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Mapping the Viral Sequences Conferring Leukemogenicity and Disease Specificity in Moloney and Amphotropic Murine Leukemia Viruses

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The Moloney murine leukemia virus (MuLV) is a highly leukemogenic virus. To map the leukemogenic potential of Moloney MuLV, we constructed chimeric viral DNA genomes in vitro between parental cloned infectious viral DNA from Moloney and amphotropic 4070-A MuLVs. Infectious chimeric MuLVs were recovered by microinjection of recombinant DNA into NIH/3T3 cells and tested for their leukemogenic potential by inoculation into NIH/Swiss newborn mice. Parental Moloney MuLV and amphotropic 4070-A MuLV induced thymic and nonthymic leukemia, respectively, when inoculated intrathymically. With chimeric MuLVs, we found that the primary determinant of leukemogenicity of Moloney and amphotropic MuLVs lies within the 1.5-kilobase-pair *Cla*I-*Pvu*I long terminal repeat (LTR)-containing fragment. The presence of additional Moloney *env-pol* sequences with the Moloney LTR enhanced the leukemogenic potential of a chimeric MuLV significantly, indicating that these sequences were also involved in tumor development. Since parental viruses induced different forms of leukemia, we could also map the viral sequences conferring this disease specificity. We found that the 1.5-kilobase-pair *Cla*I-*Pvu*I LTR-containing fragment of Moloney MuLV was necessary and sufficient for a chimeric MuLV to induce thymic leukemia. Similarly, the same LTR-containing fragment of amphotropic MuLV was necessary and sufficient for a chimeric MuLV to induce nonthymic leukemia. Therefore, our results suggest that specific sequences within this short LTR-containing fragment determine two important viral functions: the ability to transform cells in vivo (leukemic transformation) and the selection of a specific population of cells to be transformed (disease specificity).

Several strains of replication-competent murine leukemia viruses (MuLVs) have been isolated from normal or malignant tissues of laboratory mice and wild mice. Most of the oncogenic virus isolates, such as AKR, Gross passage A, radiation leukemia, and Moloney, induce T cell leukemia in their natural host or after inoculation into susceptible mice (12). A few other isolates induce completely different diseases. For example, Friend and Rausher MuLVs induce erythroleukemia (12), whereas wild-mouse amphotropic and neurotropic MuLVs have been reported to induce B cell (or null cell) lymphoma (10, 15). None of these nondefective retroviruses seem to harbor a cell-derived oncogene of the type frequently found in oncogenic defective retroviruses. Therefore, other types of mechanism must be operating to induce transformation. The existence of numerous MuLVs with a wide range of leukemogenic potential and with different cell specificities strongly suggests that specific viral sequences determine their oncogenic potential and their target cell specificity. Indeed, the viral sequences responsible for the leukemogenic potential of two AKR-derived MuLVs, the Gross passage A (8; submitted for publication) and the SL-3-3 (20, 21) MuLVs, have been identified. The long terminal repeat (LTR) tandem direct repeat was shown to be the primary determinant of leukemogenicity (20; submitted for publication), with the p15E region having an additive effect (8). The viral sequences determining the specificity for the target cell has been found to be at the 3′ end of the Moloney MuLV genome (3). Moreover, the thymotropism of some of these viruses has been mapped within the LTR tandem direct repeats (7).

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The Moloney MuLV is a highly leukemogenic nondefective retrovirus initially isolated from tumor tissue of a laboratory mouse (12). Clonal isolates of this virus were shown to induce leukemia in nearly 100% of the inoculated mice after a 3- to 6-month latent period (17). Although Moloney MuLV has been extensively used as a model system to study the biological and molecular characteristics of retroviruses, and although the complete nucleotide sequence of its genome has been known for some time (36), the molecular events leading to T cell transformation and the viral sequences necessary to induce this leukemic transformation in susceptible mice are still unknown. The genomes of wild-mouse amphotropic MuLVs share several identical restriction endonuclease sites with the genome of Moloney MuLV (4). However, very little is known about the leukemogenicity of these viruses, except that they induce nonthymic B cell or null cell leukemia after a long latent period in a low percentage of mice inoculated intraperitoneally (10).

To map the Moloney MuLV sequences responsible for its leukemogenic potential, we constructed chimeric viral genomes between parental cloned viral DNA from Moloney MuLV and from a weakly leukemogenic amphotropic MuLV. These chimeric viral genomes were transfected into NIH/3T3 cells to recover infectious viruses, which were then tested for their leukemogenic potential by inoculation into newborn mice. Because intrathymic inoculation of parental Moloney and amphotropic MuLVs lead to the appearance of thymic and nonthymic leukemia, respectively, in a significant percentage of the treated mice, this strategy allowed us to map the primary determinant of leukemogenicity of both Moloney and amphotropic MuLVs, using the same chimeric MuLVs. Moreover, we could identify the viral sequences conferring the disease specificity to these viruses.

MATERIALS AND METHODS

Cells and viruses. The origins of NIH/3T3 and XC cells have been given elsewhere (16). The infectious Moloney MuLV and 4070-A amphotropic MuLV were recovered after microinjection of NIH/3T3 cells with *Hin*dIII-cleaved and religated cloned viral DNA from pMo DNA and with *Eco*RI-cleaved and religated p4070-A DNA, respectively. The cloned Moloney MuLV genome (pMo) (37) was a generous gift from David Baltimore (Massachusetts Institute of Technology). The cloned amphotropic MuLV genome (p4070-A) (4) was kindly provided by Allen Oliff (Memorial Sloan-Kettering Cancer Center). Parental and chimeric MuLVs were all propagated in NIH/3T3 cells. Viruses were titered on NIH/3T3 cells by the XC assay (35) for the XC^+ viruses and by endpoint dilution and the reverse transcriptase assay for the XC^- viruses, as described before (16). Cells were grown in Dulbecco modified Eagle medium supplemented with 5% calf serum (GIBCO Laboratories, Grand Island, N.Y.).

Mice. NIH/Swiss mice were from our breeding colony, which was originally obtained in 1980 from the National Institute of Health, Bethesda, Md. Supernatants from infected NIH/3T3 cells were harvested, titered, and filtered through a 0.45-μm HAWP nitrocellulose filter (Millipore Corp. Bedford, Mass.) before intrathymic inoculation (0.05 ml) of newborn (<24 h old) mice. Inoculated mice were observed daily for signs of disease. Diseased mice were sacrificed when they showed signs of advanced disease.

