis

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Received 2 February 1994/Accepted 28 March 1994

Rapidly progressive T-cell lymphomas were observed in 3 of 10 rhesus monkeys several months after autologous transplantation of enriched bone marrow stem cells that had been transduced with a retroviral vector preparation containing replication-competent virus (R. E. Donahue, S. W. Kessler, D. Bodice, K. McDonagh, C. Dunbar, S. Goodman, B. Agricola, E. Byrne, M. Raffeld, R. Moen, J. Bacher, K. M. Zsebo, and A. W. Nienhuis, J. Exp. Med. 176:1124–1135, 1992). The animals with lymphoma appeared to be tolerant to retroviral antigens in that their sera lacked antibodies reactive with viral proteins and contained 10^4 to 10^5 infectious virus particles per ml. By molecular cloning and DNA sequencing, we have now demonstrated that the serum from one of the monkeys contained a replication-competent retrovirus that arose by recombination between vector and packaging encoding sequences (vector/helper [V/H] recombinant) in the producer clone used for transduction of bone marrow stem cells. Southern blot analysis demonstrated 14 or 25 copies of this genome per cell where present in two animals. The genome of a second replication-competent virus was also recovered by molecular cloning; it arose by recombination involving the genome of the V/H recombinant and endogenous murine retroviral genomes in the producer clone. Twelve copies of this amphotropic virus/mink cell focus-forming virus genome were present in tumor DNA of one animal, but it was not found in tumor DNA of the other two animals with lymphoma. Southern blot analysis of DNA from various tissues demonstrated common insertion site bands in several samples of tumor DNA from one animal, suggesting clonal origin of the lymphoma. Our data are most consistent with a pathogenic mechanism in which chronic productive retroviral infection allowed insertional mutagenesis of critical growth control genes, leading to cell transformation and clonal tumor evolution.

Retrovirus-mediated gene transfer has become one of the most widely applied methods for introduction of genes into primary cells (20, 25, 26, 28). This method has already been used successfully for clinical investigation and therapeutic applications (4, 7, 8). Attractive features of retroviral vectors include flexibility, that is, the variety of coding sequences that can be transferred, high although variable transduction efficiency, and stability of the proviral genome once integrated into a host cell chromosome. The greatest concern about the use of retroviral vectors for clinical applications has been their potential to induce or contribute to the neoplastic transformation. This concern was enhanced by the development of rapidly progressive, fatal lymphomas in three nonhuman primates subjected to autologous transplantation with retrovirus-transduced bone marrow cells (12). The disease pattern, latency period, and high titer of replication-competent retroviruses in the sera of these animals are highly reminiscent of the features of retrovirus-induced lymphomas in rodent species (41).

The packaging cell lines that constitutively synthesize retroviral proteins and the vectors in current use were derived from components of various proviral genomes (25, 26, 28). Packaging lines that yield viruses with ecotropic specificity are based on components of the Moloney murine leukemia virus (Mo-MuLV), whereas amphotropic packaging lines were engineered with the envelope gene from the 4070A virus, a naturally occurring murine retrovirus with amphotropic host range. The retroviral vectors and packaging components used to develop producer clones for clinical applications have been designed to minimize the potential for mutational events that could give rise to a replication-competent retrovirus (11, 23, 25–29). However, emergence of replication-competent viruses was common with earlier-generation packaging cell lines.

Our goal has been to utilize a nonhuman primate model to develop strategies for gene transfer into repopulating hematopoietic stem cells. Methods developed in murine models have been applied with variable success. Bodine et al. (5) found that rhesus monkey stem cells could be transduced by using a high-titer amphotropic producer line that generated an estimated 10^{10} infectious vector particles per ml of culture medium. The vector used, N2, has two regions of sequence identity with the RNA transcript in PA317 packaging cells that encodes the retroviral structural proteins. The high-titer amphotropic producer clone used for the stem cell transfection experiments had been developed by ping-pong amplification between a lower-titer PA317 producer clone and the GP+ E86 ecotropic packaging line (5). Such coculture of amphotropic and ecotropic producer cells is also likely to amplify any replication-competent virus that arises by recombination between vector and packaging RNA transcripts (2). Indeed, the high-titer N2 producer cell line was shown to generate replication-competent retroviruses with a titer of approximately 10^4/ml of culture medium (5).

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Early safety studies had suggested that amphotropic MuLV of the type likely to be generated in the N2 producer clone was nonpathogenic, either when infused intravenously or when derived from infected, autologous fibroblasts transplanted subcutaneously (9, 10). However, 3 of 10 animals that received autologous bone marrow cells transduced with preparations from the high-titer N2 producer line developed a rapidly progressive T-cell lymphoma involving the thymus, lymph nodes, liver, spleen, and bone marrow (12). Replication-competent retrovirus was found in the sera of two animals studied, and between 10 and 50 copies of a proviral genome were present in DNA from tumor tissues of each of the three animals.

Replication-competent retroviruses such as MoMuLV are known to be oncogenic (41). Chronic productive infection in newborn or immunocompromised mice or rats leads to the emergence of tumors in many animals after several months latency. Recombinant retroviruses containing envelope sequences derived from endogenous proviruses often arise during chronic productive infection in mice and may contribute to oncogenesis (41). These viruses have an expanded host range and transform mink cells in culture, hence the name mink cell focus-forming (MCF) virus. Such viruses have not been observed in rats. Both the recombinant MCF and MoMuLV replication-competent retroviruses are oncogenic, with a long latency period before the onset of the disease (41). The mechanism of oncogenesis is by insertional mutagenesis, either by inactivation of a tumor suppressor gene or by activation of a proto-oncogene. Some viruses induce tumors in rodents with short latency (3). In the genomes of such acutely oncogenic retroviruses, viral sequences have been replaced with protein-coding sequences derived from cellular proto-oncogenes. Usually these acutely oncogenic viruses are replication defective and require a complementing replication-competent virus for propagation.

