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Regions of the Moloney Murine Leukemia Virus Genome Specifically Related to Induction of Promonocytic Tumors

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Moloney murine leukemia virus (MuLV) can be a potent inducer of promonocytic leukemias in mice that are undergoing a chronic inflammatory response. The neoplasms are, at least in part, associated with insertional mutagenesis of the c-*myb* locus. Evidence is presented for the existence of at least two genetic elements of the virus that are crucial to induction of this disease but are not required for viral replication in hematopoietic tissues or induction of lymphoid disease. These genetic elements were detected by testing the pathogenicity of recombinants between Moloney and Friend MuLVs, the latter of which is nonleukemic to myeloid cells under these conditions, and by testing Moloney MuLV-based viruses that have nonretroviral sequences inserted at specific endonuclease sites in their long terminal repeats (LTRs). Analysis of the Moloney/Friend recombinants showed that there are sequences within the structural gene domain of Moloney, but not Friend, MuLV that are necessary for promonocytic leukemia, whereas the LTRs of the MuLVs are equally effective for promonocytic tumor formation and insertional mutagenesis of the c-*myb* gene. Experiments with viruses which were mutagenized in the LTR by insertions demonstrated that there is a specific genetic element in the U3 region of the LTR of Moloney MuLV, upstream of the 75-base-pair enhancer which, when interrupted, results in loss of leukemogenicity for cells in the monocytic lineage but not cells in the lymphoid lineage. We conclude, therefore, that promonocytic leukemia induction, in Moloney MuLV-infected mice undergoing a chronic inflammatory response, requires specific sequences in the structural gene region of Moloney MuLV as well as other sequences in the regulatory region of the virus.

Rapid and reproducible induction of promonocytic leukemia in BALB/c and DBA/2 mice by Moloney murine leukemia virus (Moloney MuLV) has been reported (19, 20). These neoplasms, called MML (for Moloney-induced myeloid leukemia), are a consequence of intravenous inoculation of the virus in conjunction with intraperitoneal injection of pristane. Tumor cells become detectable in the peritoneal cavity during the acute phase of the disease, and their development has been demonstrated to be dependent on the pristane-induced inflammatory granuloma. These tumors appear to be identical to other tumors reported in pristane-treated mice that had been inoculated with Moloney MuLV in conjunction with Abelson MuLV (7, 9, 13); however, our previous studies and those of others indicate that Abelson MuLV was not involved in the development of these neoplasms (7, 14).

All Moloney-MuLV-related promonocytic tumors tested to date have two or more proviruses present in their genomes (unpublished data; 7), and one is consistently found to be integrated into the c-*myb* locus, causing alternatively spliced RNAs and truncation of the c-*myb* gene product at the amino terminus (7, 14).

Recent experiments in our laboratory indicate that another replication-competent virus, Friend MuLV, does not produce monocytic leukemia under conditions identical to those described above. This led us to consider the possibility that specific viral sequences found in some replication-competent viruses, but not others, may be required for induction of promonocytic tumors. The analysis of such regions should assist in the understanding of the neoplastic process associated with this leukemia as well as provide insight into functions of retroviral genes. In order to begin to determine genetic elements of Moloney MuLV that may be critical to MML induction, the pathogenic effects of reciprocal recombinants of Friend and Moloney MuLVs and recombinant Moloney-based MuLVs that have alterations in their long terminal repeats (LTRs) were tested. These experiments, which are presented here, indicate that there are two elements in the genome that may be critical to the specific induction of these myeloid leukemias.

MATERIALS AND METHODS

Viruses and animal experiments. Friend MuLV was molecularly cloned and transfected into cells by Oliff et al. (10). Moloney MuLV was prepared from a molecular clone (15), as previously described (19). Both viruses were propagated in NIH 3T3 cells. FM-I2 (1) and MF-3 (2) MuLVs, referred to in this report as FM-MuLV and MF-MuLV, were kindly provided by N. Hopkins (Massachusetts Institute of Technology) and J. Hartley (National Institute of Allergy and Infectious Diseases) as chronically infected Sc-1 and NIH 3T3 cells, respectively. Mo+PyF101 and PyF101+Mo MuLVs (3, 4) and M-MuLV^{sup} (11) were kindly provided by H. Fan (University of California, Irvine) and R. Jaenisch, respectively, as chronically infected NIH 3T3 cells. Cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