Construction of DNA recombinants. The infectious viral DNA genomes pMo and p4070-A from Moloney and amphotropic MuLV, respectively, were described previously (4, 37). For subcloning, 5 to 20 μg of viral DNA was cleaved with restriction endonucleases. The digestion conditions were those recommended by the suppliers (Boehringer Mannheim Biochemicals, Montréal, Canada and New England Biolabs, Inc., Beverly, Mass.). The desired fragments were separated on 1.2% agarose gels and isolated by electroelution (6). These fragments were then ligated with T4 DNA ligase (New England Biolabs) at 12°C for 16 h in 0.0l ml of a solution containing 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 20 mM dithiothreitol, and 1 mM ATP (ligase buffer) and used to transform (23) *Escherichia coli* JF1161 (40). Colonies were screened by the method of Grunstein and Hogness (13) with ^{32}P-labeled (33) purified homologous viral DNA fragments. Positive clones were isolated and grown in mass culture. Plasmid DNA was extracted and molecularly analyzed by appropriate restriction endonucleases by using agarose gels as previously described (6).

Microinjection procedure. The viral inserts from various recombinant DNAs (2 μg) were excised from pBR322 by digestion with *Eco*RI (p4070-A, pMoA-2, pMoA-4) or *Hin*dIII (pMoA-1, pMoA-3), phenol and chloroform extracted, ethanol precipitated, and ligated with T4 DNA ligase (50 U) in 0.1 ml of the ligase buffer as previously described (6). The ligated chimeric viral genomes were phenol extracted and ethanol precipitated. DNA was adjusted to a concentration of 200 μg/ml in 1% KCl and microinjected into 500 to 1,000 NIH/3T3 cells as described before (6). Infectious viruses were recovered from the culture supernatant 5 to 10 days later.

Preparation of unintegrated viral DNA. NIH/3T3 cells (8×10^6) were cocultivated with chronically infected cells (4×10^6) in the presence of 2 μg of polybrene per ml. Hirt supernatant DNA was prepared after 48 h of cocultivation as previously described (16, 30). Restriction analysis of unintegrated viral DNA was done by the procedure of Southern (38) with ^{32}P-labeled cloned viral DNA probes as described elsewhere (31).

RESULTS

Characteristics of parental MuLV used to construct chimeric viruses. Chimeric MuLVs were constructed with viral genomes from Moloney MuLV and from wild-mouse amphotropic MuLV. Moloney MuLV, one of the most oncogenic nondefective murine retroviruses (25), has been reported to induce leukemia of T cell origin, in both thymic and nonthymic forms, in a variety of mouse strains after a relatively short latent period of 3 to 6 months after intraperitoneal inoculation (1, 2, 17, 29). Intrathymic inoculation of Moloney MuLV induced mainly a thymic form of leukemia after a short latent period (Table 1; see also Fig. 4). These were characterized by bilateral gross enlargement of the thymus, very often accompanied with enlarged spleen (90%) and lymph nodes (90%). About 20% of the diseased mice had nonthymic leukemia (see Fig. 5); in these mice, the thymus had a normal size, but the spleen (three of three) and the lymph nodes (one of three) were grossly enlarged.

The 4070-A amphotropic MuLV, cloned by cell culture techniques, has previously been reported to be weakly leukemogenic, inducing leukemia of B cell or null cell origin in a low percentage of mice 10 months after intraperitoneal inoculation (10). The amphotropic 4070-A MuLV used in our study was molecularly cloned (4). This virus isolate did not induce disease in any of the 11 NIH/Swiss mice inoculated intraperitoneally 12 months postinoculation (unpublished data). However, when inoculated intrathymically, it induced leukemia in five of seven mice after a long latent period (Table 1). Most of the mice had a nonthymic form of leukemia characterized by a gross enlargement of the spleen (three of five) or the lymph nodes (one of five) and a normal-sized thymus (see Fig. 5). One mouse of seven inoculated with amphotropic MuLV had a thymic form of leukemia after a long latent period (Table 1; see Fig. 4). Therefore, these parental viruses appeared to have sufficiently distinct biological characteristics to identify the viral sequences responsible for their leukemogenic potential and for the specific type of leukemia (thymic or nonthymic) they induce.

Construction and characterization of chimeric viral genomes. The construction of chimeric viral genomes from parental viral DNA of Moloney and amphotropic 4070-A MuLVs was facilitated by the fact that they share several identical restriction endonuclease sites (4, 11). Recombinant pMoA-4 was constructed by inserting the shorter *Pvu*I-*Cla*I fragment from Moloney MuLV DNA into a partially digest-

TABLE 1. Biological characterization of chimeric MuLVs in NIH/Swiss mice

Virus inoculated	XC titer (PFU/ml) ($\times 10^6$)	*Fv-1* tropism determinant	No. mice with leukemia/no. mice inoculated	
			Thymic	Nonthymic
pMO	10	NB	7/8	1/8
	0.5	NB	5/7	2/7
p4070-A	2	N	1/7	4/7
pMoA-1	10	N	12/17	4/17
pMoA-2	5	NB	2/5	0/5[a]
pMoA-3	10	N	3/19	13/19
pMoA-4	10	N	3/7	2/7
	0.5	N	4/9	0/9

[a] Five mice were lost accidentally during the experiment.

ed *Pvu*I-*Cla*I fragment from 4070-A MuLV DNA obtained by the ligation of three independently purified fragments (Fig. 1). This short Moloney MuLV DNA fragment contains essentially the LTR and the 5′ end leader sequences. Recombinant pMoA-1 was constructed by substituting the *env-pol* region of pMoA-4 DNA for the homologous sequences derived from Moloney MuLV DNA. This chimeric genome harbors the carboxy terminus of *pol*, all of *env*, the LTR, and the 5′ end leader sequences from Moloney MuLV DNA (Fig. 1). Recombinant pMoA-3 was constructed the same way as pMoA-1, except that its shorter *Cla*I-*Sal*I fragment was derived from p4070-A DNA (Fig. 1). This chimeric genome contains all the amphotropic sequences, except those between the *Cla*I and *Sal*I sites flanking the *pol-env* region. Recombinant pMoA-2 was constructed by ligating the 4.9 kilobase-pair (kbp) *Cla*I-*Sal*I *pol-gag* LTR-containing frag-

