Escape from In Vivo Restriction of Moloney Mink Cell Focus-Inducing Viruses Driven by the Mo+PyF101 Long Terminal Repeat (LTR) by LTR Alterations

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Mo+PyF101 M-MuLV is a variant Moloney murine leukemia virus containing polyomavirus F101 enhancers inserted just downstream from the M-MuLV enhancers in the long terminal repeat (LTR). The protein coding sequences for this virus are identical to those of M-MuLV. Mo+PyF101 M-MuLV induces T-cell disease with a much lower incidence and longer latency than wild-type M-MuLV. We have previously shown that Mo+PyF101 M-MuLV is defective in preleukemic events induced by wild-type M-MuLV, including splenic hematopoietic hyperplasia, bone marrow depletion, and generation of recombinant mink cell focus-inducing viruses (MCFs). We also showed that an M-MCF virus driven by the Mo+PyF101 LTR is infectious in vitro but does not propagate in mice. However, in these experiments, when a pseudotypic mixture of Mo+PyF101 M-MuLV and Mo+PyF101 MCF was inoculated into newborn NIH Swiss mice, they died of T-cell leukemia at times almost equivalent to those induced by wild-type M-MuLV. Tumor DNAs from Mo+PyF101 M-MuLV–Mo+PyF101 MCF-inoculated mice were examined by Southern blot analysis. The predominant forms of Mo+PyF101 MCF proviruses in these tumors contained added sequences in the U3 region of the LTR. The U3 regions of representative tumor-derived variant Mo+PyF101 MCFs were cloned by polymerase chain reaction amplification, and sequencing indicated that they had acquired an additional copy of the M-MuLV 75-bp tandem repeat in the enhancer region. NIH 3T3 cell lines infected with altered viruses were obtained from representative Mo+PyF101 M-MuLV–Mo+PyF101 MCF-induced tumors, and mice were inoculated with the recovered viruses. Leukemogenicity was approximately equivalent to that in the original Mo+PyF101 M-MuLV–Mo+PyF101 MCF viral stock. Southern blot analysis on the resulting tumors now predominantly revealed loss of the polyomavirus sequences. These results suggest that the suppressive effects of the PyF101 sequences on M-MuLV-induced disease and potentially on MCF propagation were overcome in two ways: by triplication of the M-MuLV direct repeats and by loss of the polyomavirus sequences.

Replication-competent (nonacute) retroviruses such as Moloney murine leukemia virus (M-MuLV) induce leukemias with a long latency. Work by many investigators has identified several important features of MuLV leukemogenesis (reviewed by Fan et al. [14]). These include (i) transcriptional activation of cellular proto-oncogenes by enhancers in the MuLV long terminal repeat (LTR) (7, 8, 15, 24, 29) and (ii) generation of mink cell focus-inducing virus (MCF) recombinants in the animal during the leukemogenic process (7, 12, 13, 24). MCFs are *env* gene recombinants between infecting MuLV and endogenous polytropic MuLV-related viruses (12, 26). Several experiments have indicated the importance of MCF recombinants in MuLV leukemogenesis (4, 5, 7, 24).

Our studies of M-MuLV leukemogenesis have been facilitated by an LTR variant, Mo+PyF101 M-MuLV. This virus contains enhancer sequences from the F101 strain of polyomavirus inserted downstream of the M-MuLV enhancers (9, 22). We previously showed that Mo+PyF101 M-MuLV has greatly decreased leukemogenicity (4, 9). Comparative studies with this virus indicated that wild-type M-MuLV induces a preleukemic state of hematopoietic hyperplasia of the spleen which may result secondarily from defects in the bone marrow (10, 20, 21). The bone marrow defects may result from combined infection of stroma by M-MuLV and M-MCF (20). Mo+PyF101 M-MuLV does not induce early hematopoietic hyperplasia, which is in parallel with its low leukemogenicity (10).

We recently discovered that Mo+PyF101 M-MuLV does not generate MCF recombinants in vivo, which provided an explanation for its low leukemogenicity (4). This could result from (i) an inability of Mo+PyF101 M-MuLV to generate MCF recombinants at all or (ii) an inability of the Mo+PyF101 LTR to propagate MCF recombinants once they are generated. We therefore generated in vitro an M-MCF driven by the Mo+PyF101 LTR (Mo+PyF101 MCF) by molecular cloning and transfection in NIH 3T3 fibroblasts. When Mo+PyF101 MCF was injected into newborn mice, it failed to propagate (4), supporting the second possibility. Presumably, the transcriptional milieu of cells that propagate MCF viruses early during infection are incompatible with expression from the Mo+PyF101 LTR.