For leukemogenesis studies, 4- to 7-week-old female BALB/cAnPt mice from Hazelton Laboratories (contract NO1 CB-710-85; National Cancer Institute) were primed by intraperitoneal injection with 0.5 ml of pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.), and after 3 or 4 weeks, they were inoculated intravenously (i.v.) through the tail vein with 5×10^5 to 5×10^6 PFU of virus as determined by the XC plaque assay described below. Mice were monitored by preparing smears of cells from the peritoneal cavity fluid and staining them with Diff-Quik (American Scientific Products, McGaw Park, Ill.). Mice developed either

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TABLE 1. Induction of tumors by recombinant MuLVs[a]

Virus[b]	Values for:			
	Promonocytic tumors		Lymphoid tumors	
	Incidence (%)[c]	Latency (avg)[d]	Incidence (%)[c]	Latency (avg)[d]
M-MuLV Experiment 1	14/26 (54)	53–102 (71)	2/26 (8)	102–113 (107)
M-MuLV Experiment 2	6/13 (46)	85–112 (106)	4/13 (30)	90–141 (113)
F-MuLV	0/25 (0)	NA[e]	0/25 (0)	NA
MF-MuLV	15/25 (60)[f]	63–137 (113)	1/25 (4)	98
FM-MuLV	0/23 (0)	NA	17/23 (74)	98–122 (111)
M-MuLV^{sup}	0/33 (0)	NA	11/33 (33)	112–166 (146)
PyF101+Mo M-MuLV	0/30 (0)	NA	2/30 (7)	161 (161)
Mo+PyF101 M-MuLV	0/21 (0)	NA	0/21 (0)	NA

[a] All mice were treated intraperitoneally with pristane and inoculated i.v. with virus as described in Materials and Methods. Mice were observed for 6 months except for mice in experiment 1, inoculated with Moloney MuLV, which were observed for 4 months.
[b] For the amount of virus inoculated, see Table 2. For viruses not shown in Table 2, the amount ranged from 5×10^5 to 5×10^6 PFU per mouse. M-MuLV, Moloney MuLV. F-MuLV, Friend MuLV.
[c] Values for incidence are given as the number of mice with tumors to the number of mice inoculated with virus.
[d] Latency postinfection is expressed in range of days, with average latency shown in parentheses.
[e] NA, Not applicable.
[f] Promonocytic tumors induced by MF-MuLV are called MF-ML.

promonocytic or lymphoid tumors, and these tumor types were diagnosed as follows. Promonocytic tumors were diagnosed by characteristic morphology in smears (which at the time of acute disease was a very homogenous population of cells) (19), by massive tumor formation which emerged as an outgrowth of the oil-induced granuloma tissue, and by lack of an enlarged mesenteric lymph node, thymus, or other lymph nodes. Lymphoid tumors were diagnosed by the presence of an enlarged mesenteric node, other enlarged lymph nodes, enlarged spleen, and enlarged thymus (all the mice with FM-MuLV-induced tumors had an enlarged thymus but the only mouse with a lymphoid tumor induced by the MF-MuLV did not).

Quantitation of virus and infectious centers. Viruses were quantified by using the UV-XC syncytial plaque assay (12). For quantitation of infectious centers (number of cells producing virus), spleen or bone marrow cells were plated onto NIH 3T3 cells; the number of infected cells was subsequently determined by the UV-XC plaque assay.

Southern blot hybridization. DNA was extracted and analyzed by Southern blot hybridization as previously described (19). The Friend virus U3 LTR probe used was a 319-bp fragment of DNA from a *Hin*fI site to a *Bgl*II site, beginning 56 bp upstream of the LTR and including approximately 260 bp of LTR sequence. This probe is specific for Friend MuLV and does not cross-hybridize under stringent conditions to endogenous or Moloney MuLVs (unpublished data). The locations of c-*myb* probes A and B are shown (see Fig. 3).