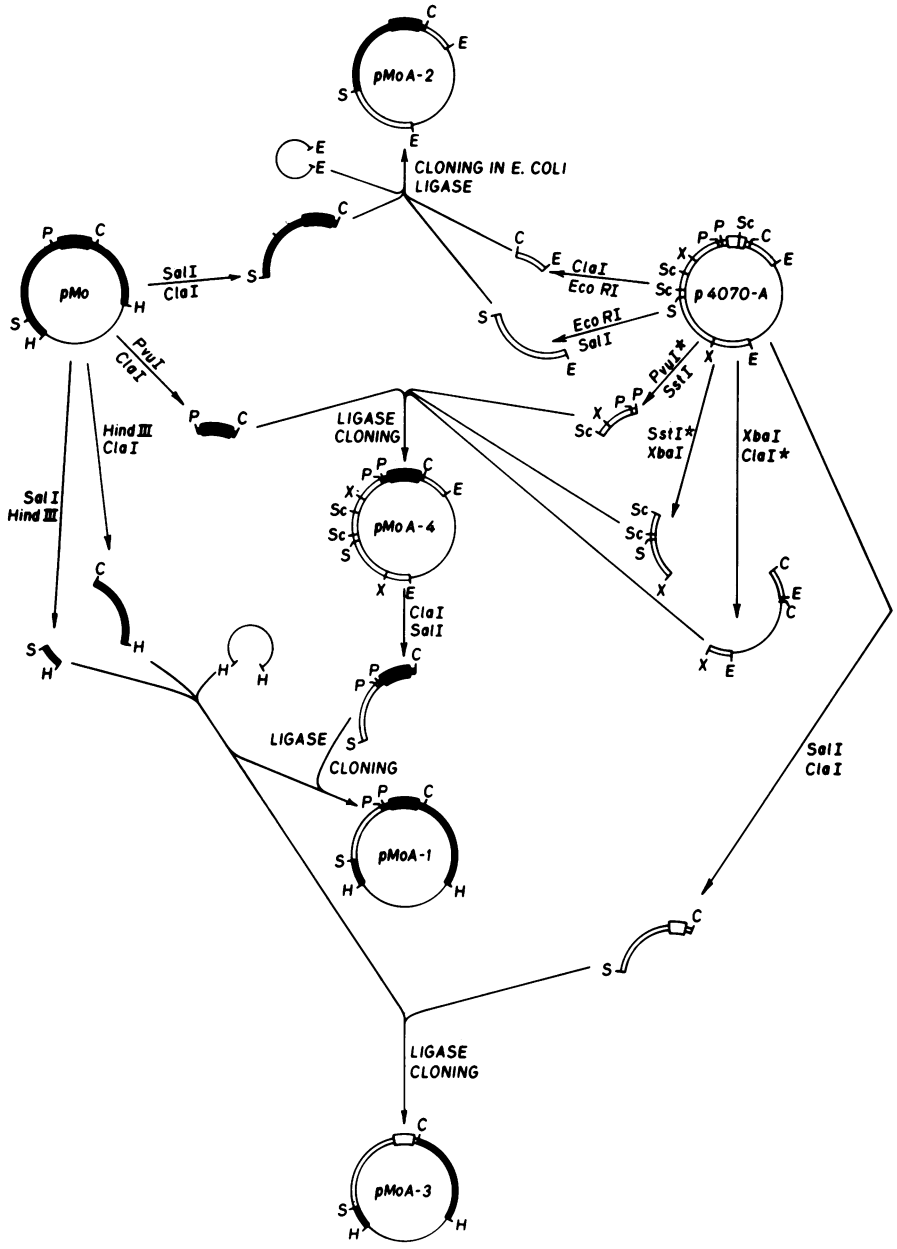

FIG. 1. Construction of chimeric MuLV genomes. The 4.8-kbp *Cla*I-*Sal*I fragment from pMo and the complementary 2.8-kbp *Sal*I-*Eco*RI and 1.2-kbp *Eco*RI-*Cla*I fragments from p4070-A were ligated with T4 DNA ligase in the presence of *Eco*RI-cleaved pBR322 and cloned in *E. coli* to generate pMoA-2. The small 1.5-kbp *Cla*I-*Pvu*I fragment from pMo and the complementary 2.1-kbp *Pvu*I-*Sst*I, 2.9-kbp *Sst*I-*Xba*I, and 6.6-kbp *Xba*I-*Cla*I (containing pBR322) fragments from p4070-A were ligated and cloned in *E. coli* to generate pMoA-4. The 1.0-kbp *Sal*I-*Hin*dIII and the 3.0-kbp *Cla*I-*Hin*dIII fragments from pMo were ligated with the pMoA-4 4.8-kbp *Cla*I-*Sal*I or the p4070-A 4.2-kbp *Cla*I-*Sal*I fragment in the presence of *Hin*dIII-cleaved pBR322 and cloned in *E. coli* to generate pMoA-1 and pMoA-3, respectively. The solid areas indicate fragments from pMo; the open areas indicate fragments derived from p4070-A; the lines indicate pBR322. Boxes represent the LTRs. The asterisk represents partial digestion. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*I; S, *Sal*I; Sc, *Sst*I; X, *Xba*I.