Prior work had documented the presence of replication-competent virus in the sera of two monkeys with lymphoma, and we suggested that insertional mutagenesis could be the mechanism by which the lymphoma developed (12). Another possibility to be considered is that transduction of a cellular gene in murine or primate cells during prolonged viral replication had created a novel, acutely oncogenic virus that caused lymphoma in these animals. Our experiments were undertaken to determine the nature of the replication-competent retroviruses that were capable of productively infecting the experimental animals. Specifically, we wish to learn whether MCF-type or acutely oncogenic viruses with transduced murine or primate cellular coding sequences were present in the monkeys with lymphoma.

MATERIALS AND METHODS

Genomic Southern analysis. DNA was isolated from either monkey tumor tissue or tissue culture cells by using sodium dodecyl sulfate (SDS) and proteinase K essentially as described by Maniatis et al. (21). Ten micrograms of genomic DNA was digested with the enzymes indicated and electrophoresed on a 0.8% agarose gel. Following transfer and binding of the DNA to Hybond-N+ (Amersham Corp.), the filters were prehybridized for 2 h in Hybrisol I (Oncor) at 42°C. Hybridization was done at 42°C with 2×10^6 to 5×10^6 cpm of random-primer-labeled probe (Pharmacia) per ml of hybridization solution (Hybrisol I) for 16 h. The washes were done at 65°C, first with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% SDS (twice for 30 min each time) and then with 0.5× SSC containing 0.5% SDS (twice for 30 min each time). The envelope probe used in our initial genomic Southern analysis as well as the screening of the λZAPII libraries and for the genomic Southern analysis shown in Fig. 2 was the 1,277-bp *Eco*RI-*Cla*I fragment derived from pPAM3. The amphotropic virus-specific probe used in the integration site genomic Southern analysis was a 970-bp *Eag*I-*Bgl*I fragment from pPAM3. The MCF virus-specific probe was a 510-bp *Pvu*II fragment which contains the MCF virus-specific sequences described by O'Neill et al. (32). In all cases, the probes were used on normal monkey genomic DNA to ensure that there was no hybridization to any monkey-specific sequences under the conditions described above.

TABLE 1. Primers used for PCR analysis

Envelope	PCR oligonucleotide	Size (bp)
AMP/MCF virus	ATGATTCCTCAGTGGTCTCCAGTG TTCGTTGAGGTCTGTCTGGATAGC	979
MCF virus	GGACTYGGGTGTCGCACTCCC ACTGGAGACCRCTGAGGAATC	245
Xenotropic virus	GGATGACCCAGAACCCGATATTGG ATCATAACAGGGGCCCTGATCCTT	249

Isolation of proviral genomes. Hirt DNA (15) was isolated from infected murine NIH 3T3 or mink CCL64 cells, digested with *Nhe*I, ligated to λZAPII *Xba*I arms, and packaged by using Gigapack II Gold λ packaging extracts (Stratagene instruction manual; catalog no. 200216). The libraries were screened as previously described (21, 30). The Bluescript SK^- phagemid was rescued from each of the purified λZAPII clones as described in the Stratagene instruction manual.

DNA sequencing. Sequencing was done by using the Taq DiDeoxy Termination Cycle Sequencing kit and protocol from Applied Biosystems (part no. 901497); the reactions were analyzed by using Applied Biosystems model 373A DNA sequencer. Oligonucleotide primers were synthesized on an Applied Biosystems model 380B DNA synthesizer, using phosphoramidite chemistry. Before use, the oligonucleotides were purified by Sephadex G-24 chromatography (Pharmacia NAP-24 columns).

PCR analysis. Previously described primers (5) were used to amplify a 227-bp fragment in the amphotropic envelope. The primers used to assay for AMP (amphotropic)/MCF, MCF, and xenotropic virus envelope sequences in genomic DNA and the sizes of the amplified fragments are shown in Table 1.

The primers used for the AMP/MCF virus envelope were determined by using PRIMER 2 (a primer designer program from Scientific and Educational Software, State Line, Pa.). The MCF- and xenotropic virus-specific primers were designed by first aligning all of the previously sequenced MCF or xenotropic virus *env* genes and subsequently choosing sequences which were in conserved regions of each class.

The conditions used for the amplification were 94°C for 1 min (denaturation), 65°C for 1 min (annealing), and 72°C for 1.5 min (elongation) for a total of 24 cycles. Genomic DNA (400 ng) was used as the template, and 0.1 μl of [^{32}P]dCTP (3,000 Ci/mmol; Amersham) was added in a total volume 100 μl.

RESULTS

Characterization of proviral genomes in tumor DNA. Our initial hypothesis was that recombination between the N2 vector transcript and the RNA transcript encoding viral proteins in PA317 cells had generated a replication-competent

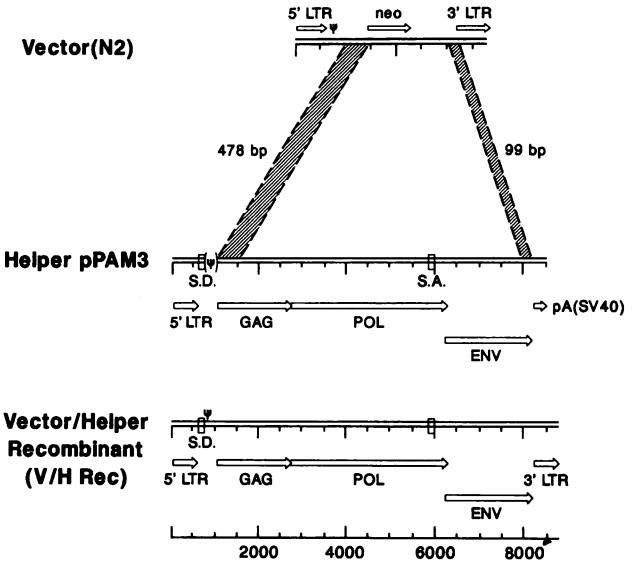


FIG. 1. Potential recombination between the sequences of the vector and packaging genomes in the producer clone. The retroviral LTRs and phosphotransferase (*neo*) gene are shown as boxed areas; ψ denotes the retroviral packaging signal. The two hatched areas between N2 and pPAM3 indicate the locations and sizes of the regions of sequence identity. The truncated 5′ LTR, *gag*, *pol*, *env*, and simian virus 40 polyadenylation site [PA(SV40)] of pPAM3 are indicated as horizontal arrows. S.D. and S.A. indicate the positions of the retroviral splice donor and acceptor sites, respectively. The horizontal arrows below indicate the positions of the *gag*, *pol*, and *env* genes and LTRs of the integrated form of the predicted provirus.