RESULTS

Leukemogenesis studies with Friend MuLV and Friend/Moloney reciprocal recombinants. In order to determine if Moloney MuLV was unique in causing promonocytic tumors, another virus, Friend MuLV, was tested for its ability to cause tumors in pristane-primed adult BALB/c mice. This virus was chosen because it was known to replicate well in myeloid lineage cells and to cause myeloid neoplasms under certain circumstances (8, 17). Surprisingly, as shown in Table 1, Friend MuLV was nonpathogenic when inoculated intravenously in adult BALB/c mice treated with pristane. For this experiment, mice were given 0.5 ml of pristane intraperitoneally and then 3 weeks later were inoculated i.v. with 0.5 ml of culture fluid containing more than 10^6 PFU of Friend MuLV. None of the 25 mice inoculated with virus developed myeloid disease, even though the same procedure with Moloney MuLV resulted in a 46 to 54% incidence of myeloid disease.

Because Friend MuLV was nonpathogenic to monocytic cells under these conditions, it was possible to utilize recombinant viruses containing sequences from both Friend and Moloney MuLVs to begin to determine areas of the Moloney MuLV genome that make it particularly well suited for induction of promonocytic tumors. MF- and FM-MuLVs, recombinant viruses that have a reciprocal exchange of LTR and structural genes between Moloney MuLV and Friend MuLV (Fig. 1), were kindly provided by Nancy Hopkins and Janet Hartley. These were tested in mice with the same pristane-priming conditions under which Moloney and Friend MuLVs had been tested, and the results of the experiment are shown in Table 1. MF-MuLV, which has the Friend sequences downstream from the *Cla*I site in the *env* gene, including the LTR, and Moloney structural genes up to the *Cla*I site, produced promonocytic tumors in 60% of the mice tested, indicating that the structural gene(s) of Moloney MuLV and not the promotor-enhancer region of the virus was required for monocytic tumor induction. These critical sequences are not present in Friend MuLV, since the reciprocal recombinant, FM-MuLV, which has the Friend structural genes, did not produce promonocytic tumors. The latter virus caused exclusively lymphoid disease, which was not surprising because Moloney MuLV causes lymphoid as well as promonocytic disease in adult BALB/c mice (Table 1), and the lymphoid specificity of Moloney MuLV has been shown to be determined by the LTR (1, 2, 6).

Morphologically, the promonocytic tumors induced by the MF-MuLV (called MF-ML) resembled the monocytic MMLs previously shown to be induced by Moloney MuLV. Because the MMLs were shown to consistently have proviral integrations within the c-*myb* locus (9, 13, 14; unpublished data), the MF-ML tumors were examined for the presence of a rearranged 10.8-kilobase *Bam*HI c-*myb* fragment (all Moloney MuLV proviruses previously mapped in