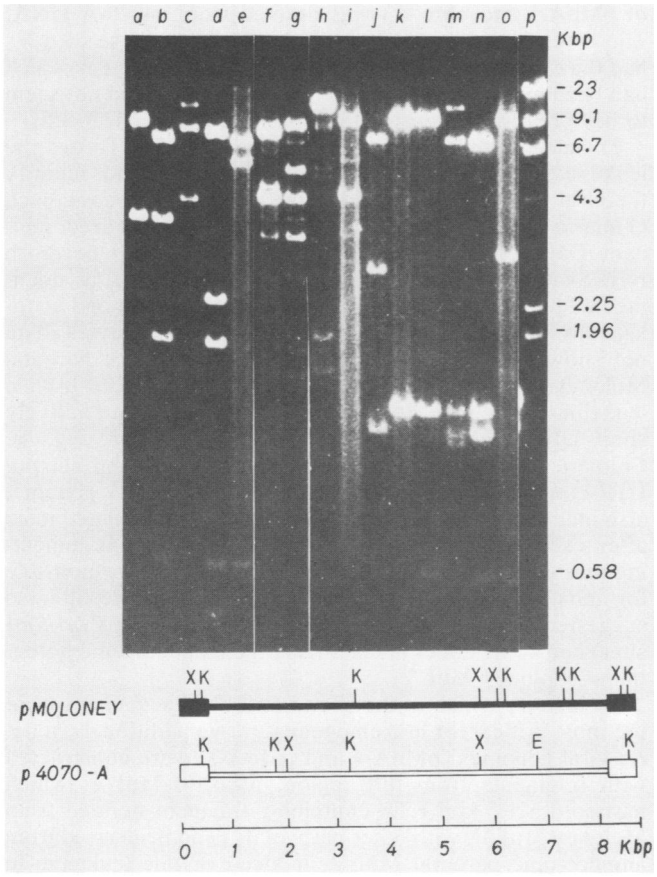

FIG. 2. Molecular characterization of recombinant DNAs. Parental pMo, p4070-A, and recombinant pMoA-1, pMoA-2, pMoA-3, and pMoA-4 DNAs (2 μg) were digested with restriction endonucleases, and the fragments were separated on 1.4% agarose gel as previously described (8). The gel was stained with ethidium bromide (5 μg/ml), and DNA was revealed by UV illumination. pMo (lane j), p4070-A (lanes a, f, and k), pMoA-1 (lanes d, h, and n), pMoA-2 (lanes e, i, o), pMoA-3 (lanes c and m), and pMoA-4 (lanes b, g, and l) DNAs were digested with *Xba*I (lanes a through e), *Eco*RI (lanes f through i), and *Kpn*I (lanes j through o). Marker, *Hin*dIII-digested lambda DNA (lane p). Bottom, Partial restriction map of parental genomes. Boxes represent the LTRs. E, *Eco*RI; K, *Kpn*I; X, *Xba*I.

ment from Moloney MuLV DNA with *Eco*RI-*Cla*I and *Eco*RI-*Sal*I fragments from p4070-A DNA in the presence of *Eco*RI-cleaved pBR322. Ligated DNAs were transfected into *E. coli*, and colonies were screened with ^{32}P-labeled homologous viral DNA fragments.

Several individual recombinant DNAs from each class were analyzed by restriction endonucleases. One clone DNA from each class of recombinants which appeared to have the desired structure was further studied to ascertain the origin of each parental fragment and prove the chimeric structure of the viral insert in each recombinant. The *env* gene of the two parental viral genomes could be distinguished by the presence of an *Eco*RI site (at 6.9 kbp) in the amphotropic MuLV genome (Fig. 2, lane f) and by the presence of additional *Kpn*I sites (at 6.25 and 7.45 kbp) in the Moloney genome, generating two 1.2-kbp comigrating fragments (lane j). pMoA-4 and pMoA-2 DNAs had the *Eco*RI site in their viral genome (lanes g and i) but no additional 1.2-kbp *Kpn*I fragments (lanes l and o), indicating that their *env* gene was derived from the genome of the p4070-A MuLV. pMoA-3 and pMoA-1 DNAs had additional *Kpn*I sites in their genome (lanes m and n), indicating that their *env* gene was derived from the genome of Moloney MuLV.

The *gag-pol* region of the two parental viral genomes could be distinguished by the presence of an additional *Xba*I site (at 2.1 kbp) and *Kpn*I site (at 1.9 kbp) in the p4070-A MuLV genome, thus generating a 3.7-kbp fragment (Fig. 2, lane a) and two comigrating 1.4-kbp fragments (lane k), respectively. *Xba*I digestion of pMoA-4 DNA generated the 3.7-kbp fragment (lane b); *Xba*I digestion of pMoA-3 and pMoA-1 DNAs generated a 8.0-kbp fragment because of the presence of pBR322 sequences at the *Hin*dIII site (lanes c and d), thus indicating that the *gag-pol* region on these viral genomes was derived from the parental p4070-A MuLV. This was confirmed by digestion with *Kpn*I, which generated the 1.4-kbp fragments from pMoA-4, pMoA-3, and pMoA-1 DNAs (lanes l, m and n). pMoA-2 DNA neither harbored the *gag-pol Xba*I site (lane e) nor the additional *Kpn*I sites (lane o), indicating that this region was derived from the Moloney MuLV genome.

The LTR region of the two parental genomes could be distinguished by the presence of two LTR copies in pMo and only one LTR copy in p4070-A DNA. Therefore, a restriction enzyme cleaving within the LTR of both genomes, such as *Kpn*I, would generate a complete permutated LTR fragment of 0.6 kbp after cleaving pMo DNA (Fig. 2, lane j) but not after cleaving p4070-A DNA (lane k). Moreover, *Xba*I cleaves once within the Moloney LTR but does not cleave p4070-A LTR sequences. *Xba*I and *Kpn*I digestion of pMoA-4 (lanes b and l), pMoA-1 (lanes d and n), and pMoA-2 (lanes e and o) DNAs generated the 0.6-kbp fragment typical of Moloney LTR sequences. Digestion of pMoA-3 DNA with *Xba*I or *Kpn*I did not generate such fragments (lanes c and m), and the presence of a 4.1-kbp *Xba*I fragment showed the absence of the *Xba*I site within the LTR of pMoA-3, indicating that its LTR was derived from the p4070-A genome (lane c). The presence of two *Xba*I fragments of 1.8 and 7.1 kbp (pMoA-4) (lane b), 1.8 and 2.4 kbp (pMoA-1) (lane d), and 5.5 and 7.1 kbp (pMoA-2) (lane e) also indicated the presence of a *Xba*I site within the LTR of these DNAs, as is found within the Moloney MuLV LTR. The presence of two LTR copies could also be documented in *Eco*RI-digested pMoA-4 and pMoA-2 DNAs, which revealed a 8.8-kbp fragment instead of the 8.2-kbp fragment generated by *Eco*RI digestion of p4070-A DNA (lanes f, g, and i). Therefore, these results indicated that each chosen recombinant had the expected structure.

Recovery and characterization of infectious chimeric MuLVs. The viral genomes excised from pMoA-1 and pMoA-3 DNAs with *Hin*dIII and from pMoA-2 and pMoA-4 with *Eco*RI were ligated with T4 DNA ligase (to generate nonpermuted forms of viral DNA) and microinjected into NIH/3T3 cells. Infectious viruses were recovered from the culture supernatant a few days later and titered on NIH/3T3 cells by an XC assay for XC$^+$ chimeric MuLVs or by endpoint dilution for XC$^-$ chimeric MuLVs.

