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A Multistep Process of Leukemogenesis in Moloney Murine Leukemia Virus-Infected Mice That Is Modulated by Retroviral Pseudotyping and Interference

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Mixed retroviral infections frequently exhibit pseudotyping, in which the genome of one virus is packaged in a virion containing SU proteins encoded by another virus. Infection of mice by Moloney murine leukemia virus (M-MuLV), which induces lymphocytic leukemia, results in a mixed viral infection composed of the inoculated ecotropic M-MuLV and polytropic MuLVs generated by recombination of M-MuLV with endogenous retroviral sequences. In this report, we describe pseudotyping which occurred among the polytropic and ecotropic MuLVs in M-MuLV-infected mice. Infectious center assays of polytropic MuLVs released from splenocytes or thymocytes of infected mice revealed that polytropic MuLVs were extensively pseudotyped within ecotropic virions. Late in the preleukemic stage, a dramatic change in the extent of pseudotyping occurred in thymuses. Starting at about 5 weeks, there was an abrupt increase in the number of thymocytes that released nonpseudotyped polytropic viruses. A parallel increase in thymocytes that released ecotropic M-MuLV packaged within polytropic virions was also observed. Analyses of the clonality of preleukemic thymuses and thymomas suggested that the change in pseudotyping characteristics was not the result of the emergence of tumor cells. Examination of mice infected with M-MuLV, Friend erythroleukemia virus, and a Friend erythroleukemia virus-M-MuLV chimeric virus suggested that the appearance of polytropic virions late in the preleukemic stage correlated with the induction of lymphocytic leukemia. We discuss different ways in which pseudotypic mixing may facilitate leukemogenesis, including a model in which the kinetics of thymic infection, modulated by pseudotyping and viral interference, facilitates a stepwise mechanism of leukemogenesis.

Cells chronically infected by murine leukemia viruses (MuLVs) continuously synthesize the Env protein (SU), which saturates the pool of cell surface receptor molecules utilized during infection. Reinfection of these cells is then blocked for MuLVs which recognize the same receptor but not for MuLVs which recognize distinct receptors. This phenomenon of viral interference has defined subgroups of MuLVs on the basis of their recognition of different cellular receptors (1, 9, 42, 43, 57). Three classes of MuLVs have been identified in inbred mouse strains. Ecotropic MuLVs are infectious for murine cell lines but not for cell lines of heterologous species. They are found as endogenous viruses transmitted vertically in the germ line of some mouse strains or as exogenous MuLVs transmitted horizontally by infection. Xenotropic viruses are infectious for heterologous cell lines but do not infect murine cells and are found as endogenous viruses in some mouse strains. A third subgroup of MuLVs, the polytropic MuLVs, infect both murine and heterologous cell lines; however, they utilize a cellular receptor which is distinct from that used by either the ecotropic or xenotropic MuLVs. Infectious polytropic MuLVs have not been identified in the germ lines of mice. Rather, these viruses arise by recombination of ecotropic MuLVs in infected mice with members of a large family of closely homologous endogenous retroviral gene sequences present in inbred mouse genomes (2, 41, 52–54). The recombination always involves a replacement of an *env* gene sequence (6, 7, 19, 21, 22, 44) encoding ecotropic receptor binding with an endogenous sequence encoding polytropic receptor binding.

Polytropic MuLVs have been implicated in the induction of lymphocytic leukemia in highly leukemic mouse strains, such as AKR/J and HRS/J mice, which harbor endogenous ecotropic MuLVs (13, 29, 38, 39), and also in mice which have been inoculated with the exogenous Moloney MuLV (M-MuLV) (4, 16, 45, 55). Brightman et al. (4) have described a mutant of M-MuLV which efficiently infects mice but is deficient in its ability to generate polytropic viruses. This variant does not induce lymphocytic leukemia, suggesting that the generation and spread of polytropic MuLVs may be necessary for disease progression. The principal mechanism of oncogenesis appears to be integration of MuLV proviruses near genes which influence cellular proliferation (proto-oncogenes), resulting in activation of the cellular gene by viral transcriptional regulatory sequences (15, 16, 34, 45, 51, 55). Although polytropic MuLV proviruses are frequently integrated near proto-oncogenes in M-MuLV-induced tumors, a substantial proportion of the tumors exhibit ecotropic MuLV proviruses integrated near proto-oncogenes (16, 45, 55). This suggests that the polytropic MuLV may not be essential for transcriptional activation in M-MuLV-induced tumors and may have additional roles in the induction of lymphocytic leukemia in these mice.

A number of other studies also suggest that polytropic MuLVs may play a role other than transcriptional activation in earlier stages of disease (17, 18, 28, 33). One possibility is that polytropic MuLVs serve a trafficking function in infected mice. The generation of recombinant viruses in mice results in a mixed infection of MuLVs which utilize different cellular receptors. Mixed MuLV infection frequently results in virus pseudotyping, in which an RNA genome encoding an SU protein of a particular tropism is encapsulated in a virion (the viral structural particle) containing an SU protein of a different tropism (28, 31, 56, 58). Pseudotyping could facilitate infection

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of cells not normally susceptible to infection, as well as enable multiple infections of cells by MuLVs of the same group which would otherwise be blocked by viral interference. In this report, we have examined polytropic and ecotropic pseudotyping in mice throughout the preleukemic period after neonatal infection by M-MuLV. We describe a dramatic change in pseudotyping late in the preleukemic period which may facilitate superinfection of thymocytes to influence malignant transformation.

MATERIALS AND METHODS

Mice and viruses. The mice utilized in this study were NFS/N mice maintained as an inbred colony at Rocky Mountain Laboratories. Ecotropic MuLVs utilized in this study were M-MuLV$_{1387}$ (23), Friend erythroleukemia virus 57 (F-MuLV$_{57}$) (40), and the chimeric virus FM-I2 (5). The M- and F-MuLVs were originally obtained from E. M. Scolnick (Merck Sharp and Dohme Research Laboratories, West Point, Pa.). FM-I2 was originally obtained from N. Hopkins (Massachusetts Institute of Technology).

MAbs, in vitro cell lines, and virus assays. The monoclonal antibodies (MAbs) utilized in this study were MAb 516 (8) and Hy 7 (12), which are specifically reactive with the SU proteins of polytropic MuLVs; MAb 48 (10), which is specifically reactive with the SU protein of F-MuLV; and MAb 538 (generously provided by Bruce Chesebro, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Mont.), which is specifically reactive with the SU protein of M-MuLV. Titration of virus stocks and infectious center (IC) assays were performed by a focal immunofluorescence assay as previously described (50). Briefly, dilutions of virus stocks or cells from dissociated tissues were used to infect in vitro cell cultures seeded 1 day previously at cell concentrations determined to reach confluence in 5 days. When the infected cultures reached confluence, they were incubated with the appropriate MAb and then treated with fluorescein isothiocyanate goat anti-mouse immunoglobulin G (IgG), IgA, and IgM (Cappel Organon Teknika Corp.; 55499) diluted 1:200 to detect MAb binding. Foci of infected cells were visualized by fluorescence microscopy. The in vitro cell lines used in this study were NIH 3T3 cells and a mink lung fibroblast line (ATCC CCL64) (30).