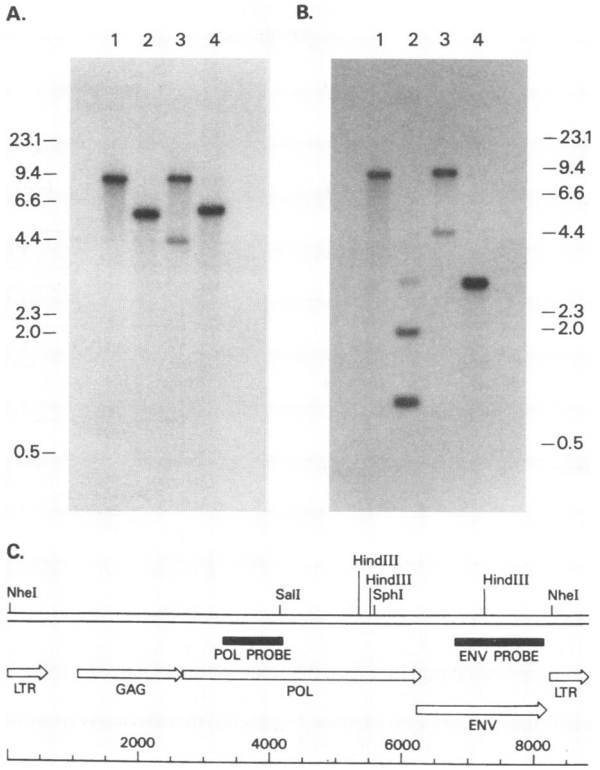


FIG. 2. Southern blot analysis of tumor DNA. Lanes in panels A and B correspond to digestion of DNA with *Nhe*I (lane 1), with *Nhe*I and *Hin*dIII (lane 2), with *Nhe*I and *Sal*I (lane 3), and with *Nhe*I and *Sph*I (lane 4). The blot in panel A was probed with the *pol* fragment (indicated in panel C), and that in panel B was probed with the *env* fragment (indicated in panel C). Sizes are indicated in kilobases. Shown in panel C is a map of restriction sites in the predicted V/H recombinant proviral integrant (Fig. 1).

virus as depicted in Fig. 1. Accordingly, we chose to perform Southern blot analysis of monkey tumor DNA from animal 15445 with enzymes that would give fragment lengths predicted from the restriction map of this vector/helper (V/H) recombinant provirus (Fig. 2). All of the anticipated fragments were present in a series of single and double digestions, but in addition, a 2.7-kb fragment not predicted from the map was present in DNA doubly digested with *Nhe*I and *Hin*dIII. A portion of the *Nhe*I fragment of genomic length remained in double digests with *Sal*I, but this may reflect partial methylation of the *Sal*I site, with resulting inhibition of cutting. Of relevance to our cloning strategy is the fact that all proviral sequences that annealed to probes derived from *pol* and *env* sequences when digested with *Nhe*I were 8.3 kb in length, as predicted from the map of the V/H recombinant (Fig. 2).

Cloning of proviral genomes from virus present in monkey serum. The scheme for cloning genomes of replication-competent viruses is shown in Fig. 3. Serum containing 10^5 infectious particles per ml from a monkey with lymphoma was used to infect both mouse 3T3 and mink CCL64 cells in an effort to amplify amphotropic-, xenotropic-, and MCF-type viruses, if present. Hirt-extracted DNA was prepared only 48 h after infection to enhance the probability that the spectrum of proviral genomes in infected cells was representative of the population of infectious virus in serum. Earlier work had shown that replication-competent retroviruses of amphotropic host range derived from vector producer clones by recombination were sensitive to complement (9, 10). One additional hypothesis tested was whether a complement-resistant variant virus had arisen through mutation in the infected monkeys and accounted for the infectious particles present in serum. Serum from animal 15445 was diluted 1:10 with human plasma, incubated for 1 h at 37°C, and then used to infect 3T3 and CCL64 cells. Because the titer of virus was reduced to 1% of the initial level by this complement treatment, the cells initially infected with the complement-treated serum were cultured for 14 days in order to expand any remaining replication-competent retrovirus. The cells then were cocultured with naive cells for 48 h, and DNA was isolated by Hirt extraction for molecular cloning.

Extracted DNA was digested with *Nhe*I and subcloned into the *Xba*I site of λZAPII. Each library was screened with the *env* probe (Fig. 3), and the purified clones were probed with the *pol* fragment. A total of 34 vector phage clones were isolated from the four libraries with the *env* probe, and 33 of these were found to hybridize to the *pol* probe.

The Bluescript phagemid containing the inserted retroviral sequences was excised from each of these 33 clones and analyzed by digestion with *Hin*dIII, *Sal*I, and *Sst*II. Twenty-nine of the thirty-three clones had the restriction enzyme pattern A or B shown in Fig. 4. These patterns are identical to the map expected for the *Nhe*I fragment of the V/H recombinant cloned in either of the two possible orientations (Fig. 4). Clone 24 had restriction pattern C (Fig. 4); it was approximately the same size as the V/H recombinant, but the *Hin*dIII site in the *env* gene was not present and a new *Sst*II site was observed in this same region. Details of the sequence of this clone are presented below. Clones 11 and 33 (Fig. 2) had restriction pattern D. A 2,039-bp deletion beginning immedi-