The recovered viruses were further characterized by analysis of their unintegrated viral DNA purified by Hirt extraction of acutely infected cells. Hirt supernatant DNAs were cleaved with various restriction endonucleases and analyzed by the agarose gel-Southern transfer procedure. Virus-specific DNA fragments were detected with ^{32}P-labeled cloned viral DNA. Parental viral DNAs share only a few similar restriction endonuclease sites, so the structure of the chimeric viral genomes was therefore easily recognizable by restriction analysis. The presence of a *Xba*I site in the LTR

and of two *Kpn*I sites in the *env* gene are typical of Moloney MuLV DNA. The presence of an *Eco*RI site at 6.9 kbp, a *Kpn*I site at 1.9 kbp, and an *Xba*I site at 2.1 kbp are typical of p4070-A MuLV DNA. pMoA-2, pMoA-4, and pMoA-1 (Fig. 3, lanes g through i) DNAs (linear and supercoiled) generated a 600- and a 300-base-pair LTR fragment after *Xba*I digestion, indicating the presence of a Moloney MuLV-derived LTR (lane k). pMoA-3 (lane j) DNA did not have this site. The pMoA-4, pMoA-1, and pMoA-3 (lanes h through j) genomes also harbored the *gag Xba*I site of p4070-A MuLV DNA. Indeed, *Xba*I digestion generated two fragments of 3.7 and 2.1 kbp (pMoA-3) (lane j) or 3.7 and 1.8 kbp (pMoA-4 and pMoA-1) (lanes h and i). This smaller 1.8-kbp fragment confirmed that the LTR of these recombinants was derived from Moloney MuLV. pMoA-2 DNA (lane g) did not harbor this *gag Xba*I site and showed the 5.5-kbp *Xba*I fragment derived from Moloney MuLV DNA (lane k). Also, digestion of pMoA-2 (lane b) and pMoA-4 (lane a) DNAs with *Eco*RI generated a 1.9-kbp fragment, indicating that the *env* sequence was derived from p4070-A MuLV (lane c). Digestion of pMoA-1 (lane d) and pMoA-3 (lane e) DNAs with *Kpn*I generated the typical 1.2-kbp Moloney *env* fragment (lane f). The shorter 1.1-kbp pMoA-3 fragment confirmed that its LTR was derived from p4070-A DNA. Moreover, the presence of a 1.4-kbp *Kpn*I fragment confirmed that the *gag* gene of pMoA-1 and pMoA-3 was derived from p4070-A DNA. These results indicated that the genomes of these MuLVs had the expected structures of the chimeric molecules which had been microinjected. These chimeric DNAs did not seem to have rearranged during their passage in NIH/3T3 cells.

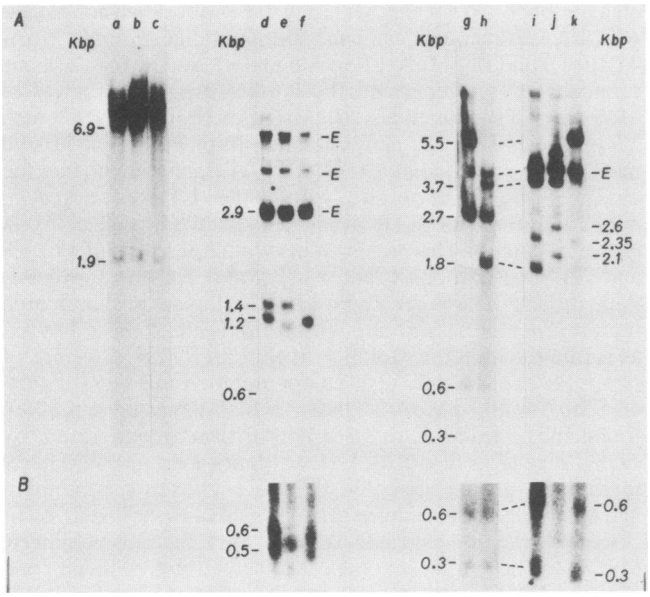

FIG. 3. Agarose gel electrophoresis of unintegrated viral DNA from NIH/3T3 cells infected with parental or chimeric MuLVs. The recombinant viral DNAs were microinjected into NIH/3T3 cells, and infectious viruses spread throughout the culture within a few days after microinjection. These chronically infected cells (4×10^6) were cocultivated with NIH/3T3 cells (8×10^6) in the presence of 2 μg of polybrene per ml for 48 h. Hirt supernatant DNA was then extracted. Hirt DNAs were digested with restriction endonucleases, and DNA fragments, separated on 1.4% agarose gel and transferred to nitrocellulose filters, were hybridized with ^{32}P-labeled 8.2-kbp cloned DNA. Virus-specific DNA was detected by autoradiography. (A) Hirt supernatant DNA from cells infected with pMo (lanes f and k), p4070-A (lane c), pMoA-1 (lanes d and i), pMoA-2 (lanes b and g), pMoA-3 (lanes e and j), and pMoA-4 (lanes a and h) MuLVs was digested with *Eco*RI (lanes a through c), *Kpn*I (lanes d through f) and *Xba*I (lanes g through k). Several endogenous (E) sequences also hybridized to this probe. (B) Part of the same filters after a longer exposure.

Thymic leukemia induced by chimeric MuLVs. To test the leukemogenic potential of chimeric MuLVs and thus identify the viral sequences involved in the malignant process, each chimeric MuLV, recovered after microinjection of recombinant DNA, was inoculated intrathymically into newborn NIH/Swiss mice. Thymic or nonthymic leukemia, as documented pathologically by gross organ enlargement and histologically by the presence of transformed lymphocytes (data not shown), were induced by these viruses. The parental Moloney MuLV inoculated intrathymically induced thymic leukemia in the majority (80%) of the mice after a relatively short latent period of 70 to 105 days (Table 1; Fig. 4). Chimeric pMoA-1 MuLV, which carried a 5.5-kbp *env-pol* LTR-containing fragment from the Moloney MuLV genome, also induced thymic leukemia in most of the inoculated mice after a slightly longer latent period (Table 1; Fig. 4), indicating that these Moloney MuLV sequences were responsible for most of its leukemogenic potential. This result also suggested that the *gag-pol* region (between the *Pvu*I-*Sal*I sites) had some effect in enhancing the leukemogenic potential of Moloney MuLV.