Analyses of DNAs by blot hybridization. DNAs were isolated from dispersed thymocytes and purified with a DNA isolation kit (Gentra Systems, Inc.; D5000A) according to the manufacturer's instructions. Restriction endonuclease cleavage of DNAs was carried out for 4 to 12 h with 10 U of enzyme per μg for all enzymes except *Bss*HII (4 U/μg). Digested DNAs and molecular weight marker DNAs (5 ng of digoxigenin [DIG]-labeled lambda DNA cleaved with *Hin*dIII from Boehringer Mannheim; 1218590) were electrophoresed on 22-cm-long agarose gels in Tris-Borate-EDTA buffer (36) at 85 V. The duration of the electrophoresis was 19 h for 0.5% gels and 17 h for 0.8% gels. Following electrophoresis, DNAs were transferred to positively charged nylon membranes (Boehringer Mannheim; 1417240) with a vacuum blotting apparatus (Pharmacia; VacuGene XL). During the blotting procedure, the DNAs were depurinated by incubation with 0.25 N HCl for 25 min, denatured and cleaved by incubation with 0.5 N NaOH–1.5 M NaCl for 25 min, neutralized with 1.0 M Tris HCl–1.5 M NaCl (pH 8.0) for 25 min, and transferred to the membranes with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) for 1 h, according to the manufacturer's instructions. The membranes were placed on Whatman 3MM paper which had been saturated with 2× SSC, and the DNAs were cross-linked to the membranes by exposing both sides to 0.12 J of UV irradiation with a Stratalinker UV Crosslinker, model 2400 (Stratagene). Following cross-linking, the gels were baked for 1 h in an 80°C oven.

The ecotropic *env* gene probe was prepared by excision of a 498-bp *Hpa*I-to-*Age*I fragment from a plasmid containing the M-MuLV genome (see Fig. 6) (48). The excision was performed sequentially by excision and purification of an *Hpa*I-to-*Hpa*I fragment which encompasses the probe sequences and then by excision and purification of the *Hpa*I-to-*Age*I fragment. The polytropic *env* gene probe was excised from a plasmid containing a 620-bp *Bam*HI-to-*Eco*RI fragment cloned from a polycythemia-inducing strain of the Friend spleen focus-forming virus (35). The defective spleen focus-forming virus corresponds to a deleted polytropic virus (24), and the *Bam*HI-to-*Eco*RI fragment is homologous to an identically sized fragment in polytropic genomes (see Fig. 6). All DNA fragment purifications were performed by agarose gel electrophoresis and electroelution. Labeling of the probes for subsequent detection by chemiluminescence was performed by random primed synthesis with a DIG-labeled dUTP with a DIG DNA labeling kit (Boehringer Mannheim; Genius 2 kit, 1175033) according to the manufacturer's instructions. Twenty-microliter reaction mixtures were precipitated with ethanol and digested with 0.1 mg of proteinase K in 50 μl of 1 mM $CaCl_2$–5 mM Tris HCl (pH 8.0). The membranes were prehybridized for at least 3 h at 65°C in 5× SSC–0.8× maleic acid buffer (0.15 M NaCl, 0.1 M maleic acid [pH 7.5])–0.1% *N*-lauroylsarcosine–0.2% sodium dodecyl sulfate (SDS)–2% (wt/vol) blocking reagent (Boehringer Mannheim, 1096176)–200 μg of sheared, denatured salmon sperm DNA per ml. The membranes were then hybridized for 10 to 12 h with denatured probe diluted in the prehybridization buffer at the concentrations indicated in the figure legends. After hybridization, the membranes were washed three times at room temperature for 15 min in 2× SSC–0.1% SDS and then washed twice for 1 h each time in 0.1× SSC–0.1% SDS at 67°C. All subsequent treatments were at room temperature. After equilibrating for 5 min with maleic acid buffer containing 0.3% Tween 20 (washing solution), the membranes were incubated for at least 3 h in maleic acid buffer containing 5% blocking reagent (blocking solution). They were then incubated for 30 min with a preparation of anti-DIG Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim; 1093274) diluted 1:20,000 in blocking solution and subsequently washed two times in washing solution for 2 h. The blots were equilibrated in 0.1 M NaCl–0.1 M Tris HCl (pH 9.5) for 5 min, transferred to a plastic zipper bag, and incubated with 1 ml of 0.25 mM CDP-Star per 20 cm^2 of membrane (Tropix; MS250R). Excess substrate was removed, the membranes were placed on film (Kodak; BioMax MR, 8715187), and the films were exposed for the times indicated in the figure legends. Membranes to be rehybridized with a second probe were rinsed in H_2O, treated with 0.4 N NaOH–0.1% SDS for 30 min two times at 37°C, and rinsed in 2× SSC. The membranes were then prehybridized and hybridized to the second probe as described above. Densitometric analyses were performed on an LKB Ultroscan XL enhanced laser densitometer.

RESULTS

Detection of polytropic MuLVs from M-MuLV-infected animals with mouse or mink indicator cell lines. Comparisons of infectivity on mouse and mink cells have been used as a measure of pseudotyping of polytropic MuLV genomes in ecotropic MuLV virions with the implicit assumption that the polytropic MuLVs in a given system are largely homogeneous and are equally infectious for mouse and mink cell lines. It is clear, however, that polytropic MuLVs exhibit a great deal of heterogeneity in their in vitro infectivities, with mouse-to-mink infectivity ratios ranging from 10^{-3} in the case of a group of recombinant viruses isolated from F-MuLV-infected mice (21) to 10^3 in the case of class II polytropic MuLVs from AKR mice (20, 25). We have recently reported the occurrence of two antigenic subclasses of polytropic MuLVs in M-MuLV-infected NFS/N mice defined by the MAbs 516 and Hy 7 (32). Preliminary evidence suggests that the Hy 7-reactive polytropic MuLVs from M-MuLV-infected mice are variable in their in vitro tropisms and are frequently much less infectious for mink cells than for mouse cells. In contrast, polytropic MuLVs reactive with MAb 516 are approximately equally infectious for mouse and mink cells (22).

The observation that M-MuLV-infected mice contain two major groups of polytropic MuLVs and the possibility that these populations may exhibit large quantitative differences in their in vitro host ranges prompted us to reexamine pseudotyping in M-MuLV-infected mice utilizing assays which distinguish the two polytropic groups. Thymus cells from NFS/N mice infected neonatally with M-MuLV were assessed as ICs on murine cell lines or on mink CCL64 cells with MAb 516 or Hy 7 in parallel assays. These analyses indicated that MAb 516-reactive polytropic MuLVs released from thymocytes were detected much more readily on murine cells than on mink cells (Fig. 1). This difference was even more pronounced for polytropic MuLV released from the splenocytes of the infected mice. Polytropic ICs from splenocytes were readily detected with NIH 3T3 cells, although the titers were somewhat lower (10- to 100-fold) than thymocyte titers; however, the splenocytes from all but one mouse failed to score as polytropic ICs on mink cells (Fig. 2).

Similar results were seen with Hy 7-reactive MuLVs; however, the titers on mink cells for this subclass of polytropic MuLVs were quite low with both thymocytes and splenocytes (data not shown).

Interference of polytropic MuLV infection by prior infection of NIH 3T3 cells with ecotropic M-MuLV. The results noted above, that polytropic MuLVs from spleens of M-MuLV-infected mice were rarely detected on mink cells, suggested vir-

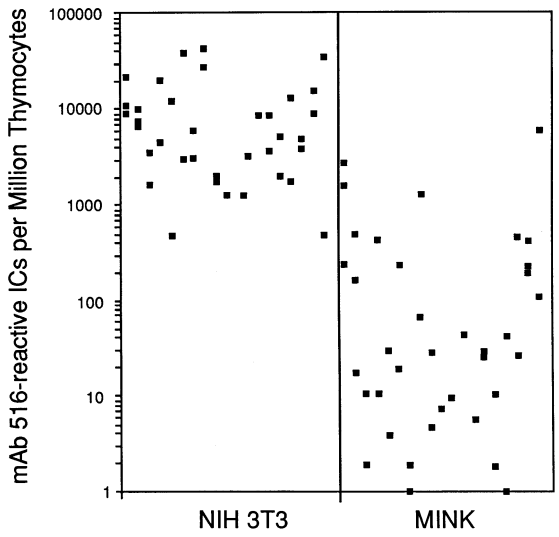

FIG. 1. Detection of MAb 516-reactive polytropic MuLVs from thymocytes of M-MuLV-infected NFS/N mice. Mice were inoculated neonatally with M-MuLV and sacrificed between 27 and 58 days of age. The thymocytes were assayed as ICs with MAb in parallel assays on NIH 3T3 cells and mink cells. Each point on the graph corresponds to the IC titer from a single mouse.