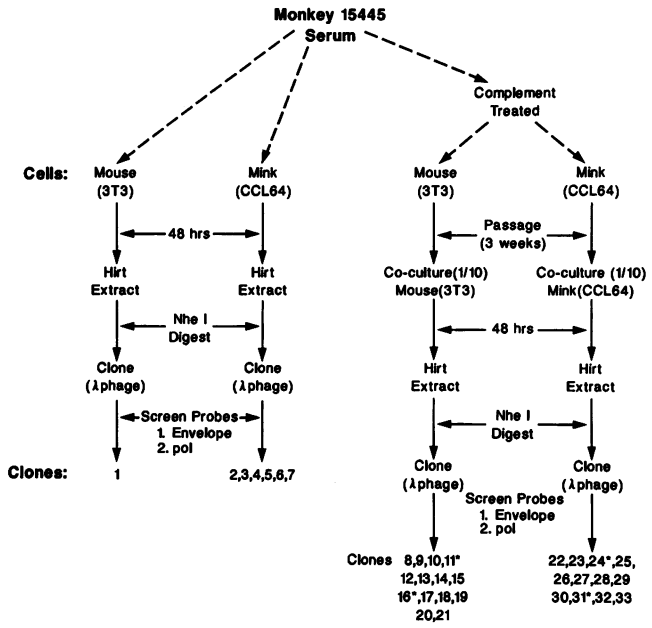


FIG. 3. Molecular cloning strategy. The steps in the cloning procedure are indicated together with the relevant time intervals. The clones isolated in each arm are numbered consecutively; those indicated by asterisks gave restriction enzyme fragments that deviated from those predicted for the V/H recombinant (Fig. 1). See text for details.

TABLE 2. Degree of homology between the *env* gene in isolated clones and expected *env* gene sequence[a]

Clone	Homology (%)	Description of differences
1	100	
2	100	
4	100	
9	99.69	1 6-bp deletion, 4 base pair differences (three flanking deletion)
13	100	
16	99.85	2 base pair differences, 1 base pair addition
23	100	
25	99.75	5 base pair differences
26	100	
27	100	
31	100	

[a] The degree of homology between the sequence of the *env* gene for the clone indicated and that expected for the N2-pPAM3 recombinant is expressed as a percentage. This value was calculated by excluding any additions and deletions within the *env* gene sequence of the isolated clone. Any differences between the *env* of the isolated clone and that expected for the N2-pPAM3 recombinant are also indicated.

ately 3′ to the 5′ long terminal repeat (LTR) and extending into the *gag* gene was identified by sequencing of clone 31 (data not shown). Clone 16, which was most likely a cloning artifact, had restriction pattern E (Fig. 4). It contained approximately 200 bp of extraneous DNA added as a *Nhe*I fragment to the 3′ end of the V/H genome during molecular cloning. A search of the GenBank data base revealed no known homology.

Sequence of the envelope-coding region of cloned proviruses. The envelope-coding regions of 11 clones (clones 1, 2, 4, 9, 13, 16, 23, 25, 26, 27, and 31) were sequenced. Eight were identical to the predicted sequence of the V/H recombinant. Clone 9 had a 6-bp deletion and a 3-bp substitution flanking this deletion and another single nucleotide substitution upstream from the deletion (Table 2). As a result of these mutations, amino acids 100 and 168 of the *env* gene were changed and amino acids 166 and 167 were deleted. Clone 16 had an insertion of a T residue between codons 100 and 101 of the *env* gene, forming a termination codon (TAG) at the site of insertion, and nonconservative substitutions at codons 15 and 295. Clone 25 had five nonconservative substitutions: codons 81, 100, 102, 295, and 629.

Characterization of clone 24. Because of the differences in the restriction enzyme map noted above (Fig. 4), this clone was

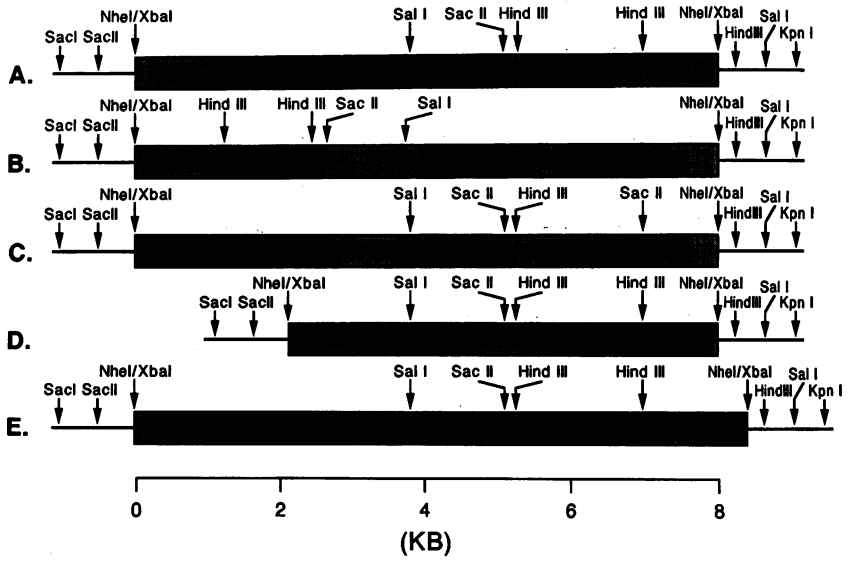


FIG. 4. Restriction enzyme maps of different types of cloned proviral genomes. The *Sst*I and *Kpn*I sites as well as the *Hin*dIII, *Sac*II, and *Sal*I sites within the Bluescript SK$^-$ polylinker are shown. The restriction enzyme sites within the polylinker are not shown to scale.

TABLE 3. Degree of homology between AMP/MCF *env* gene and other *env* gene sequences[a]

Type	Virus	Reference(s)	Homology (%) at Nucleotide positions:		
			1–1049	1050–1209	1210–1911
MCF	Friend MCF, FrNx strain	1	99.1	77.0	78.6
	Friend MCF, NIH leukemic Swiss mice	19	97.3	97.5	80.6 (1)
	Moloney MCF, isolate 81	6	96.2	80.0	81.5
	Friend MCF, Eveline	19	95.3	76.4	69.7
	MCF murine leukemia virus, CI-3 isolate	22	96.0 (1)	91.3	76.5
	MCF-247	16,18	95.0	81.4	74.6
	Rauscher MCF	42	92.2 (2)	78.3	79.1
Xenotropic	NZB-9-1 murine leukemia virus	31	91.5 (2)	98.8	76.4
	CWM murine leukemia virus	24	91.7 (2)	96.3	76.4
Amphotropic	10A1 murine leukemia virus	33	79.1 (6)	72.7	96.0
	4070A murine leukemia virus	33	72.2 (14)	74.5	98.7
Ecotropic	Moloney murine leukemia virus	36	66.4 (37)	79.5	81.5
	AKV murine leukemia virus	13,14	58.6 (15)	80.8	75.6

[a] The degree of homology between the AMP/MCF the *env* gene sequence and previously reported *env* gene sequences is expressed as a percentage. The *env* gene is divided into three segments as indicated in Fig. 4A. A number in parentheses indicates the number of gaps introduced to obtain the value indicated.