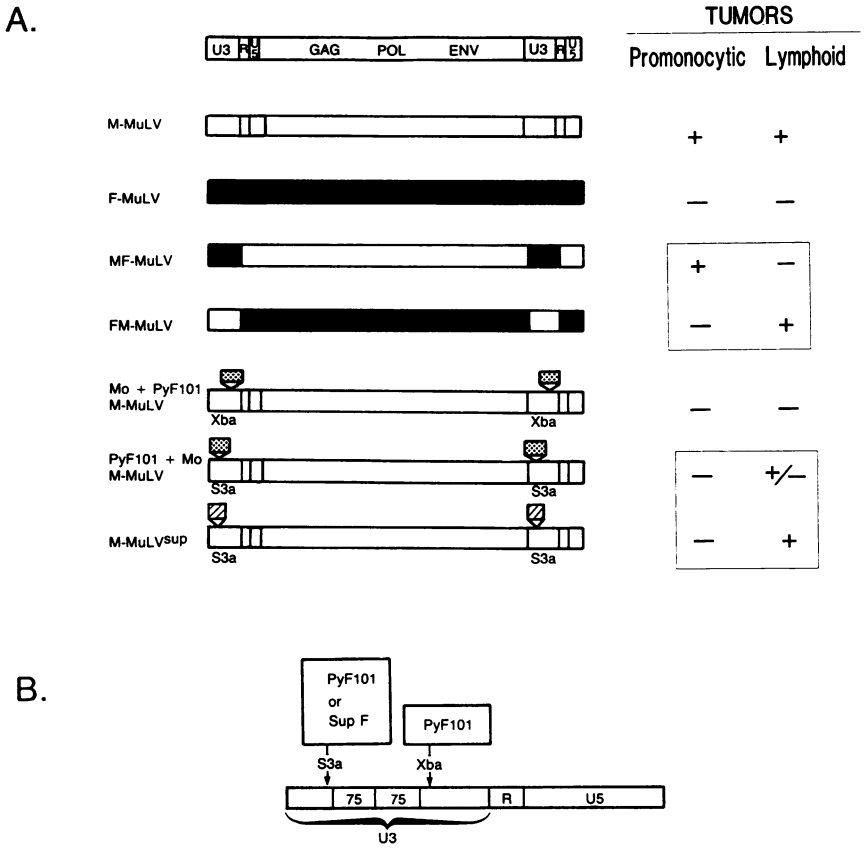

FIG. 1. Genetic composition of recombinant retroviruses used in leukemogenesis studies. (A) Organization of viruses and summary of results of pathogenesis experiments. (B) Diagram of the Moloney LTR showing locations of inserted sequences relative to the 75-bp repeat. Symbols: □, Moloney MuLV sequences; ■, Friend MuLV sequences; ▩, polyomavirus enhancer sequences; ▨, *supF* sequences. S3a and Xba, *Sau*3AI and *Xba*I restriction endonuclease sites, respectively. Detailed results of leukemogenesis experiments are given in Table 1.

MML tumors were found to be located within this fragment). As demonstrated in Fig. 2, 7 of 9 MF-ML DNAs examined had a rearranged c-*myb* allele. In most instances, the rearranged allele was smaller than the normal allele because of the presence of *Bam*HI sites in the genome of Moloney MuLV. Two tumors (26A1-8 and 26A2-21), however, had a larger rearranged band, indicating that the viral *Bam*HI sites were lost through deletion. Two monocytic tumors did not have a rearranged c-*myb* fragment. One of these tumors (26B2-18) had a different morphology from the other tumors in this experiment; the cells had a more mature macrophage phenotype resembling tumors previously obtained in our laboratory after infection of mice with a c-*myc*-containing retrovirus vector (18, 19). This FM-ML tumor may, therefore, have arisen by a different mechanism. The FM-ML tumor, 26B2-17, does not appear to have any integrated proviral DNA as indicated by Southern blot analysis with the virus-specific probes used as described below (data not shown). This tumor may have been produced spontaneously by a mechanism which did not involve virus integration.

The MF-MLs in which c-*myb* rearrangements could be detected were examined for the presence of MF-MuLV proviral integrations by digesting tumor DNA with *Eco*RI, which does not cut within the virus, and hybridizing the subsequent blot with a probe from the U3 region of the Friend MuLV LTR (probe described in Materials and Methods). All of the MF-MLs had multiple proviral integrations (Fig. 3, panel A). For each tumor, one of the integrations could be correlated in size with a rearrangement of one of two c-*myb* *Eco*RI fragments as shown in the additional panels of the same figure. Panels B and C of Fig. 3 were prepared by rehybridizing the blot in panel A with c-*myb* probe A or B, respectively.

The location of provirus integrations in six tumors is indicated in Fig. 4. The three downstream proviruses, located within the 4.2-kilobase *Eco*RI fragment, were mapped to specific sites by using *Cla*I plus *Eco*RI-digested DNA and hybridization with c-*myb* probe A (data not shown). The *Cla*I site is approximately 700 bp upstream from the 3′ end of the virus; therefore, the distance of the provirus from the *Eco*RI site can be calculated by subtracting 700 bp from the size of the *Cla*I-*Eco*RI band.

It was concluded from the testing of Friend/Moloney recombinants that Friend MuLV, like Moloney MuLV, has an LTR that is functional for promonocytic leukemia induction but that unlike Moloney MuLV, it is lacking something present in the structural gene region which is required for disease.