tually complete pseudotyping of polytropic MuLVs by ecotropic virions in this tissue. Thymocytes exhibited detectable titers on mink cells but a much higher polytropic MuLV titer on mouse cells, which also suggested a high degree of pseudotyping. To further evaluate pseudotyping by ecotropic virions, parallel IC assays using NIH 3T3 cells or NIH 3T3 cells that had been previously infected with the ecotropic M-MuLV were performed. Polytropic MuLV genomes which are pseudotyped within ecotropic MuLV virions should be blocked from infection of M-MuLV-infected cells because of ecotropic interference. Marked reductions in the levels of both MAb 516- and Hy 7-reactive polytropic MuLVs from either spleen or thymus cells were observed when assayed on M-MuLV-infected NIH 3T3 cells compared with results from assays with uninfected cells (Table 1). In control experiments, Hy 7- or MAb 516-reactive virus isolates, free of the ecotropic M-MuLV (and thus, not pseudotyped), infected both NIH 3T3 and M-MuLV-infected NIH 3T3 cells with approximately the same level of efficiency (data not shown).

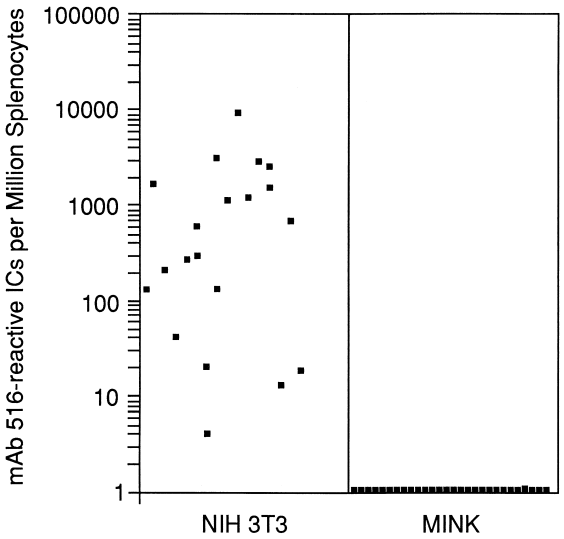

FIG. 2. Detection of MAb 516-reactive polytropic MuLVs from splenocytes of M-MuLV-infected NFS/N mice. Mice were inoculated neonatally with M-MuLV and sacrificed between 27 and 58 days of age. The splenocytes were assayed as ICs with MAb 516 in parallel assays on NIH 3T3 cells and mink cells. Each point on the graph corresponds to the IC titer from a single mouse.

The results of our experiments confirmed the results of a previous study (49) of M-MuLV-infected mice that reported a high proportion of polytropic MuLV genomes released as pseudotypes from spleen and thymus cells, and our results extended those of the earlier study to include both of the major polytropic MuLV groups present in these mice.

Increase in number of thymocytes releasing polytropic virions during the late preleukemic period. In the analyses presented above, ICs of polytropic MuLVs on mink cells were restricted to thymocytes, which are the major oncogenic targets of M-MuLV. A substantial degree of variation in polytropic titers from thymocytes, was observed among different mice. MAb 516-reactive IC titers on mink cells ranged from 0 to 6,500 ICs per million thymocytes (Fig. 1). Analyses of these values as a function of time after inoculation revealed an interesting correlation. Up to 40 days after infection, thymocytes releasing MAb 516-reactive polytropic MuLVs were detected at very low levels on mink cells (Fig. 3A), even though substantial titers of these viruses were detected on NIH 3T3 cells (see Fig. 5). However, levels of MAb 516-reactive MuLVs detected on mink cells increased abruptly between 40 and 50 days after infection, which indicated an abrupt increase in the number of thymocytes producing sufficient polytropic virions to score as ICs in our assays. This was not simply the result of an increased number of polytropic MuLV-infected cells. The percentage of polytropic-MuLV-infected cells that release nonpseudotyped polytropic virions also increased abruptly (Fig. 3B). A similar result was seen with Hy 7-reactive polytropic MuLVs; however, the increase in thymus titers of this subclass of polytropic MuLVs was not as great as that found with MAb 516-reactive MuLVs (data not shown).

Pseudotyping of ecotropic M-MuLV genomes by polytropic MuLV virions during preleukemic stages of disease. It was possible that the increase in polytropic virions late in preleukemia might result in the pseudotyping of ecotropic genomes within polytropic virions. This possibility was examined in parallel IC assays on mink cells using MAb 538, which is an antibody reactive with the ecotropic M-MuLV but unreactive with all polytropic MuLVs examined. Assays of thymocytes of M-MuLV-infected mice using MAb 538 (Fig. 4) revealed an increase in the number of ecotropic MuLV ICs on mink cells which closely paralleled the increase of polytropic ICs on mink cells (Fig. 3A). MAb 538-reactive MuLVs were isolated from infected mink cells and found to be identical to the inoculated M-MuLV in terms of their reactivities to different MAbs, host ranges, and RNase T_1-resistant oligonucleotide fingerprints (data not shown). These results demonstrated an abrupt increase in titers of pseudotyped ecotropic MuLV during the late preleukemic period. Furthermore, since pseudotypes arise only in cells coinfected with both viruses, the increased level of polytropic virions during late preleukemia was not simply the result of cells infected with and producing only polytropic MuLVs.

Spread of MuLVs in thymuses of late-preleukemic mice. Ecotropic MuLV expression by thymocytes of M-MuLV-infected mice is first observed about 1 week after infection and reaches maximum levels by approximately 2 weeks (18, 26), at or before the time polytropic MuLV expression is first observed in the thymus. Since the vast majority of polytropic

TABLE 1. Polytropic ICs from spleens and thymuses of M-MuLV-infected mice on NIH 3T3 cells or NIH 3T3 cells chronically infected with M-MuLV

Mouse[a]	IC titer of foci/10^6 cells of the indicated tissue on infected or uninfected NIH 3T3 cells with the specified MAb[b]							
	Thymus				Spleen			
	NIH 3T3 cells		M-MuLV-infected NIH 3T3 cells		NIH 3T3		M-MuLV-infected NIH 3T3 cells	
	Hy 7	MAb 516	Hy 7	MAb 516	Hy 7	MAb 516	Hy 7	MAb 516
1	8,300	3,800	13	57	2,000	600	0	1
2	10,000	2,100	69	22	4	4	0	0
3	15,000	2,100	310	81	2,300	3,200	0	0
4	25,000	5,900	480	290	1,200	1,100	8	62
5	6,900	4,600	12	7	110	680	0	0
6	730	490	0	0	7	18	0	0

[a] Mice were infected neonatally with M-MuLV and assayed 27 to 37 days postinoculation.
[b] IC assays were performed on serial dilutions of thymocyte or splenocyte suspensions.

MuLVs in the early preleukemic stage were pseudotyped within ecotropic virions, the spread of polytropic MuLVs in the thymus would be expected to be substantially arrested because of interference by ecotropic MuLV infection. Ecotropic and polytropic MuLVs released by thymocytes which scored on mink cells in the late preleukemic period were packaged in polytropic virions and would not be blocked by ecotropic interference. This could facilitate the spread of both ecotropic and polytropic viruses by superinfection of M-MuLV-infected cells. Analyses of MAb 516-reactive ICs revealed an increase in the number of MAb 516-reactive ICs scored on mouse cells (permissive for all polytropic MuLVs, regardless of pseudotyping), suggesting an infectious spread of these viruses in the late preleukemic period (Fig. 5). A large degree of variability was observed in the mice in these analyses, particularly during the late preleukemic period (42 and 47 days). The increase in thymus titers was significant when all of the mice assayed between 30 and 40 days postinfection were compared with mice assayed between 40 and 50 days. We were unable to document a significant spread of Hy 7-reactive polytropic MuLVs or of ecotropic M-MuLV in parallel analyses.