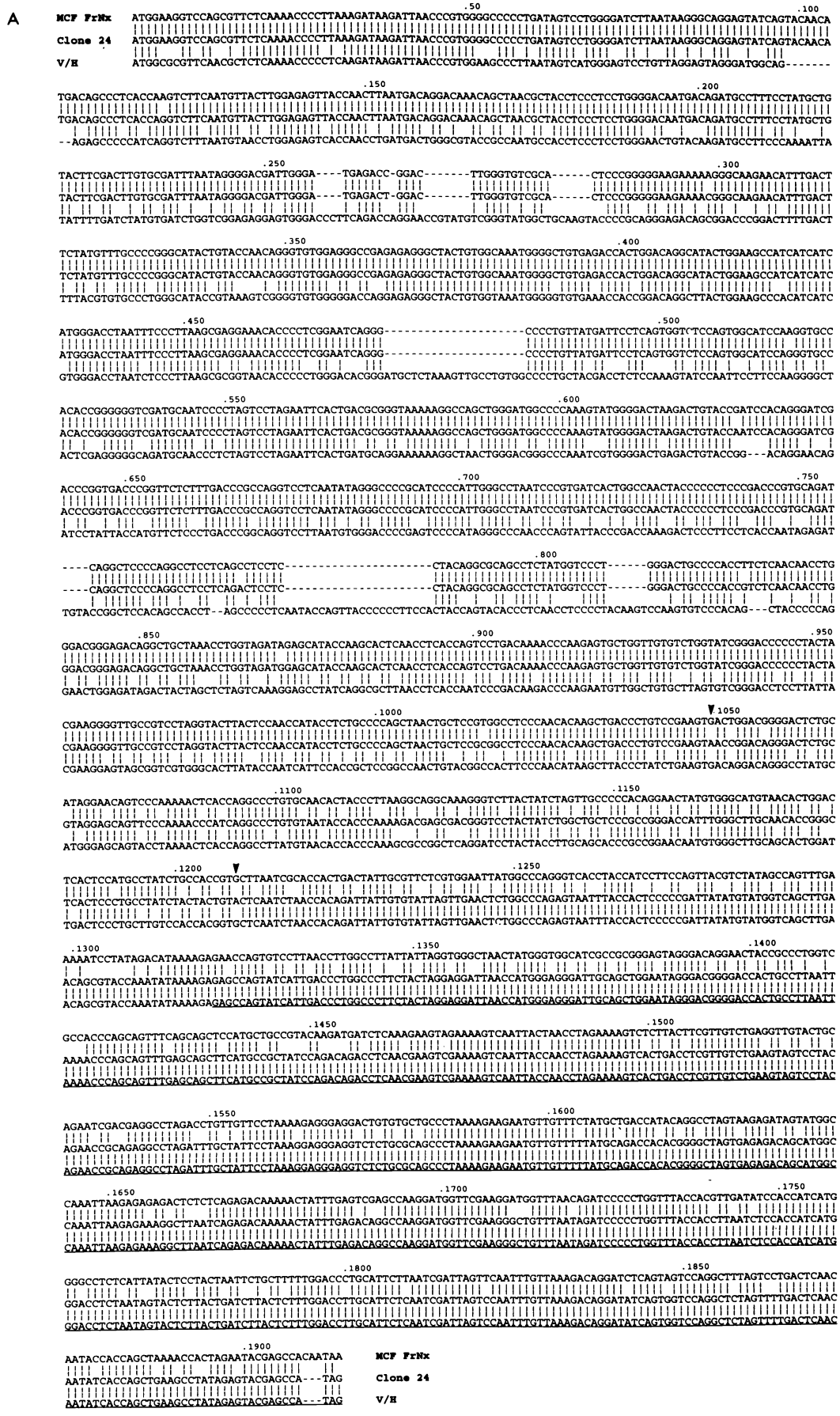
sequenced in its entirety. The 3′ end of the *pol* gene and the 5′ two-thirds of the *env* gene diverged significantly from the sequence of the V/H recombinant (Fig. 5); 1,536 bp of the V/H genome were replaced with 1,456 bp of extraneous sequence. The reading frames of both the *pol* and *env* genes were preserved in this novel proviral genome. Comparison of the *env* gene sequence of clone 24 with previously published *env* gene sequences indicated that the majority of the divergent region was most highly homologous to the Friend FrNx strain MCF *env* gene (1). The alignment of the *env* sequences from clone 24, the FrNx strain MCF, and the V/H recombinant (Fig. 5A) indicated that a central segment of 160 bp of the clone 24 *env* sequence had relatively low homology to either the FrNx strain MCF or the V/H recombinant. Therefore, the *env* gene of clone 24 was divided into three segments, and each segment was compared with the previously sequenced *env* genes (Table 3). The sequence from nucleotides 1 to 1049 the clone 24 *env* gene is more homologous to the Friend FrNx strain MCF sequence (99.9%) than to any of the other sequences. Between nucleotides 1050 and 1209, the sequence of the clone 24 *env* gene had a greater degree of homology with the NZB-9-1 xenotropic murine leukemia virus *env* sequence (31) than with the other *env* sequences. For the remainder of the *env* gene (nucleotides 1210 to 1911), the sequence of clone 24 is identical to the analogous region of the V/H recombinant. The homology, at the nucleotide level, between clone 24 and the V/H recombinant, excluding the LTRs, was 99.8% 5′ to the divergent region and 100% 3′ to it. The U3 segment of the LTR of clone 24 has a 23-bp insertion compared with the sequence of the V/H recombinant (Fig. 5B); this insertion is within the 5′ member of the two 75-bp direct repeats which comprise the enhancer region of U3 (37, 38). The inserted segment is a direct copy of the 23 bp 3′ to it. We designated the novel virus potentially encoded by clone 24 as the AMP/MCF virus.