Tumor induction studies utilizing Moloney MuLV-based recombinants containing insertions in the LTR. Although the Moloney MuLV LTR can be substituted by at least one other LTR and is not necessarily unique in its ability to function in promonocytic leukemia induction, it is still possible that this LTR and other MuLV LTRs have proper-

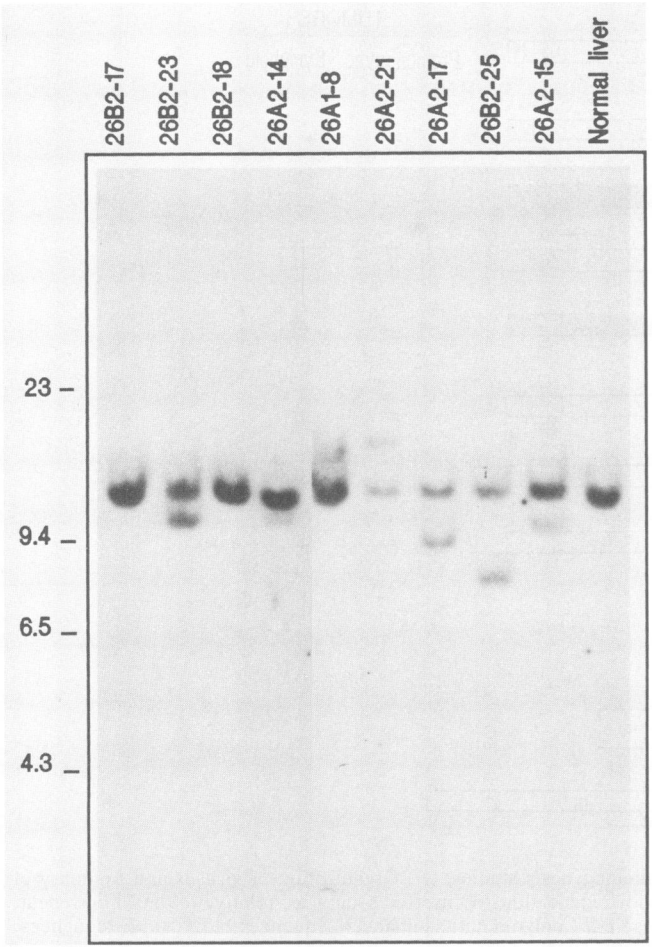

FIG. 2. c-*myb* rearrangements in MF-ML tumors. Tumor DNAs were extracted and digested with *Bam*HI. They were then separated on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled c-*myb* probe A (*Bgl*II fragment; see the map of the murine c-*myb* locus in Fig. 4).

ties contained within them which are specific for promonocytic disease. In an attempt to address this issue, three viruses having insertions of nonretroviral sequences were tested for pathogenicity in pristane-primed adult mice. Two of the viruses, Mo+PyF101 MuLV and PyF101+Mo MuLV, were generously provided by Hung Fan (4), and the M-MuLV^{sup} was kindly provided by Rudolf Jaenisch (11). Two of the constructed viruses had either sequences from the polyomavirus enhancer or *supF*, the amber suppressor gene, inserted at the *Sau*3AI site 13 bp upstream of the 75-bp repeat region (Fig. 1, A and B). These viruses, called PyF101+Mo MuLV and M-MuLV^{sup}, did not induce promonocytic tumors when injected into pristane-primed adult mice, as shown in Table 1, but were still capable of causing lymphoid tumors. For this experiment, the mice were inoculated i.v. with 2.0×10^6 to 11×10^6 PFU per mouse, amounts comparable to the amounts of Moloney MuLV inoculated in previous experiments. The M-MuLV^{sup} produced lymphoid tumors at a rate comparable to that of production by Moloney MuLV (for example, see Table 1, M-MuLV Experiment 2), and this virus replicated well in the mice. The ability to replicate was demonstrated through the use of infectious center assays which were carried out on bone marrow and spleen cells of M-MuLV^{sup}-infected mice

TABLE 2. Replication of viruses in hematopoietic tissues of mice[a]