Polytropic and ecotropic proviruses in late-preleukemic and leukemic thymuses. The extent of MuLV infection in thymocytes should be reflected in the level of proviruses integrated into genomic DNA. We attempted to quantify ecotropic and recombinant polytropic proviruses in thymocytes from late-preleukemic and leukemic mice by blot hybridization analyses of genomic DNA preparations cleaved with four different restriction endonucleases. The enzymes chosen enabled the analysis of ecotropic and polytropic MuLV proviruses on the same blot. The levels of ecotropic M-MuLV proviruses were analyzed by blot hybridization with a probe corresponding to an

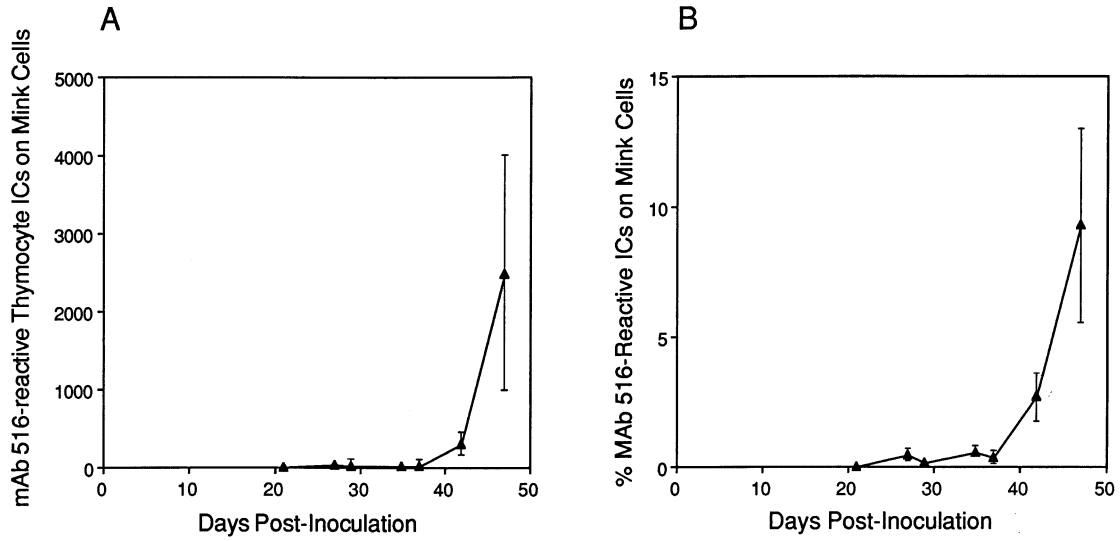

FIG. 3. Thymocytes releasing nonpseudotyped MAb 516-reactive polytropic MuLVs during the preleukemic period. (A) NFS mice were inoculated neonatally with M-MuLV, and the thymocytes were assayed as ICs with MAb 516 on mink cells to quantify thymocytes producing nonpseudotyped polytropic MuLVs. The IC titers were plotted as a function of time after inoculation. Each datum point represents the mean of the individual titers obtained from four to eight animals. The error bars represent the standard errors of the mean (SEM). The increase in titers after 40 days compared with titers measured between 30 and 40 days after inoculation was found to be very significant ($P = 0.0012$) (37). (B) The percentages of polytropic MuLV-producing thymocytes that release nonpseudotyped polytropic MuLVs were determined by comparing the IC titers measured on mink cells (nonpseudotyped polytropic MuLV) with the IC titers measured on NIH 3T3 cells in parallel assays (pseudotyped plus nonpseudotyped polytropic MuLV). Each datum point represents the mean percentage determined in assays with four to eight animals. The error bars represent the SEM. The increase in the percentage of polytropic MuLV-infected thymocytes producing nonpseudotyped viruses was extremely significant ($P = 0.0008$) (37).

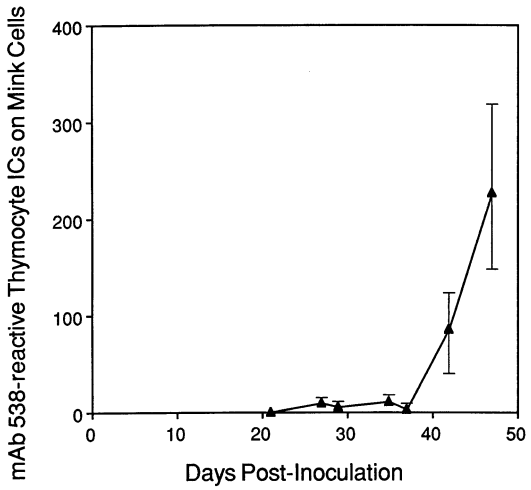

FIG. 4. Pseudotyping of M-MuLV ecotropic genomes within polytropic virions. IC assays of thymocytes were performed on thymocytes of infected mice on mink cells with MAb 538 to detect ecotropic M-MuLV pseudotyped by polytropic virions. Each datum point represents the mean average obtained from four to eight mice. The error bars represent the standard errors of the mean. The increases in titers after 40 days compared with titers measured between 30 and 40 days after inoculation was found to be significant ($P = 0.008$) (37).

*Hpa*I-to-*Age*I restriction endonuclease digestion fragment of the M-MuLV SU protein-encoding sequences (Fig. 6). Cleavage of the DNAs analyzed on the blot yielded a 3.0-kbp fragment complementary to the ecotropic probe from a *Bam*HI site located in the *pol* gene of M-MuLV to a *Bam*HI site located 760 bp from the 5′ end of the SU protein-encoding sequences (Fig. 6 and 7A). Densitometric analyses of the bands in Fig. 7A suggested a small but statistically significant increase (ca. 60%, $P = 0.03$) in the level of ecotropic M-MuLV proviruses between 42 and 52 days postinoculation and a substantial increase (ca. fivefold, $P = 0.01$) in M-MuLV proviruses in DNA from thymomas, compared with the thymic DNA from mice 52 days postinoculation. Some tumor DNAs (Fig. 7A, lanes 9 to 11) had bands in addition to the expected band at 3.0 kbp, indicating the presence of genetically altered ecotropic proviruses. These could be the result of point mutations or, perhaps more likely, the generation of recombinant MuLVs which have retained the 5′ end of the ecotropic SU gene.

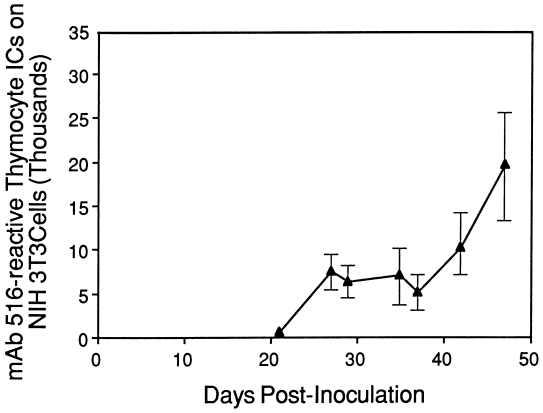

FIG. 5. Spread of thymus infection by MAb 516-reactive polytropic MuLVs during the preleukemic period. IC assays of thymocytes were performed on thymocytes of infected mice as indicated in the legend to Fig. 3. The data are expressed as IC titers on NIH 3T3 cells, which are a measure of the total number of thymocytes productively infected with polytropic MuLVs, irrespective of pseudotyping. Each datum point represents the mean average obtained from four to eight mice. The error bars represent the standard errors of the mean. The increase in titers after 40 days compared with titers measured between 30 and 40 days after inoculation was found to be significant ($P = 0.0341$) (37).