Occurrence and formation of AMP/MCF virus (clone 24). The isolation of clone 24 from Hirt DNA of CCL64 (mink) cells exposed to serum of monkey 15445 raises a number of questions. First, is the AMP/MCF provirus present and integrated in the tumor DNA of monkey 15445? Second, is the AMP/MCF provirus present in the DNA of the other two monkeys that developed lymphoma? Third, where did the AMP/MCF virus genome originate?

PCR analyses of DNA samples from the monkey 15445 as well as the other two monkeys that developed lymphoma, using primers specific for the amphotropic virus and AMP/MCF virus *env* genes, are shown in Fig. 6. As demonstrated previously (12), amphotropic virus envelope sequences were present in DNA from all monkeys (Fig. 6A). Primers that span the 3′ recombination junction and therefore are specific for the AMP/MCF virus *env* gene were devised. An amplified fragment of the expected size was present on analysis of thymic DNA from monkey 15445, but no such product was detected in DNA samples from the other two animals (Fig. 6B).

DNA from the three monkeys was also analyzed with primer pairs specific for MCF virus and xenotropic virus *env* encoding sequences. These primers are homologous to conserved segments of these genes that differ among the various classes of *env* genes (32). An amplified product with the MCF virus primers was seen with template DNA from monkey 15445, presumably derived from the AMP/MCF virus genome (Fig. 6C), but no other amplified products were observed (Fig. 6C and D). Southern analysis using both MCF virus and xenotropic virus *env*-specific probes (32) confirmed the PCR results (data not shown); specific bands were seen with the MCF virus probe on analysis of DNA from monkey 15445 but not with DNA from the other two monkeys.

The number of integrated copies of the AMP/MCF and V/H recombinant proviruses were determined by both integration site analysis (Fig. 7) and quantitative genomic Southern blotting (data not shown), using probes which are specific for the MCF virus or amphotropic virus envelope-coding sequence. In the integration site analysis, the genomic DNA was digested with a restriction enzyme which has a single site in proviral DNA yielding a unique band for each integration position. Multiple hybridizing fragments were seen with the amphotropic probe on *Bgl*II-digested thymic DNA of monkeys 15445 and 88049 (Fig. 7A), consistent with a large number of integrated proviral copies. With the MCF virus-specific probe, 11 to 12 bands were seen on analysis of genomic DNA from monkey 15445 (Fig. 7B). For quantitative genomic Southern analysis, specific amounts (corresponding to variable copy equivalents) of plasmid DNA containing the appropriate *env* genes (clone 24 for AMP/MCF virus and clone 25 for the V/H recombinant) were added to a specific amount of control monkey DNA. The signal intensity obtained on analysis of


A
MCF FrNx
Clone 24
V/H

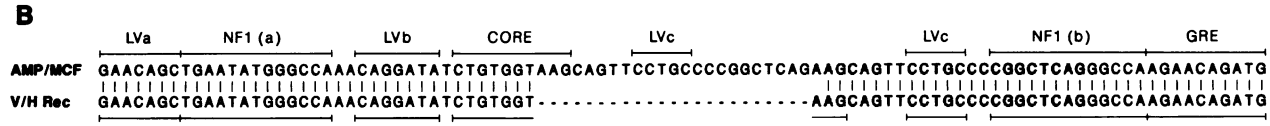


FIG. 5. Comparison of the *env* sequence of clone 24 with those of the MCF FrNx endogenous provirus and the V/H recombinant. (A) Numbering reflects the nucleotides from the *env* translational initiation codon of clone 24. The two vertical arrows delineate the region of the clone 24 *env* gene between nucleotides 1050 and 1209 that is more homologous to NZB-9-1 *env* sequence than to that of either the FrNx MCF virus or the V/H recombinant (see Table 2 and Discussion). The underlined sequence indicates the segment of the *env* gene that encodes p15E. (B) Sequences of segments of the U3 regions of the LTRs. Sequences corresponding to binding sites for transcription factors are overlined (AMP/MCF virus) or underlined (V/H recombinant [Rec]). Designations: LVa, LVb, and LVc, binding sites for leukemia factors a, b, and c; NF1, nuclear factor 1; CORE, is a binding motif found in the simian virus 40 enhancer (37); GRE, glucocorticoid response element.

DNA from monkeys 15445 and 88049 was compared against standards, using a PhosphorImager for quantitative analysis. Tumor DNA from monkey 15445 contained an estimated 12 copies of the AMP/MCF proviral genome and about 24 copies of the V/H genome. DNA from monkey 88049 contained 14 integrated copies per genome of the V/H recombinant provirus (data not shown).

The AMP/MCF virus arose during passage of the A2 producer line. DNA was isolated from cells in culture at 2, 19, 22, 24, and 33 months after isolation of the clone. The specific product amplified with the AMP/MCF virus primer pair was first detected in the DNA from the cells at 24 months and was of greater intensity when DNA was analyzed from the passage at 33 months. Culture medium from A2 cells after the 33-month passage transferred the AMP/MCF viral genome into mink cells (Fig. 8).

Tumor clonality demonstrated by integration site analysis. DNA samples from several tissues infiltrated with tumor were available from monkey 15445. The pattern of integration site fragments was virtually identical on Southern blot analysis of each of the samples, using an MCF virus *env*-specific probe or an amphotropic virus-specific probe (Fig. 9).

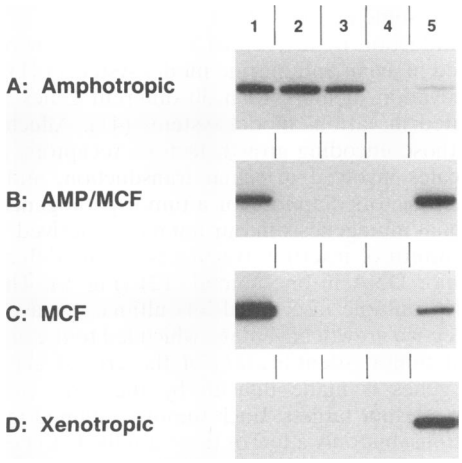


FIG. 6. Analysis of tumor DNA for amphotropic, AMP/MCF, MCF, and xenotropic virus envelope sequences by PCR. Lanes: 1 to 3, results of analysis of tumor DNA from monkeys 15445, 88049, and 88053 (12), respectively; 4, results obtained with negative control monkey DNA; 5, results with a relevant positive control. Positive control DNAs were recovered from CCL64 cells infected with 4070 (amphotropic), 247 (MCF), or NZB-9-1 (xenotropic) virus or with monkey serum (AMP/MCF virus).