Virus (PFU[b] injected per mouse)	Animal no.	Infectious centers[c] per 10^5 cells	
		Bone marrow	Spleen
Moloney MuLV (4.4×10^6)	1	34,000	4,800
	2	61,000	25,000
	3	17,000	71,000
M-MuLV^{sup} (1.1×10^7)	1	5,700	2,000
	2	11,100	630
	3	2,000	6,500
PyF101+Mo MuLV (2.0×10^6)	1	450	25
	2	2	60
	3	3,000	2,600
	4	28	5
Mo+PyF101 MuLV (1.3×10^7)	1	<1[d]	<1
	2	<1	<1
	3	<1	<1

[a] Mice were treated with pristane 3 weeks prior to inoculation of virus, and assays were performed 3 weeks postinoculation of virus.
[b] PFU were determined in the UV-XC plaque assay.
[c] Infectious centers were determined with the UV-XC plaque assay.
[d] No XC plaques were detected when 10^5 cells were plated. Therefore, if there were any infected cells in the tissues, there had to be less than 1 in 10^5.

(Table 2). Mice infected with PyF101+Mo MuLV had a lower incidence of lymphoid tumors than mice infected with Moloney MuLV had, but this could be explained by the fact the virus replicated poorly in mice. Only one of four mice infected with the virus demonstrated significant levels of infectious centers in their bone marrow or spleen. We concluded from the animal experiments with M-MuLV^{sup} and PyF101+Mo MuLV as infectious agents in pristane-primed mice that disruption of the sequences around the *Sau*3AI site results in loss of leukemogenicity for cells in the myeloid lineage but not for cells in the lymphoid lineage.

Another virus that was tested in pristane-primed BALB/c mice, Mo+PyF101 MuLV, was nonpathogenic. It was subsequently determined that the virus is nonpathogenic in BALB/c mice because it does not replicate in the hematopoietic organs of these mice. Virus was not detected in 10^5 spleen or bone marrow cells plated in an infectious center assay (Table 2). The BALB/c strain may be unique in resisting the replication of this virus, since, in previous studies in another laboratory (3), this virus was shown to replicate in hematopoietic tissues of NIH Swiss mice.

DISCUSSION

The present study indicates for the first time that the Moloney murine leukemia genome can lose its ability to induce promonocytic tumors (MML) while remaining capable of replicating and inducing lymphoid tumors. This suggests that some genetic elements of the viruses are specifically related to induction of promonocytic tumors or to tumors in the myeloid lineage.

Experiments with Friend/Moloney recombinant viruses show that Moloney MuLV has a genetic element(s) in the structural gene region of the virus, (including GAG, POL, and ENV) that is not found in Friend MuLV but which is required for MML disease induction. At present, it is unknown what these genetic elements are, but it could be speculated that they are required for activation of the c-*myb* gene. The MF-ML promonocytic tumors derived from infection of mice with the MF-MuLV virus were shown to have