The membrane was stripped and rehybridized with a DNA probe homologous to a 620-bp 5′ *Bam*HI-to-*Eco*RI segment of the polytropic SU protein-encoding sequences (Fig. 6). The analysis of polytropic MuLV proviruses is complicated by the presence of 20 to 30 endogenous polytropic sequences in the NFS mouse genome (see Fig. 9, lane 16). This problem was circumvented by digestion of the DNA with *Bam*HI, *Pst*I, *Sac*I, and *Bss*HII. Most endogenous polytropic *env* sequences complementary to the probe were cleaved to a 1.28-kbp *Bam*HI-to-*Sac*I fragment which corresponds to nearly the entire SU protein-encoding sequence or to a 1.3-kbp *Bam*HI-to-*Pst*I fragment which corresponds to all of the SU protein-encoding sequences (Fig. 6) (52). All recombinant MuLVs between M-MuLV and endogenous polytropic genes thus far examined have undergone recombination within the SU protein-encoding sequences to include the 5′ polytropic *Bam*HI site but not the *Sac*I or *Pst*I sites (3, 22, 46). Cleavage of these recombinant viruses at the 5′ polytropism-derived *Bam*HI site in the SU protein-encoding sequences and a *Bss*HII site in the M-MuLV-derived long terminal repeat sequence yielded a fragment of 2.4 kbp (Fig. 6 and 7B). Densitometric analyses of the blot normalized to the endogenous band at 1.9 kbp (Fig. 7B) did not reveal a significant difference in intensities between 42 and 52 days; however, a substantial increase was detected in the thymoma DNAs (ca. 2.5-fold, $P = 0.02$).

The results of these analyses suggest a slight increase in the level of ecotropic infection during the late preleukemic period; however, in contrast to the IC results presented above, we did not observe a difference in the levels of polytropic proviruses during the preleukemic stage in these analyses. This could be due to the small sample size in these analyses and the large degree of variability in late-preleukemic mice. The thymomas exhibited large increases in the levels of polytropic and ecotropic proviruses compared with the thymuses of the preleukemic mice. This could be the result of infectious spread of MuLVs during the late preleukemic stage and/or the selection of cells in the tumors which exhibit a higher ecotropic and polytropic MuLV burden.

The increase in polytropic virions during the late preleukemic stage does not appear to be the result of emerging populations of tumor cells. It is possible that the change in pseudotyping observed late in the preleukemic period was simply the result of an initial outgrowth of tumor cells which exhibit such properties. None of the late-preleukemic thymuses in our analyses exhibited enlargement (<50 mg), and the earliest time after inoculation that we have observed an enlarged thymus is 54 days. However, it is conceivable that a thymus of normal size is leukemic if the initial outgrowth replaces normal cells. Two lines of evidence suggested that the changes observed late in the preleukemic stage were not the result of an outgrowth of tumor cells in the thymuses. First, if the changes were the result of emerging tumor cells, a high percentage of thymoma cells which score as polytropic ICs should release polytropic virions infectious for mink cells. However, assays of thymocytes derived from thymomas did not consistently reveal a high percentage of ICs which infect mink cells (Table 2). Of the five thymomas examined, only one (Table 2, no. 4) exhibited a high level of infectivity on mink cells. Two thymomas (no. 1 and 2) exhibited intermediate levels of infectivity, while polytropic viruses from the remaining two thymomas were not detected on mink cells.

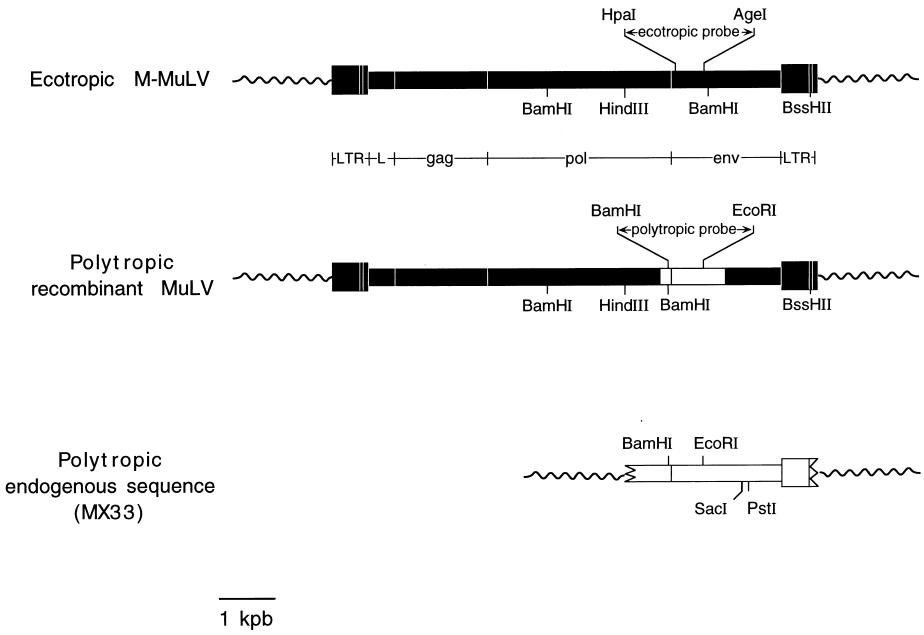

FIG. 6. Restriction endonuclease cleavage sites of proviruses examined by blot hybridization. Cleavage sites on the M-MuLV provirus and MX33 endogenous polytropic sequence provirus were determined from the published sequences (47, 52). Only those sites pertinent to the analyses described in the text are represented. The bars represent proviral or endogenous retroviral sequences, and the wavy lines represent adjacent genomic sequences. LTR, long terminal repeat; L, leader sequence.

A second line of evidence came from analyses of clonality in thymuses from preleukemic and leukemic mice. Clonality in mouse thymomas is established and can be demonstrated by the presence of discrete proviral integration sites detected by blot hybridization analysis of genomic DNA (16, 45, 54, 55). Retrovirus integration occurs in a multitude of sites in the chromosomal DNA of infected cells, and discrete sites cannot be detected in cell populations unless a substantial proportion of the population is clonal (i.e., derived from a single infected cell). We examined the clonality of late-preleukemic and leukemic mice with respect to both ecotropic M-MuLV and recombinant polytropic MuLV integration sites (Fig. 8 and 9). Discrete integration sites of polytropic and ecotropic MuLV proviruses were not detected in thymus DNAs from preleukemic mice at 42 days postinfection, when the initial increase of polytropic virion expression was observed (Fig. 3), or 10 days later, 52 days postinfection. In contrast, multiple polytropic and ecotropic integration sites were readily apparent in all of the thymomas examined.

The extent of pseudotyping late in the preleukemic stage correlates with pathogenicity. Sitbon et al. (49) have reported a high degree of pseudotyping of polytropic MuLVs in mice infected with F-MuLV$_{57}$. We have also examined pseudotyping in F-MuLV$_{57}$-infected mice and found virtually complete pseudotyping of polytropic MuLV genomes within ecotropic MuLV virions (data not shown). This finding was consistent for both the spleens and thymuses of all F-MuLV-infected mice throughout the course of disease. M-MuLV and F-MuLV exhibit very distinct pathogenicities. M-MuLV induces lymphocytic leukemia, whereas F-MuLV induces erythroleukemia. Differences in the extents of pseudotyping in thymuses between F-MuLV and M-MuLV could relate to differences in their pathogenicities. A chimeric virus in which the U3 and a portion of the R region of F-MuLV are replaced with the homologous region of M-MuLV exhibits altered pathogenicity (5). This chimera, termed FM-I2, induces a high incidence of lymphocytic leukemia, similar to that induced by M-MuLV, rather than erythroleukemia. We have compared the pseudotyping of MuLVs in thymuses of M-MuLV-, F-MuLV-, and FM-I2-infected mice in the late preleukemic period (Table 3). Thymocytes from FM-I2-infected mice released ecotropic and polytropic genomes within polytropic virions (infectious for mink cells), similar to thymocytes from M-MuLV-infected mice, whereas thymocytes from F-MuLV-infected mice did not. These results suggested that the presence of polytropic virions late in the preleukemic stage correlated with the pathogenicity of the ecotropic MuLV.