During experiments on Mo+PyF101 MCF, we observed that pseudotypic mixtures of Mo+PyF101 M-MuLV–Mo+PyF101 MCF showed significantly greater leukemogenicity than either virus alone. A study of this phenomenon is described here. The results provide information about the nature of secondary LTR alterations that allow Mo+PyF101 MCF viruses to escape restriction in the animal.

MATERIALS AND METHODS

Viruses and inoculation of mice. Mo+PyF101 M-MuLV and Mo+PyF101 MCF have been previously described (4). All viral stocks were culture supernatants derived from productively infected NIH 3T3 cells. 28T and 28S cell lines were established by overlay of tumor thymocytes and splenocytes,

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FIG. 1. (a) Proviral maps of Mo+PyF101 M-MuLV and Mo+PyF101 MCF. Mo+PyF101 M-MuLV contains the 199-bp *Pvu*II-4 enhancer fragment of polyomavirus F101 inserted at the *Xba*I site of the M-MuLV LTR U3 region downstream from the M-MuLV enhancers (22). For the Mo+PyF101 MCF construct, the *Xho*I-*Cla*I MCF envelope-containing fragment of Mo-MCF₁ (1) was substituted into Mo+PyF101 M-MuLV plasmid DNA at the corresponding restriction sites. X, *Xba*I; Xh, *Xho*I; Cl, *Cla*I. (b) Pathogenicity of Mo+PyF101 M-MuLV-Mo+PyF101 MCF in NIH Swiss mice. Mo+PyF101 M-MuLV-Mo+PyF101 MCF (●) was inoculated subcutaneously into neonatal NIH Swiss mice. Pathogenicity is shown in comparison with those of wild-type M-MuLV (■) and Mo+PyF101 M-MuLV alone (▲; Mo+PyF101 M-MuLV does not propagate MCFs in vivo [4]). The number of mice inoculated is indicated in parentheses.

respectively, onto NIH 3T3 cells in 2 μ g of Polybrene per ml for 24 h. The infected 3T3s were passed two times (in the presence of 20 μ g of Polybrene per ml for 1 h) to obtain confluent infected cultures. Titration of viral stocks was either by UV-XC plaque-forming assay (23) for ecotropic virus or by reverse transcriptase assay for MCFs (16). Titer of Mo+PyF101 M-MuLV-Mo+PyF101 MCF clone 3 viral stock was $\sim 1 \times 10^6$ reverse transcriptase-inducing units/ml, and for the ecotropic virus in the stock, titer was 8×10^5 XC PFU/ml. XC titers for the 28S and 28T cell lines producing altered Mo+PyF101 M-MuLV-Mo+PyF101 MCF were 5×10^3 and 9×10^2 , respectively. Viral stocks (0.2 ml) were inoculated subcutaneously into NIH Swiss mice 1 to 2 days after birth, giving 10^2 to 10^5 XC PFU per animal.

Southern blot analysis. DNA was obtained from tumors as described previously (2, 3). Southern blot procedures were performed by using Gene Screen Plus (New England Nuclear, Boston, Mass.) as previously described (4). Random primer 3' *env* probe was prepared by using the 1.1-kb *Bam*HI-*Cla*I fragment of M-MuLV by the standard protocol (28). Polyomavirus enhancer random primer probe was prepared from the *Pvu*II-4 polyomavirus F101 enhancer fragment inserted at the

*Xba*I site of Mo+PyF101 M-MuLV as previously described (22). For some analyses, a polyomavirus-specific oligomer from the B core of the polyomavirus enhancer region, 5'-CAGTGTGGTTTTGCAAGAGGAAGCAAAAAG-3', which was synthesized on an Applied Biosystems DNA synthesizer, was used. The oligomer was end labelled with [γ - 32 P]ATP and T4 polynucleotide kinase by standard protocols (27). Hybridization with this probe was performed at 55°C in 5 $\times$ Denhardt's solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400), 5 $\times$ SSPE (0.9 M NaCl, 50 mM Na₂H₂PO₄, 5 mM EDTA), 0.5% sodium dodecyl sulfate (SDS), 100 μ g of denatured salmon sperm DNA per ml, and 4×10^5 cpm/ml of probe. The blots were then washed in 6 $\times$ SSC (0.9 M NaCl plus 0.09 M sodium citrate) plus 0.5% SDS three times at room temperature for 15 min, once at 55°C for 1.5 min, and once at 55°C for 1 min; the blots were given a final wash in 2 $\times$ SSC for 2 min at room temperature.