To identify which of the 3′ end *Sal*I-*Pvu*I sequences were the most critical for leukemogenicity, two additional chimeric viral genomes, pMoA-4 and pMoA-3, were constructed with fragments from this region. pMoA-4 MuLV, which harbored a 1.5-kbp LTR-containing fragment derived from Moloney MuLV, with other parts of its genome derived from amphotropic parental MuLV, induced thymic leukemia in ca. 40% of the inoculated mice after a longer latent period (80 to 280 days) (Table 1; Fig. 4), indicating that this fragment harbored sequences sufficient to confer some leukemogenic potential to this chimeric virus. To study the contribution of the Moloney *env* region alone in transferring the high leukemogenic potential of Moloney MuLV, we used pMoA-3 MuLV, which harbored only the *pol-env* region from Moloney MuLV, with all additional sequences derived from the amphotropic MuLV. This virus induced thymic leukemia in a very low percentage of the treated mice after a very long latent period (Table 1; Fig. 4), indicating that this fragment was not sufficient to transfer the leukemogenic potential of Moloney MuLV to this chimeric MuLV. To study the contribution of the *gag-pol* region in the leukemogenic potential of Moloney MuLV more precisely, we constructed pMoA-2 MuLV, which harbored the Moloney 4.9-kbp *gag-pol* LTR-containing fragment. Despite the low number of mice studied, this virus appeared to be as leukemogenic as pMoA-4 MuLV, suggesting that the Moloney *gag-pol* region was not contributing significantly to its leukemogenic potential. Taken together, these results indicated that the primary determinant of leukemogenicity of Moloney MuLV lies within the 1.5-kbp *Cla*I-*Pvu*I LTR-containing fragment. The association of the Moloney *pol-env* sequences with this LTR-containing fragment enhanced the leukemogenic potential of chimeric MuLV. The presence of the Moloney *gag-pol* region seemed to have some effect only in the presence of the Moloney *pol-env* sequences.

Nonthymic leukemia induced by chimeric MuLVs. When inoculated intrathymically, the parental 4070-A amphotropic MuLV induced nonthymic leukemia in ca. 60% of the inoculated mice after a very long latent period (100 to 360 days) (Table 1; Fig. 5). Only one treated mouse had thymic

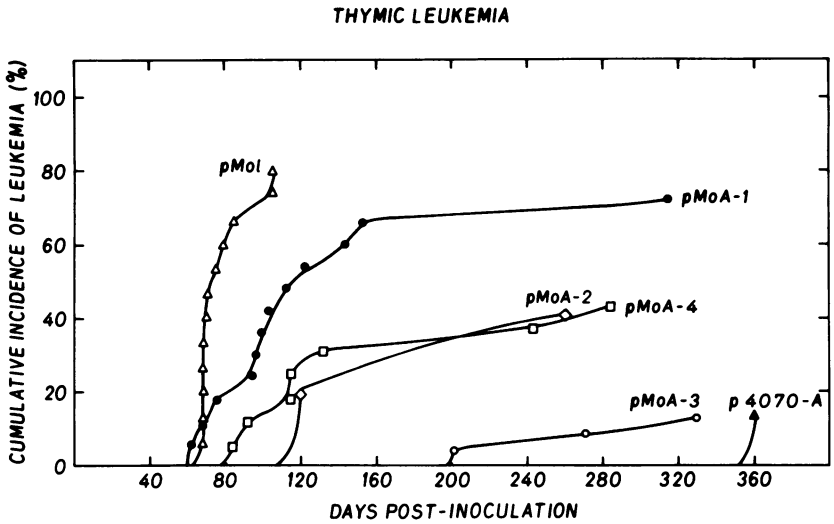

FIG. 4. Incidence of thymic leukemia induced by intrathymic inoculation of parental and chimeric MuLVs into NIH/Swiss mice. Animals were sacrificed when they showed signs of advanced disease. The data are from Table 1.

leukemia (Fig. 4). About 20% of the mice inoculated with Moloney MuLV developed nonthymic leukemia after a very short latent period (Table 1; Fig. 5). Inoculation of mice with pMoA-1 MuLV (whose genome harbored the *gag-pol* region from the amphotropic MuLV), with pMoA-4 MuLV (whose genome harbored 7.3 kbp of *gag-pol-env* sequences derived from amphotropic MuLV), and with pMoA-2 MuLV (whose genome harbored the *pol-env* sequences from amphotropic MuLV) induced nonthymic leukemia in a low percentage of the mice after a relatively short latent period (Table 1; Fig. 5), suggesting that these amphotropic sequences were not sufficient by themselves to transfer the nonthymic form of the leukemogenic potential of the amphotropic MuLV to these chimeric MuLVs. However, the pMoA-3 MuLV (whose genome harbored the LTR-*gag-pol* region from the amphotropic MuLV genome) induced nonthymic leukemia in a significant percentage (68%) of the inoculated mice after a latent period similar to the one seen with the parental amphotropic MuLV (Table 1; Fig. 5). This result indicated that the primary determinant of 4070-A amphotropic MuLV lies within this LTR-*gag-pol* region. Since pMoA-1 and pMoA-4 MuLVs did not appear to confer the leukemogenic potential of parental amphotropic MuLV and since both viruses harbored the same amphotropic *gag-pol* fragment found in pMoA-3, this *gag-pol* fragment was unlikely to harbor the leukemogenic determinant of the amphotropic MuLV. Therefore, the amphotropic 1.5-kbp LTR-containing *Cla*I-*Pvu*I fragment was likely to harbor this determinant.