DISCUSSION

The initial results of these studies confirmed a previous report that polytropic viruses from spleens or thymuses of M-MuLV-infected mice are extensively pseudotyped within ecotropic virions (49) and confirmed the results of earlier studies in other systems which also described extensive pseudotyping of polytropic MuLVs by ecotropic MuLVs (28). However, in this study we have considered the different tropisms of polytropic MuLVs and the pseudotyping of ecotropic MuLVs within polytropic virions and have monitored these parameters during the course of disease development. In these analyses we found that polytropic MuLVs from splenocytes are extensively pseudotyped within ecotropic virions throughout the course of infection, while polytropic MuLVs from thymocytes are extensively pseudotyped during the initial 5 weeks of infection. During the late preleukemic stage (5 to 7 weeks postinfection), an abrupt increase in the number of thymocyte ICs which score on mink cells occurs, indicating an increased number of cells releasing polytropic virions. This includes genomes of both the ecotropic M-MuLV and polytropic MuLVs packaged within polytropic virions (Fig. 3 and 4). It is not clear what triggers the increase of polytropic virions. It is important to note, however, that there is not only an increase in the number of cells releas-

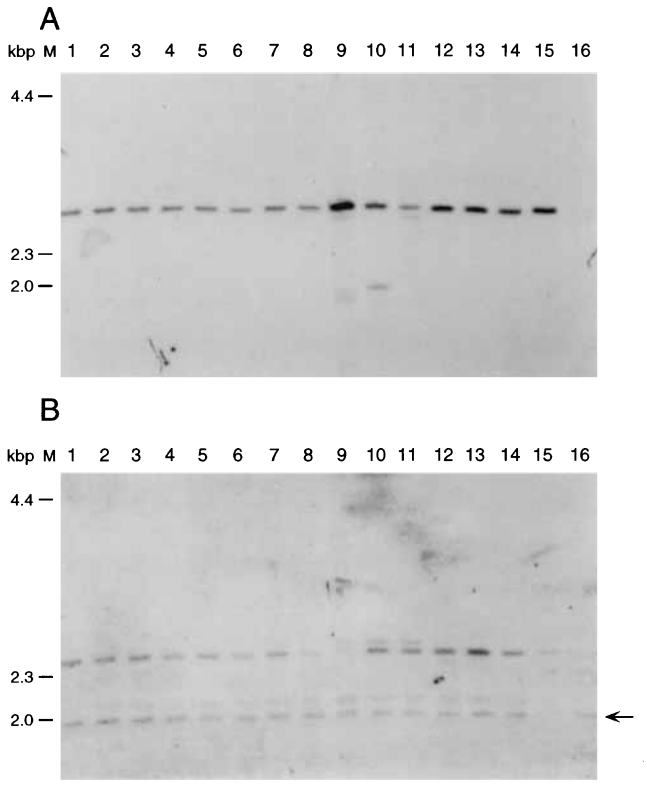

FIG. 7. Comparison of ecotropic M-MuLV and polytropic MuLV provirus levels in DNAs from preleukemic and leukemic thymuses of mice infected by M-MuLV. DNAs were extracted from the thymuses of M-MuLV-infected NFS/N mice at different times postinoculation. Samples (2 μg) of DNA from individual mice were digested by *Bam*HI, *Bss*HII, *Sac*I, and *Pst*I before electrophoresis was performed on a 0.8% agarose gel for 17 h at 85 V. (A) Ecotropic MuLV provirus analyses. The gel was blotted and hybridized with 1.5 ng of DIG-labeled *Hpa*I-*Age*I M-MuLV probe (ecotropic probe) per ml and subsequently treated for chemiluminescence detection, and the film was exposed for 8 h. Lanes: M, molecular size markers; 1 to 4, DNAs from the thymuses of preleukemic mice 42 days postinoculation; 5 to 8, DNAs from the thymuses of preleukemic mice 52 days postinoculation; 9 to 15, DNAs from thymomas of mice 63 to 70 days postinoculation; 16, DNA from the thymus of an uninfected 54-day-old mouse. The band observed corresponds to the 3.0-kbp *Bam*HI-*Bam*HI fragment of M-MuLV (Fig. 6). (B) Polytropic MuLV provirus analyses. The membrane was stripped, rehybridized with 3 ng of DIG-labeled *Bam*HI-*Eco*RI spleen focus-forming virus probe (polytropic probe) per ml, and treated as described above, and the film was exposed for 8 h. The upper band corresponds to the 2.4-kbp *Bam*HI-*Bss*HII fragment of polytropic MuLV (Fig. 6). The band indicated by an arrow is an endogenous polytropic band used as an internal control for densitometric analysis.

ing polytropic virions but also an abrupt increase in the percentage of polytropic-MuLV-infected cells that release polytropic virions (Fig. 3B). This implies that either the infected cells undergo a phenotypic change or the composition of the population of infected cells changes, perhaps by infection of new cells in the thymus or by migration of infected cells to the thymus from other tissues. The changes do not appear to be the result of an outgrowth of leukemic cells which exhibit different pseudotyping properties. Thymomas do not consistently exhibit such properties (Table 2), and tumor clones are not detected in preleukemic thymuses well after the time when the change in pseudotyping is observed (Fig. 8 and 9).

The sudden increase of polytropic virions may be accompanied by the infectious spread of MAb 516-reactive polytropic viruses as well as ecotropic MuLVs in thymuses, although this is not easily documented. IC assays reveal a significant increase

TABLE 2. Percentages of infected thymoma cells releasing polytropic and ecotropic genomes in polytropic virions

Thymoma	% ICs detected on mink cells that reacted with[a]:		
	MAb 516[b]	Hy 7[b]	MAb 538[c]
1	0.6	2.5	<0.1
2	1.0	<0.1	<0.1
3	<1.0	<0.2	<0.1
4	8.8	12.2	0.2
5	<5.0	<0.1	<0.1

[a] The data represent the IC titers of foci on mink cells as percentages of the titers in parallel assays using NIH 3T3 cells.
[b] The data correspond to the percentages of polytropic genomes packaged in polytropic virions.
[c] The data correspond to the percentages of ecotropic genomes packaged in polytropic virions.

in thymocytes releasing MAb 516-reactive MuLVs but not in thymocytes releasing Hy 7-reactive polytropic viruses or ecotropic M-MuLV during the late preleukemic period. The infectious spread of ecotropic genomes packaged within polytropic virions may not be reflected by an increase in the ecotropic MuLV IC titers if the spread occurs primarily by superinfection of cells already infected by M-MuLV. In this regard, a small but significant increase in M-MuLV infection during the preleukemic period was suggested by our analyses of integrated M-MuLV proviruses (Fig. 7A). The infectious spread of MAb 516-reactive but not Hy 7-reactive MuLVs is more difficult to understand. It is possible that the appearance of polytropic virions in the preleukemic period is limited to the MAb 516-reactive subgroup. If so, the release of polytropic virions containing genomes corresponding to Hy 7-reactive viruses may occur in a limited population of cells coinfected