DISCUSSION

Our molecular analysis has documented the occurrence of a recombination event between the vector and packaging genomes that has arisen in the course of derivation and passage of a high-titer retroviral producer clone. In three monkeys that developed lymphoma, this virus was able to replicate extensively in vivo, leading to a high genomic copy number in tumor tissue. A second novel retrovirus involving sequences derived from endogenous murine MCF-type viruses arose after several months of in vitro passage of the high-titer producer clone. Its genome was present in tumor tissue DNA of only one of three monkeys. This AMP/MCF virus was isolated from the arm of the experiment in which the monkey serum was treated with human complement and then used to infect CCL64 (mink)

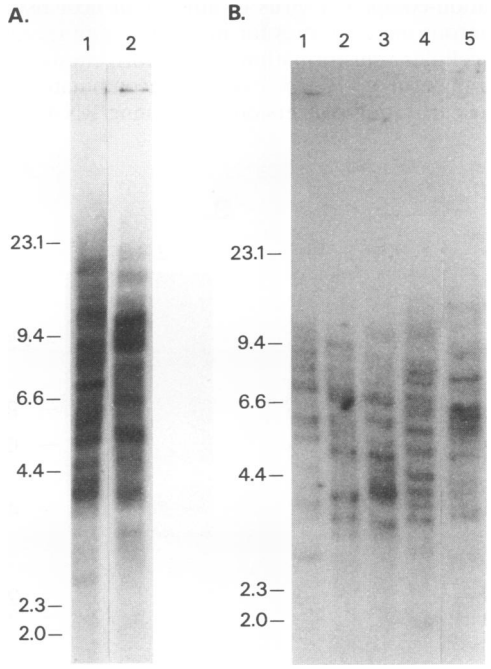


FIG. 7. Characterization of the integration bands of the V/H (A) or AMP/MCF (B) provirus in monkey tumor DNA. In each case, the probe detected bands defined by a site in the provirus and a second site in adjoining genomic DNA. (A) *Bgl*II digestion of DNA from monkeys 15445 and 88049 (lanes 1 and 2, respectively) probed with the amphotropic virus *env* probe (see Materials and Methods). (B) DNA from monkey 15445 digested with *Bam*HI, *Bgl*I, *Bgl*II, *Hin*dIII, and *Sph*I (lanes 1 to 5, respectively) probed with an MCF virus-specific probe (32). Sizes are indicated in kilobases.

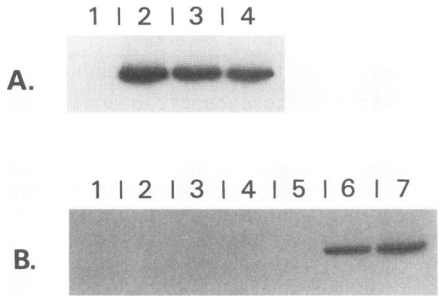


FIG. 8. The AMP/MCF virus is infectious and arose during passage of the A2 producer clone in vitro. Template DNAs were amplified with primers specific for the AMP/MCF virus *env* sequences. Template DNAs are as follows: (A) negative control DNA from CCL64 cells (lane 1), DNA from CCL64 cells exposed to serum from 15445 and medium conditioned by the A2 producer clone (lanes 2 and 3, respectively), and DNA from the thymus of monkey 15445 (lane 4); (B) DNA from NIH 3T3 cells (lane 1), DNA from the original low-titer producer line, N273 (5) (lane 2), and DNA derived from the high-titer A2 (5) producer lines 2, 19, 22, 24, and 33 months after the original isolation (lanes 3 to 7, respectively).

cells. This particular virus probably survived the complement treatment (complement treatment did not completely inactivate all of the replication-competent retroviruses present; it reduced the titer by 2 orders of magnitude [data not shown]) and because of the tropism of the envelope for mink cells was expanded. No evidence for an acutely transforming retrovirus in these animals was uncovered. We suggest that the capacity of replication-competent virus to amplify in host tissues created numerous opportunities for insertional mutagenesis with resulting cellular transformation and tumorigenesis.

Formation of novel retroviruses by recombination is most likely to occur during conversion of genomic RNA molecules

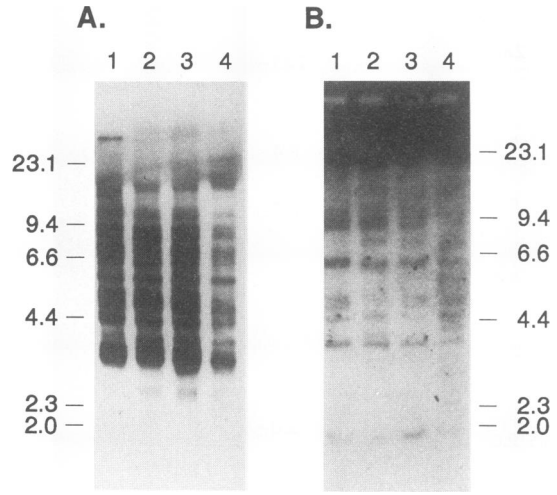


FIG. 9. Clonal origin of lymphoma. DNA from several tissues infiltrated with lymphoma from monkey 15445 was analyzed by Southern blot analysis using a probe specific for amphotropic and AMP/MCF virus envelope sequences in panels A and B, respectively. DNA samples were obtained from thymus (lane 1), spleen (lane 2), lymph node (lane 3), and bone marrow (lane 4). The DNA samples in panel A were digested with *Bgl*II, and those in panel B were digested with *Hin*dIII.

into DNA within infected cells (17, 43). The RNA that encodes the packaging genome in PA317 cells, despite its lack of a packaging signal, is likely to be copackaged with a vector RNA molecule to form a retroviral particle at some finite, albeit low frequency. Each retroviral particle is diploid in that it contains two genomic RNA molecules, and each is converted by reverse transcription into DNA. During reverse transcription, there is significant opportunity for template switching, leading to recombination and emergence of a novel retrovirus (17). Such events are approximately 100-fold more likely to occur in regions of sequence homology than between nonhomologous sequences (43). The vector and packaging RNAs in the A2 producer clone had two regions of sequence identity that favor recombination (Fig. 1). Newer packaging lines and vector combinations have largely although not completely eliminated the possibility for recombination events that lead to emergence of replication-competent virus (11, 23, 25, 26, 28).