However, the pMoA-3 MuLV induced a form of nonthymic leukemia slightly different from the nonthymic form seen

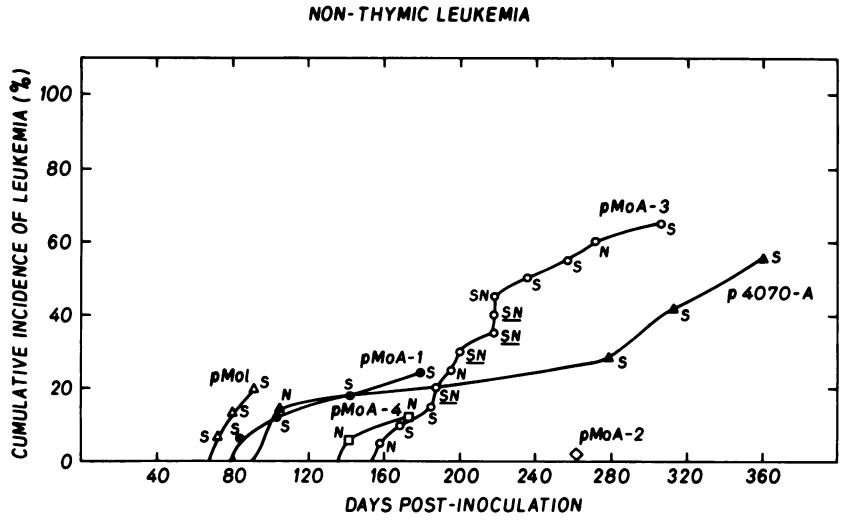

FIG. 5. Incidence of nonthymic leukemia induced by intrathymic inoculation of parental and chimeric MuLVs into NIH/Swiss mice. Animals were sacrificed when they showed signs of advanced disease. The data are from Table 1. S, Enlarged spleen without lymph node enlargement; N, enlarged peripheral and peritoneal lymph nodes without spleen enlargement; SN, enlarged spleen and lymph nodes, with thoracic lymph node enlargement when underlined.

with the parental 4070-A amphotropic MuLV. Indeed, whereas most nonthymic leukemias induced by amphotropic MuLV were characterized by only a grossly enlarged spleen, the nonthymic leukemias induced by pMoA-3 MuLVs were characterized by an enlarged spleen (5 of 13), enlarged nodes (3 of 13), or both enlarged nodes and an enlarged spleen (5 of 13). The induction of this slightly different type of nonthymic leukemia must be attributed to the contribution of the Moloney *pol-env* sequences present on the pMoA-3 genome.

Mapping the viral sequences conferring disease specificity. We showed above that the parental MuLVs induced different types of disease, namely, thymic (Moloney MuLV) and nonthymic (amphotropic MuLV) leukemia, when inoculated intrathymically. Using the same chimeric MuLVs, we were therefore able to identify which sequences from each parental genome determined this disease specificity. The most obvious result was obtained with pMoA-4 MuLV. The majority (seven of nine) of the leukemias induced by this virus were of the thymic form, indicating that the 1.5-kbp *Pvu*I-*Cla*I fragment derived from the Moloney MuLV genome was sufficient to confer on this chimeric MuLV the same disease specificity as the parental Moloney MuLV. This result was confirmed and extended with two other chimeric MuLVs, pMoA-1 and pMoA-3. Except for the 1.5-kpb LTR-containing *Pvu*I-*Cla*I fragment, these two viruses have identical genomes. Nevertheless, most (12 of 16) of the leukemias induced by pMoA-1 MuLV were of the thymic form, whereas most (13 of 16) of the leukemias induced by pMoA-3 MuLV were of the nonthymic form. This result indicated that the 1.5-kbp *Pvu*I-*Cla*I fragment of Moloney MuLV and the same fragment of 4070-A amphotropic MuLV were not only necessary but also sufficient to confer disease specificity to each of these viruses. The presence of this sequence and this sequence alone seemed to determine which type of leukemia (thymic or nonthymic) developed (Fig. 6).

DISCUSSION

Our results indicate that the Moloney sequences which allow this virus to be so highly leukemogenic are distributed all along the genome. However, the sequences which are both necessary and sufficient to induce disease, defined as the primary determinant of leukemogenicity, were shown to map within the small 1.5-kbp *Cla*I-*Pvu*I LTR-containing fragment. Within this fragment, the U3 LTR sequences were the most likely to be responsible for this effect. Indeed, similar experiments with chimeric MuLV derived from the leukemogenic Gross passage A and the BALB/c B-tropic MuLVs also revealed that the U3 tandem direct repeats were the primary determinant of their leukemogenicity (submitted for publication). The U3 LTR tandem direct repeats also appeared to be the primary determinant of the leukemogenicity of AKR SL3-3 MuLV (20). The most likely molecular mechanism by which these sequences could promote malignant transformation is through the activation of a cellular *onc* gene as previously reported for the activation of c-*myc* (14, 26–28) or c-*erb* (9) by nondefective avian retroviruses. Moloney MuLV sequences have been shown to be integrated in the vicinity of c-*myc* (39) and in the vicinity of two other specific and distinct (19, 41) loci in several Moloney MuLV-induced rat thymomas.

The addition of the Moloney MuLV *env* sequences to the Moloney 1.5 kbp *Cla*I-*Pvu*I fragment, along with the remaining amphotropic sequences, enhanced the leukemogenicity of the chimeric MuLV (compare pMoA-4 and pMoA-1 MuLVs), indicating that these Moloney *pol-env* sequences were somehow involved in tumor development. We also observed the same phenomenon with the *env* sequences from Gross passage A MuLV and we could identify the p15E *env* to be primarily responsible for this enhancing effect (submitted for publication). The mechanism by which the *pol-env* sequences might be operating is unclear. They might harbor other enhancer sequences. Alternatively, the gene products, gp70 or p15E proteins, might have a mitogenic effect on lymphoid cells as suggested before (24), or might prevent the transformed virus-producing lymphoid cells from being recognized by the immune system.

Interestingly, pMoA-1 MuLV was slightly less leukemogenic than the parental Moloney MuLV. Indeed, this chimeric MuLV induced disease in almost 100% of the inoculated