PCR cloning and sequencing. The U3 region of the Mo+PyF101 M-MuLV LTR from selected tumors was amplified by the polymerase chain reaction (PCR) with forward and reverse primers spanning the *Cla*I and *Sac*I sites (5'-ATTCTT AATCGATTAGTCCA-3' and 5'-TTTTATTGAGCTCGGG

GAGC-3', respectively). Fifty picomoles of each primer was used in a 100- μ l volume containing 0.2 mM nucleoside triphosphates and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). After denaturation at 94°C for 5 min, samples were treated for 35 cycles in a thermal cycler at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min per cycle. Amplified fragments were digested with *Cla*I plus *Sac*I, cloned into a pBR-based vector to obtain the *Cla*I-adjacent *Hind*III site, recloned as a *Hind*III-*Sac*I fragment into PUC19, and then selected by colony hybridization to the polyomavirus enhancer probe. The *Cla*I-*Sac*I insert of these clones was of a size equivalent to that of the *Cla*I-*Sac*I tumor DNA band seen by Southern blot analysis with the polyomavirus enhancer probe. The insert was then sequenced by the dideoxy chain termination method by using [α -thio-³⁵S]dATP and Sequenase (U.S. Biochemicals, Cleveland, Ohio). In addition to M13 universal primers, the following primers were synthesized on an Applied Biosystems DNA synthesizer for use in sequencing of internal nucleotides: 5'-CCATTTTGAAGGCATGG-3' (bases 189 to 206 [see Fig. 3a]), 5'-GTCATAGTAATGATTGGGTGG-3' (antisense of bases 707 to 687), and 5'-AAAGAGGATGTGCGACGG-3' (antisense of bases 543 to 526).

RESULTS

Pathogenicity of Mo+PyF101 MCF–Mo+PyF101 M-MuLV.

The genomic organizations of Mo+PyF101 M-MuLV and Mo+PyF101 MCF are shown in Fig. 1a. Mo+PyF101 M-MuLV is an LTR variant of wild-type M-MuLV, in which enhancer sequences from the F101 mutant of polyomavirus were inserted into the *Xba*I site at –150 bp (22). This is downstream of the tandemly repeated 75-bp repeats which constitute the M-MuLV enhancers—there are two copies of the 75-bp repeats in the wild-type M-MuLV LTR. Mo+PyF101 MCF is an M-MuLV MCF containing the Mo+PyF101 LTR; this virus was generated by molecular cloning (4).

In our previous study, we reported that when Mo+PyF101 MCF was injected into newborn NIH Swiss mice (either alone or as a pseudotypic mixture with Mo+PyF101 M-MuLV), the virus failed to propagate as measured by Southern blot hybridizations at 6 to 8 weeks after inoculation (4). However, the mice inoculated with the pseudotypic mixture developed leukemia with markedly accelerated kinetics (average latency, 16 weeks) compared with that of the ecotropic Mo+PyF101 M-MuLV alone (latency, 36 weeks [Fig. 1b]). (Mice inoculated with Mo+PyF101 MCF alone did not develop disease.) Gross pathology at necropsy indicated enlarged thymuses, spleens, and lymph nodes typical of lymphoblastic lymphomas induced by wild-type M-MuLV. Southern blot analysis for T-cell receptor beta gene rearrangements (2, 18) indicated that the tumors were T lymphomas, similar to the disease induced by wild-type and (with lower efficiency) Mo+PyF101 M-MuLV (data not shown). These results indicated that while an MCF driven by the Mo+PyF101 LTR did not propagate efficiently in mice when injected alone, nevertheless it appeared to participate in leukemogenesis when injected as a pseudotypic mixture with Mo+PyF101 M-MuLV.