Chronic productive infection of primates by murine retroviruses had not been observed previously despite injection of large amounts of replication-competent virus or implantation of autologous producer fibroblasts subcutaneously in normal or immunocompromised animals (9, 10). The unique feature of the animals that developed lymphoma is the apparent lack of an immunological response to the infectious virus (12). Our favored hypothesis is that a pluripotent stem cell or early committed T-cell lineage progenitor became infected with the replication-competent virus during in vitro transduction and that the progeny of this cell(s) contributed to the reestablishment of intrathymic lymphopoiesis during reconstitution. Tolerance induction to foreign antigens during reconstitution of the lymphohematopoietic system in the context of bone marrow transplantation has been well documented in experimental animals (35, 40), and its exploitation clinically has been suggested. Since only 3 of 10 animals subjected to our experimental protocol failed to mount an immune response to the virus and developed lymphoma, tolerance induction must depend on stochastic events influenced by unidentified factors. The remaining seven animals with a documented immune response to the infectious virus have remained disease free for 2 to 4 years (unpublished observations) despite the presence of the proviral genome in a very small proportion of circulating leukocytes in some animals (12).

Tumor induction by insertional mutagenesis has been well documented in avian and murine model systems (41). Disruption or activation of more than 30 different genes has been demonstrated in various model systems (41). Affected genes including those encoding growth factors, receptors, cytoplasmic molecules involved in signal transduction, and nuclear transcription factors. Support for a tumor pathogenesis based on insertional mutagenesis in our animals is derived from the common pattern of insertion fragments among different samples of tumor DNA in one animal (12) (Fig. 9). These data suggest that multiple insertional hits ultimately gave a single cell the selective growth advantage which led to the emergence of a clonal tumor. Identification of the critical activated or disrupted genes is made difficult by the large number of potentially relevant targets. Each tumor contained many proviral insertions, but only a few of these are likely to be relevant to lymphoma pathogenesis. To date, we have evaluated the c-*myc* and Ha-*ras* loci in tumor DNA from these animals without finding evidence of locus disruption (8a).

The AMP/MCF virus present in one of three animals clearly arose during passage of the retroviral producer clone in vitro (Fig. 8). Interestingly, the bone marrow cells from monkey 15445 were transduced with supernatant from the A2 producer line in which the AMP/MCF virus first appeared (Fig. 8), while

the bone marrow cells from monkeys 88053 and 88049 were transduced before and after, respectively, the appearance of the AMP/MCF virus. Therefore, the AMP/MCF virus is present in only one of two monkeys that were exposed to this particular virus. This finding together with the fact that monkey 88053 developed lymphoma indicates that the AMP/MCF virus cannot be solely responsible for the development of lymphoma in these animals. Presumably, the FrNx genome was expressed in the producer cells, allowing its RNA transcript to be copackaged with a V/H genome and leading to emergence of a novel genome by homologous recombination during a subsequent infectious cycle. Perhaps a second copackaging and recombination cycle resulted in incorporation of NZB-9-1 sequences into the AMP/MCF virus genome. This novel virus represents the second example of a chimeric amphotropic/MCF envelope gene; the first, a virus designated A10.1, arose in vivo during infection with an amphotropic virus (34). It has small segments of MCF sequences within a predominantly amphotropic envelope gene (33). The A10.1 virus has an expanded host range and is more highly oncogenic than the amphotropic virus from which it was derived. Expression of its chimeric envelope gene, presumably by infected lymphoid cells, may alter the growth properties of the cells and enhance the probability of neoplastic transformation. Although the AMP/MCF virus that we have described has an altered host range compared with the usual amphotropic virus (unpublished observations), no novel transforming properties have been noted.

Our work unequivocally establishes the ability of murine retroviruses of two types to replicate freely in nonhuman primates. The high proviral copy number in tumor DNA with a clonal pattern is consistent with an insertional mutagenic mechanism of oncogenesis. Could we have failed to detect a novel acutely transforming virus by limitations of our cloning methodology? Our basic strategy was to characterize the integrated proviral genomes by Southern blot analysis and to molecularly clone the genomes of viruses capable of infecting either murine or mink cells in vitro. A novel virus suggested by analysis of tumor DNA was recovered by molecular cloning. However, there are potential biases in that our cloning strategy favored recovery of viruses able to replicate freely in murine and mink cells whose genomes had extensive homology to the *env* probe used for library screening. Other efforts have focused on cloning of virus sequences directly from tumor tissue and the use of a more general screening methodology (33a).

What is the relevance of our observations with respect to the clinical use of retroviral vectors? It is important to realize that we have not uncovered a new risk for such vectors but rather have shown that replication-competent viruses can be oncogenic in primates. Tumor formation was observed in a unique experimental setting in which induction of tolerance to virus was possible. However, the pathogenic potential of replication-competent murine retroviruses in primate species seems established and cannot be ignored in other clinical settings. Our data provide a compelling rationale for careful screening of vector products. Acceptable evidence that clinical lots are free of replication-competent virus eliminates the risk for disease induction by the mechanism that we have observed.

ACKNOWLEDGMENTS

We thank Stephen M. Jane, John M. Cunningham, and David Bodine for helpful discussions during the course of this work. We also thank Edward Otto and Celeste Zalewski for assistance with the dideoxy dye terminator PCR sequencing, James Mason for the MCF 1381- and NZB-9-1-infected CCL64 cells, and Paul Tolstoshev, W. French Anderson, Malcolm Martin, and Damian Purcell for reviewing the manuscript.

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