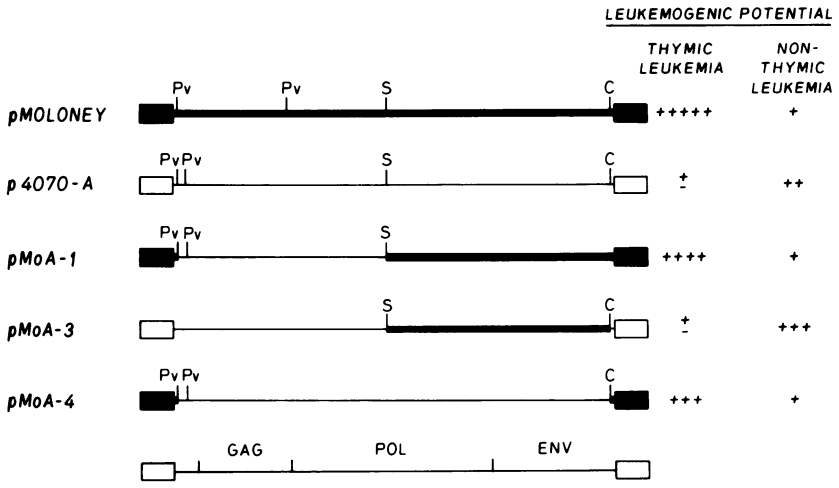

FIG. 6. Nonpermuted forms of parental and chimeric viral DNA genomes with their leukemogenic potential. The viral genomes are represented as linear double-stranded molecules flanked at their ends by LTR sequences (boxes). The positions of the *gag*, *pol*, and *env* genes are represented at the bottom. The solid areas indicate the Moloney MuLV fragments, and the lines represent p4070-A amphotropic MuLV fragments. The leukemogenic potential of each virus (high, +++++; low, +; and intermediate values) and the type of leukemia induced are summarized from Table 1 and Fig. 4 and 5. Abbreviations: C, *Cla*I; Pv, *Pvu*I; S, *Sal*I.

mice but after a longer latent period (Fig. 4). Since pMoA-1 MuLV differed from the parental Moloney MuLV by only one of its regions, the *gag-pol* sequences, we must conclude that this region was also involved in enhancing the leukemogenicity of Moloney MuLV. Because the pMoA-2 MuLV did not appear to be more leukemogenic than the pMoA-4 MuLV, the Moloney *gag-pol* region seemed to be enhancing leukemogenicity only in the presence of Moloney *pol-env* sequences. We reported before that the *gag-pol* region was significantly modified in all the leukemogenic radiation leukemia viruses we isolated from X-ray-induced C57BL/6 thymoma cells (32). Recombinant oncogenic avian leukosis viruses were also found to have this region modified (34). The molecular mechanism responsible for this enhancing effect remains obscure.

The unexpected result that the amphotropic MuLV could induce leukemia with a very distinct tissue distribution in a relatively high percentage of mice inoculated intrathymically has also allowed us to identify the amphotropic sequences responsible for the leukemogenic potential of this virus. These sequences identified mainly with pMoA-3 MuLV mapped within a 4.8-kbp *gag-pol* LTR-containing fragment. Since the *gag-pol* sequences did not appear to harbor the primary determinant of leukemogenicity of the amphotropic MuLV when studied with other chimeric MuLVs (pMoA-1, pMoA-4), the 1.5-kbp *Cla*I-*Pvu*I LTR-containing fragment was likely to be the primary determinant of leukemogenicity of amphotropic MuLV, as was found with Moloney MuLV.

The presence of additional *env-pol* sequences from Moloney MuLV with the amphotropic LTR sequences had an interesting effect. These sequences were indeed responsible for a slightly different distribution of the nonthymic leukemia induced by pMoA-3 MuLV, with a shift towards enlargment of the lymph nodes. A cell-specific enhancer sequence might be present in this Moloney region and explain this shift. Alternatively, the Moloney *env* proteins (gp70, p15E) could allow the virus to penetrate into a specific subpopulation of lymphoid cells or could facilitate growth of these transformed lymphoid cells in a different microenvironment.

The two parental viruses used in this study induced two different forms of leukemia (thymic and nonthymic) under our experimental conditions. This unexpected result allowed us to map the sequences responsible for this disease specificity within the 1.5-kbp *Cla*I-*Pvu*I LTR-containing fragment of Moloney MuLV, confirming a previous report (3). Indeed, a chimeric virus constructed with Moloney MuLV sequences and sequences from the Friend MuLV was used to map the disease specificity of Moloney MuLV within the same *Cla*I-*Pvu*I fragment (3). We extended this finding to the amphotropic MuLV by showing that the disease specificity of this virus also resides within its *Cla*I-*Pvu*I fragment, suggesting that allelic sequences on Moloney and amphotropic MuLV genomes are responsible for this phenotype. These allelic sequences are likely to be the U3 LTR tandem direct repeats since we have already shown that the thymotropism of BALB/c B-tropic MuLV and Gross passage A MuLV is determined by the U3 LTR tandem direct repeats (7). Therefore, we showed here that the same region of the genome, namely, the 1.5-kbp *Pvu*I-*Cla*I fragment, determine two important characteristics of the virus: its transformation potential and its disease specificity. From what is known from Gross passage A and BALB/c B-tropic MuLVs, the same sequences, namely, the U3 LTR tandem direct repeats, are most likely responsible for both functions. Similar sequences on other murine retroviruses have been shown to be "enhancer" elements (18, 22). An attractive model is that these enhancer sequences first allow the virus to replicate in specific target cells; the cells then become transformed when a provirus integrates in the vicinity of an *onc* gene.

Heterogeneity of the types of leukemia (thymic and nonthymic) induced by uncloned or partially cloned Moloney MuLV inoculated intraperitonealy into newborn mice has been reported previously (1, 2, 29). Both types of leukemia were shown to be of T cell origin (1, 2, 29), and the heterogeneity was reported to be under the control of cellular genes (2). We found a similar heterogeneity, but to a much lesser extent, in leukemia induced by molecularly cloned Moloney MuLV inoculated intrathymically into outbred NIH/Swiss mice. However, our results with chimeric MuLVs indicate that the viral sequences themselves are also important in determining which form of leukemia will eventually develop. Some of the previously described heterogeneity of the leukemia induced by Moloney MuLV might have been caused by a mixture of different viruses in the viral preparations inoculated.

The amphotropic 4070-A MuLV cloned by standard virus dilution techniques has previously been reported to induce leukemia of B cell or null cell origin in mice inoculated intraperitoneally (10). When inoculated intraperitoneally into newborn NIH/Swiss mice, the molecularly cloned 4070-A MuLV did not produce disease after more than 1 year (unpublished data). However, as shown here, it induced leukemia in a significant percentage of the mice inoculated intrathymically. And one of these was a typical thymoma, indicating that the route of injection can alter the target cell specificity of the virus. A similar finding was reported with Abelson MuLV: this virus complex, which usually induces B cell lymphoma when inoculated intraperitoneally, was shown to induce thymoma when inoculated intrathymically (5). We are currently investigating whether the nonthymic leukemias induced by amphotropic MuLV are of B or T cell lineage.

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