Southern blot analysis of tumor DNAs. One possible way to reconcile the inefficient propagation of Mo+PyF101 MCF in vivo with its ability to accelerate leukemogenesis could be the appearance of secondary LTR variants during growth in the animal. To investigate this, we carried out Southern blot hybridizations on tumor DNAs from animals injected with Mo+PyF101 M-MuLV–Mo+PyF101 MCF. We previously used digestion of tumor DNA with *Bam*HI plus *Xba*I and then

hybridization with an MCF-specific *env* probe to test for MCF proviruses in Mo+PyF101 M-MuLV or wild-type M-MuLV-infected animals (4). However, if the LTR were of increased size, the critical diagnostic band might comigrate with endogenous MuLV-related bands. We have found that use of a 3' ecotropic *env*-specific probe reduces the number of background bands in the region of interest. In addition, other diagnostic digests and hybridization probes were used. The first analysis used *Bam*HI plus *Xba*I digestion and then hybridization with the 3' *env* probe as shown in Fig. 2a and b. Because of a difference in location of the *env* region *Bam*HI site, as seen in Fig. 2a, *Bam*HI-*Xba*I digestion will yield a 2.3-kb fragment from an Mo+PyF101 MCF provirus and a 1.5-kb fragment from ecotropic Mo+PyF101 M-MuLV. As shown in Fig. 2b, MCF proviruses (fragments in the 2.3-kb range) showed a diagnostic band of 2.4 kb (five of six) in addition to the input 2.3-kb band (four of six). Ecotropic proviruses of equivalent increased size were also observed in the 1.5-kb range. For both MCF and ecotropic tumor proviruses, diagnostic fragments increased by ~100 bp were the predominant species.

One possible explanation for these results could have been the acquisition of an additional copy of the M-MuLV 75-bp tandem repeats in the Mo+PyF101 LTR, yielding an LTR with three M-MuLV tandem repeats upstream of the polyomavirus enhancers. Correlation between number of tandem repeats in the LTR and leukemogenicity has been reported by others (19). Since the polyomavirus sequences in the Mo+PyF101 LTR exerted a dominant negative effect (inhibition of MCF propagation, reduction in leukemogenicity [4]), it seemed possible that an additional M-MuLV tandem repeat might counteract it. We therefore examined the enhancer regions of the proviruses in the induced tumors by *Sau*3a plus *Sac*I digestion and then by hybridization with the polyomavirus-specific probe (Py) (Fig. 2a and c). This digest yielded relatively small fragments (500 bp for both Mo+PyF101 MCF and for Mo+PyF101 M-MuLV) which allowed more accurate determination of size differences. Since the polyomavirus enhancer is inserted at the *Xba*I site upstream from the *Sac*I site, this analysis also allowed us to investigate retention of polyomavirus enhancer sequences in the tumor proviruses. Five of six tumors showed a predominant polyomavirus-specific *Sau*3a-*Sac*I fragment 70 to 100 bp larger than the fragment from the original Mo+PyF101 LTR, consistent with acquisition of an additional copy of the M-MuLV 75-bp tandem repeat. In addition, one of these tumors (tumor 1) showed yet an additional fragment that might reflect an LTR with four copies of the M-MuLV 75-bp repeat.

We also performed Southern analysis with a *Sac*I digest and the 3' *env*-specific probe, as shown in Fig. 2d. As seen in Fig. 2a, this digest distinguishes Mo+PyF101 MCF from Mo+PyF101 M-MuLV proviruses, as is the case for the *Xba*I-*Bam*HI digest. The diagnostic band for the input MCF is 1.7 kb, while that of the input ecotropic virus is 6.0 kb. As for the *Xba*I-*Bam*HI analysis, in addition to diagnostic bands equivalent to the input virus, both MCF and ecotropic proviruses of increased size compared with that of input viruses were seen. For some of the tumors (tumors 2, 3, and 6), bands of faster mobility were also observed, which is indicative of deleted proviruses. Most of these bands also hybridized with the polyomavirus-specific probe (data not shown). However, for tumor 6, this MCF-related band did not hybridize with the polyomavirus probe (data not shown). Thus, it resulted from deletion of polyomavirus sequences from the Mo+PyF101 LTR. Tumor 5 appeared to have deleted Mo+PyF101 MCF provirus altogether. In the *Bam*HI plus *Xba*I analysis (Fig. 2b), the *Xba*I site separates the polyomavirus enhancer sequences