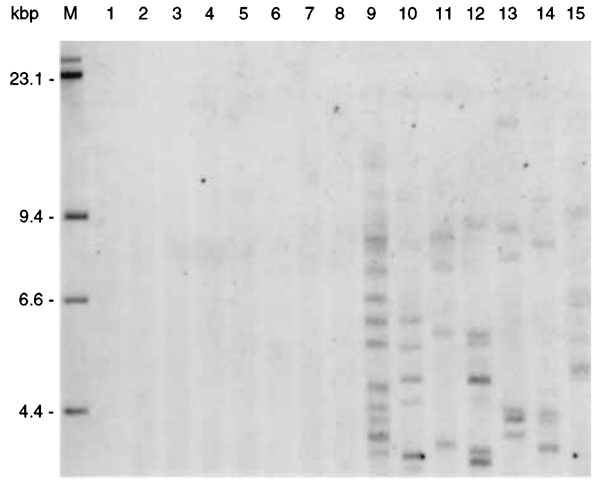

FIG. 8. Detection of M-MuLV clonality in thymuses of mice infected by M-MuLV. DNAs were extracted from the thymuses of M-MuLV-infected NFS/N mice at different times postinoculation. DNAs (6-μg samples) were digested with *Hin*dIII before electrophoresis was performed on a 0.5% agarose gel for 19 h at 85 V. The gel was blotted, hybridized with 1.5 ng of DIG-labeled *Hpa*I-*Age*I M-MuLV probe (ecotropic probe) per ml, and treated for chemiluminescence detection, and the film was exposed for 12 h. Lane M, molecular size standards; lane 1, thymus DNA of an uninfected 54-day-old mouse; lane 2 to 4, thymus DNAs of preleukemic mice 42 days postinoculation; lanes 5 to 8, thymus DNAs of preleukemic mice 52 days postinoculation; lanes 9 to 15, thymoma DNAs from leukemic mice 63 to 70 days postinoculation. The bands observed in the thymoma DNA correspond to *Hin*dIII-*Hin*dIII fragments between the M-MuLV *Hin*dIII site (*pol* gene) (Fig. 6) and the nearest endogenous *Hin*dIII site after the 3′ viral extremity.

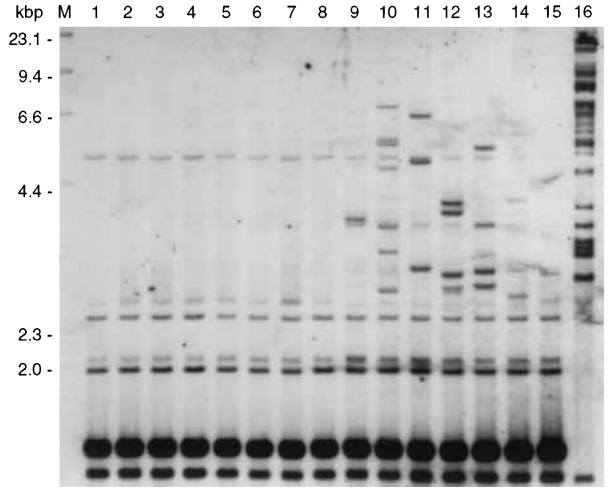

FIG. 9. Detection of polytropic MuLV clonality in thymuses of mice infected by M-MuLV. DNAs were extracted from the thymuses of M-MuLV-infected NFS/N mice at different times postinoculation. DNAs (6-μg samples) were digested with *Bam*HI and *Pst*I (lanes 1 to 15) or *Bam*HI alone (lane 16) before electrophoresis was performed on a 0.8% agarose gel for 17 h at 85 V. The gel was blotted, hybridized with 5 ng of DIG-labeled *Bam*HI-*Eco*RI spleen focus-forming virus probe (polytropic probe) per ml, and treated for chemiluminescence detection, and the film was exposed for 12 h. The samples in lanes 1 to 15 are DNAs from the mice analyzed in the experiment shown in Fig. 8. Lane M, molecular size standards; lane 1, thymus DNA of an uninfected 54-day-old mouse; lanes 2 to 4, thymus DNAs of preleukemic mice 42 days postinoculation; lanes 5 to 8, thymus DNAs of preleukemic mice 52 days postinoculation; lanes 9 to 15, thymoma DNAs from leukemic mice 63 to 70 days postinoculation; lane 16, DNA of an uninfected 54-day-old mouse digested only with *Bam*HI. The bands observed in lanes 16 correspond to endogenous polytropic sequences. Cleavage at the internal *Pst*I site reduced dramatically the number of endogenous bands (compare lane 1 with lane 16), allowing the detection of polytropic MuLV provirus integrations in the thymoma DNAs (lanes 9 to 15). The bands in the tumors correspond to *Bam*HI-*Bam*HI or *Bam*HI-*Pst*I fragments between the polytropic MuLV *Bam*HI site located at the end of the *pol* gene (Fig. 6) and the nearest endogenous *Bam*HI or *Pst*I site after the 3′ viral extremity.

with both MAb 516- and Hy 7-reactive polytropic viruses. Analyses of proviruses did not detect an increase in polytropic integration during the preleukemic period (Fig. 7B). As noted earlier, this discrepancy may be due to large variability among individual mice in the IC assays in the preleukemic period. A large increase in the number of proviral copies of both ecotropic and polytropic MuLVs was found in the thymoma DNAs compared with the DNAs from preleukemic thymuses. This may be due to the outgrowth of multiply infected clones in the tumors. Our analyses of the clonality in the tumors support this conclusion in that a large number of ecotropic and polytropic integrations (ca. eight per tumor and four to five per tumor, respectively) were observed. It is possible that a high degree of oligoclonality could account for the multiplicity of integration sites; however, examination of our ecotropic integration analyses (Fig. 8) suggests that the bands are grouped into one or two sets on the basis of their intensities, suggesting a limited number of clones.

A number of roles for pseudotyping in MuLV-infected mice have been suggested. These include escape from the immune system, protection from inactivating components in serum, and specific cell targeting (11, 28). From our analyses we propose that pseudotyping in M-MuLV-infected mice may facilitate leukemogenesis by the circumvention of viral interference. In murine systems viral interference is a very strong barrier to reinfection both in vivo and in vitro (14). Yet, all of the tumors we have examined exhibit multiple integrations of both the ecotropic and polytropic MuLVs which appear to be the result of multiple infections rather than a large number of singly infected clones. This suggests that the cells were superinfected by viral pseudotypes or that the cells were infected at a high multiplicity of infection with both ecotropic and polytropic viruses, thereby allowing multiple infections before interference was established. These two possibilities are not mutually exclusive and, in either case, multiple infections and integrations in target cells would be expected to increase the probability of an integrative event(s) resulting in malignant transformation. Considering that maximal infection of the thymus by the ecotropic M-MuLV occurs before polytropic MuLVs are detected (18, 26, 27), it would seem that most of the superinfection that occurred would be via polytropic virions if viral pseudotyping is involved.

It is well documented that leukemia induction in M-MuLV-infected mice involves the activation of proto-oncogenes by either an ecotropic or polytropic MuLV (16, 45, 55); however, it is not yet clear if a single event is sufficient for oncogenesis. If only one genetic event is required, the effect of pseudotyping may be additive and simply increase the probability of the event by increasing the viral burden in target cells. After the initial rapid infection of the thymus by the ecotropic M-MuLV, polytropic virions containing ecotropic MuLVs could continue to infect a M-MuLV-infected cell up to the time the cell became infected with polytropic MuLVs. In this regard, our data indicate very low, but detectable, levels of polytropic virions as early as 4 weeks after infection. Cells that remained uninfected with polytropic MuLVs throughout the early preleukemic period would presumably become infected, perhaps much more rapidly, during the late preleukemic period when the increase in polytropic virions is observed. This sequence of events does not adequately explain some of our observations and those of other researchers. The earliest we have observed thymic enlargement (>50 mg) in late preleukemic mice is 54 days after infection with M-MuLV. Thereafter, the incidence of leukemia, as judged by thymic enlargement, increases rapidly to include virtually all mice examined within the following 3 weeks (75 days postinoculation). If leukemia induction requires only one event, one might expect tumors earlier after infection, considering that maximal infection of thymuses oc-

TABLE 3. Pseudotyping characteristics of MuLV from thymocytes of mice infected with M-MuLV, F-MuLV, or a chimeric MuLV

Virus	Pathology induced	% MAb 516-reactive ICs detected on mink cells[a]	% Hy 7-reactive ICs detected on mink cells[a]	% Ecotropic ICs detected on mink cells[a,b]
M-MuLV	Lymphocytic leukemia	5.2 ± 2.4[c]	0.15 ± 0.04	0.12 ± 0.06
F-MuLV	Erythroleukemia	<0.2	<0.002	<0.001
FM-I2	Lymphocytic leukemia	6.7 ± 1.5	0.35 ± 0.08	0.15 ± 0.09

[a] The data represent the IC titers of foci on mink cells as percentages of the titers in parallel assays using NIH 3T3 cells.
[b] Assays were performed with MAb 538 for M-MuLV or with MAb 48 for F-MuLV and FM-I2.
[c] The average percentage ± the SEM determined in assays of thymocytes from 8 to 12 mice 40 to 50 days after neonatal inoculation.