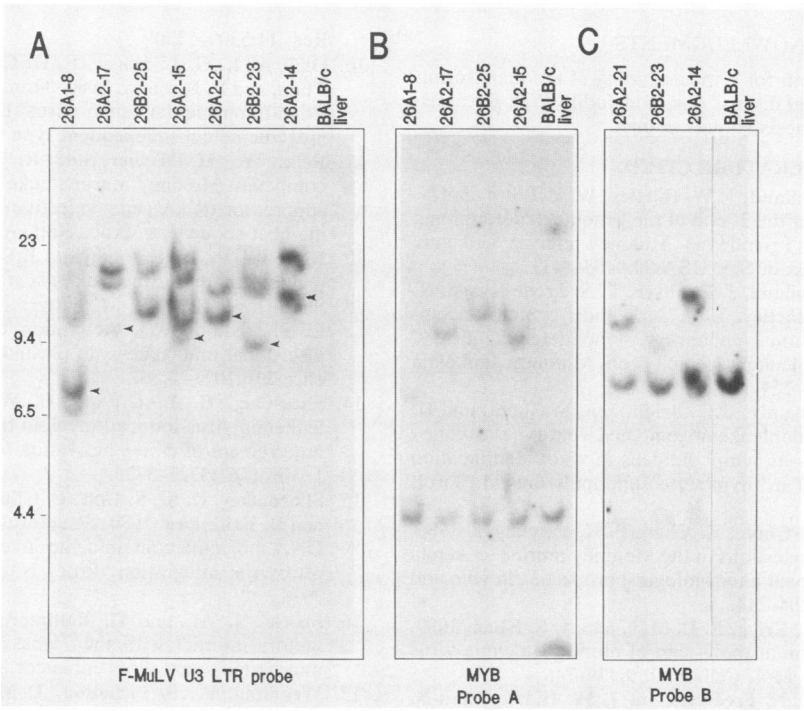

FIG. 3. Integrated proviruses in MF-ML tumors. Tumor DNAs were prepared for blot hybridization as described in Fig. 2. The same blot was rehybridized three separate times to obtain panels A, B, and C. (A) Friend MuLV U3 LTR probe (described in Materials and Methods). (B) c-*myb* probe A. (C) c-*myb* probe B (see Fig. 3 for location of probes derived from the c-*myb* locus DNA). Arrows in panel A correlate bands with rearranged c-*myb* bands in either panel B or panel C.

insertions into the c-*myb* locus similar to those observed for promonocytic tumors derived by infection with Moloney MuLV.

The fact that Friend MuLV was nonpathogenic in pristane-primed mice was unexpected, since Friend MuLV replicates well in some myeloid lineage cells and can cause tumors in them as well (8, 17). Interestingly, this suggests that Friend MuLV could be utilized as a helper virus in pristane-treated adult BALB/c mice for experiments employing vectors containing other oncogenes. For experiments in which one wishes to inject the virus i.v., Friend MuLV would be particularly useful because it would not cause the monocytic and lymphoid tumors observed when Moloney MuLV is inoculated i.v.

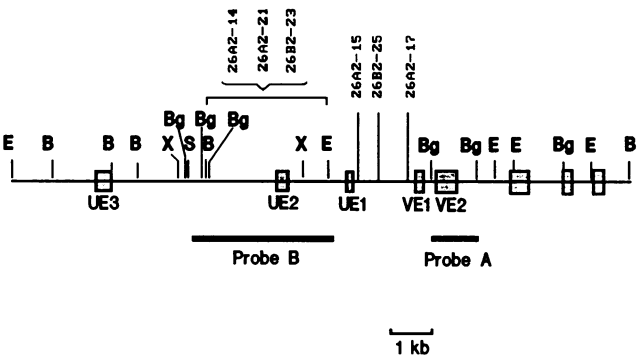

FIG. 4. Map of the murine c-*myb* locus. The map is based on those described by Lavu and Reddy (9) and Gonda et al. (7). c-*myb* probes A and B that were used for Southern blot hybridization (Fig. 2 and 4) are depicted below the map. Location of MF-MuLV provirus insertions in six MF-ML tumors are shown above the map. Restriction endonuclease sites are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sac*I; X, *Xba*I.

Additional experiments presented in this study show that PyF101+Mo MuLV was nonpathogenic to myeloid cells of BALB/c mice, even though it had been shown by Fan et al. (4) to be very pathogenic for both lymphoid and myeloid cells in newborn NIH Swiss mice. In fact, the lack of myeloid pathogenesis associated with this virus in BALB/c mice that have been pristane primed supports the observation made with M-MuLV^{sup} that promonocytic tumor induction in pristane-treated BALB/c mice requires maintenance of the region around the *Sau*3AI site upstream of the 75-bp repeat enhancer region. At present, it is unknown why this genetic region is important. It is upstream from the region where most investigation has been carried out in regard to enhancers and protein binding domains (16) of the Moloney MuLV LTR, although recently a regulatory region at the very 5′ end of retrovirus LTRs has been demonstrated by Flanagan et al. (5). It could be speculated that other enhancers or transcriptionally important sequences required for transcription in early myeloid cells may be found in this region. It should be noted that we cannot rule out the possibility that the inserted sequences themselves directly produce a negative effect on the potential of the virus to cause myeloid disease.

An interesting observation that was derived from this study was that the Mo+PyF101 MuLV was unable to replicate in mouse spleen and bone marrow cells, even though it replicated well in NIH 3T3 cells and 10^7 PFU were injected per mouse. Further studies will be required to determine the cause of this block to replication. At present, we do not know if it is a species-related or a tissue-specific phenomenon.

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