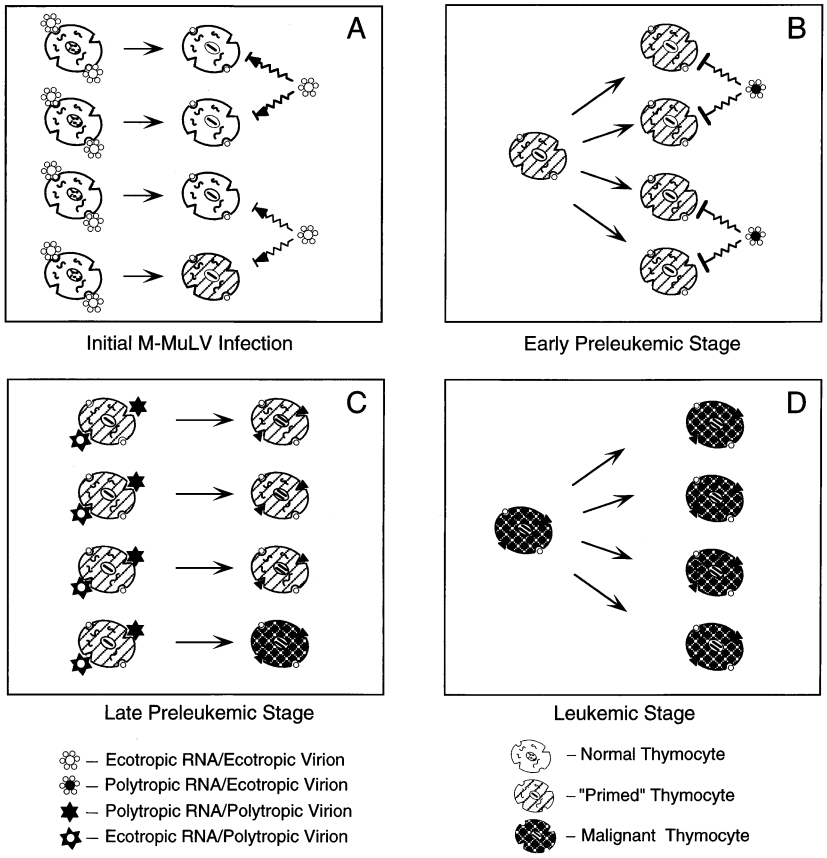

FIG. 10. Multistep process of leukemogenesis involving the modulation of thymic infection by pseudotyping. The proposed model depicts a sequence of infection of the thymus whose tempo is modulated by viral interference and pseudotyping. (A) Ecotropic M-MuLV infects normal thymocytes between 1 and 2 weeks postinoculation via the ecotropic receptor. The infected cells are blocked for reinfection by ecotropic viral interference. An event occurs in some infected cells (primed thymocytes) which is necessary, but not sufficient, for leukemogenesis. The rapid rate of infection may allow a high multiplicity of infection (indicated in the figure by the simultaneous infection of normal thymocytes), which would increase the probability of the occurrence of the initial event in leukemogenesis. (B) The primed thymocytes proliferate during the early preleukemic period (2 to 5 weeks postinoculation), increasing the number of target cells for a second event. Polytropic MuLVs which are detected during this time are pseudotyped within ecotropic virions and unable to infect primed thymocytes because of interference. (C) Polytropic virions containing polytropic or ecotropic RNA genomes emerge during the late preleukemic period (5 to 7 weeks). These virions superinfect primed thymocytes via the polytropic receptor. A second event occurs in a small number of superinfected primed thymocytes to confer full malignancy. (D) The malignantly transformed thymocyte proliferates to generate thymomas.

curs rapidly between 7 and 14 days after inoculation (18, 26, 27). Furthermore, polytropic MuLVs are not detected until after the massive early ecotropic MuLV infection of the thymus occurs, yet the generation of polytropic MuLVs appears to be necessary for the efficient induction of leukemia (4). This implies that the initial infection by M-MuLV does not result in malignant transformation and that all tumors would exhibit both ecotropic and polytropic MuLV integrations. In this regard, all of the seven tumors we have analyzed in this study and five additional tumors examined but not presented here appear to have multiple polytropic MuLV integrations. This suggests that polytropic infection occurred even three or four cellular divisions after transformation, it would not be detected as a discrete integration.

It has been suggested that malignant transformation may occur in a stepwise fashion in murine leukemias. O'Donnell et al. (38, 39), in studies of lymphomas induced by inoculation of polytropic viruses in AKR mice, demonstrated clonal populations of nonmalignant thymus cells in the preleukemic stage of disease (39) which exhibit a low frequency of c-*myc* activation compared with that of tumors from leukemic mice (38). It was suggested that the activation of uncharacterized genes may induce a preleukemic proliferative stage of disease (39) and that a subsequent integration would confer full malignancy. The early rapid infection of thymuses may be necessary for the rapid onset of leukemia in M-MuLV-infected mice (18, 26, 27) but apparently is not sufficient for rapid onset. A mutant which exhibits an early rapid infection but does not generate polytropic MuLVs fails to induce leukemia (4). If both early ecotropic infection and generation of polytropic MuLVs are required, a two-step mechanism is implied, although both steps in this case do not necessarily involve genetic alteration.

If M-MuLV-induced leukemia involves more than one genetic alteration, the change in pseudotyping we have observed could have a profound effect. The probability of more than one specific genetic event occurring simultaneously in the same cell is extremely low. However, genetic events which occur in a sequential fashion allow for the expansion of primed target cells, particularly if the initial event confers even a small selective growth advantage. A primed target cell which has undergone only 10 cellular divisions would expand the target cell pool over 1,000-fold and greatly enhance the probability of more than one genetic alteration in the same cell. Figure 10 depicts a hypothetical stepwise mechanism of leukemogenesis, illustrating the possible role of pseudotyping. In this scheme an initial burst of ecotropic infection increases the probability of

an event, perhaps a specific integration, which confers a proliferative advantage to the cells but not full malignancy. These primed target cells expand during the early preleukemic period at a time when polytropic viruses are nearly completely pseudotyped within ecotropic virions and are unable to infect ecotropic MuLV-infected thymocytes because of ecotropic viral interference. The abrupt appearance of virions which utilize the polytropic receptor later in the preleukemic stage circumvents ecotropic interference and enables a wave of superinfection of thymocytes. The rate of infection and the expanded population of primed target cells vastly increase the probability of a second event necessary for the development of frank lymphoma. The genome which is integrated in this step could be either polytropic or ecotropic, since both exhibit an increase in levels of packaging within polytropic virions (Fig. 3 and 4) and would be able to superinfect ecotropic MuLV-infected primed target cells.

A prediction of the two-step hypothesis presented above would be the existence of a population of M-MuLV-infected, polytropic MuLV-uninfected cells in preleukemic mice corresponding to primed thymocytes. We found no evidence for such a population in our analyses of total thymus DNA; however, as noted earlier, a limited expansion of such a population would have a profound effect on the efficiency of a two-step mechanism. Analyses of fractionated pools of thymocytes during the preleukemic period may provide an approach to this question.

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