FIG. 2. Southern blot analysis of tumor DNAs from Mo+PyF101 M-MuLV-Mo+PyF101 MCF-inoculated mice. (a) Restriction sites used in analysis of provirus in Mo+PyF101 M-MuLV-Mo+PyF101 MCF-induced tumors are shown for both MCF and ecotropic virus. The 3' *env* and LTR regions are identical for the two viral classes. B, *Bam*HI; Sc, *Sac*I; Sa, *Sau*3a; X, *Xba*I. The *Sac*I site in the *env* region of the MCF is absent from the ecotropic virus, and the *Bam*HI site differs by 800 bp for the two viruses. Also shown are polyomavirus enhancer (Py) and 3' *env* (*env*) region probes used in the analysis as described in Materials and Methods. (b) Southern blot analysis of tumor DNAs digested with *Bam*HI and hybridized with the 3' *env* probe. Tumor DNAs 1 to 6 (lanes 1 to 6, respectively) are shown in comparison to input Mo+PyF101 M-MuLV-Mo+PyF101 MCF (Input virus) and uninoculated mouse spleen (Uninoculated). Expected bands for input MCF (2.3 kb) and ecotropic provirus (1.5 kb) are indicated. Bands of a ~100-bp-increased size were seen for both MCF- and ecotropic-specific fragments. (c) *Sau*3a-*Sac*I digest with the polyomavirus enhancer-specific probe. Tumors 1 to 4 and 6 (lanes 1 to 4 and 6, respectively) exhibited bands of an ~75-bp increase in size compared with those of the producer cell line. (d) *Sac*I digest with the 3' *env*-specific probe. Input Mo+PyF101 MCF virus and Mo+PyF101 M-MuLV yield 1.7- and 6.0-kb diagnostic bands, respectively.

from the M-MuLV enhancer sequences; thus, Mo+PyF101 LTRs with deletions in the polyomavirus sequences would not have been distinct.

Ten different tumors were analyzed by the three digests of Fig. 2 as above. Provirus with added LTR sequences predominated in most tumors. Eight of 10 and 10 of 10 tumors contained Mo+PyF101 MCF and Mo+PyF101 M-MuLV proviruses, respectively, showing increased size in the LTR region. These LTRs retained polyomavirus sequences. Two of the 10 either contained no Mo+PyF101 MCF provirus or were deleted for the polyomavirus sequences of that virus (Fig. 2, e.g., tumor 6). In conclusion, the tumors arising from infection by

Mo+PyF101 M-MuLV-Mo+PyF101 MCF contained MCF proviruses with altered LTRs of two general varieties: those that acquired additional M-MuLV-related sequences in the enhancer region and those that deleted polyomavirus sequences. In a quantitative sense, the former class predominated.

Other digests were used to examine the U5 LTR region as well as the coding regions of the proviruses in the tumors (*gag*, *pol*, and *env*). The predominant proviruses in these tumors contained no differences in these regions in comparison to the parental Mo+PyF101 M-MuLV and Mo+PyF101 MCF proviruses (not shown). Thus, the changes were generally confined to the U3 region of the LTRs.

a	1						50
	Cla I						
	2873S	atcgattagt	ccaatttggt	aaagacagga	tatcagtggt	ccaggctcta	
	Mo+PyF101	ATCGATTAGT	CCAATTGTGT	AAAGACAGGA	TATCAGTGGT	CCAGGCTCTA	
	Consensus	ATCGATTAGT	CCAATTGTGT	AAAGACAGGA	TATCAGTGGT	CCAGGCTCTA	
	51						100
	2873S	gttttgactc	aacaatatca	ccagctgaag	cctatagagt	acgagccata	
	Mo+PyF101	GTTTTGACTC	AACAATATCA	CCAGCTGAAG	CCTATAGAGT	ACGAGCCATA	
	Consensus	GTTTTGACTC	AACAATATCA	CCAGCTGAAG	CCTATAGAGT	ACGAGCCATA	
	101						150
2873S	gataaaataa	aagattttat	ttagtctcca	gaaaaagggg	ggaatgaaag		
Mo+PyF101	GATAAAATAA	AAGATTTTAT	TTAGTCTCCA	GAAAAAGGGG	GGAATGAAAG		
Consensus	GATAAAATAA	AAGATTTTAT	TTAGTCTCCA	GAAAAAGGGG	GGAATGAAAG		
151						200	
2873S	accccacctg	taggtttggc	aagctagctt	aagtaacgcc	attttgcaag		
Mo+PyF101	ACCCACCTG	TAGGTTTGGC	AAGCTAGCTT	AAGTAACGCC	ATTTTGCAAG		
Consensus	ACCCACCTG	TAGGTTTGGC	AAGCTAGCTT	AAGTAACGCC	ATTTTGCAAG		
201						250	
2873S	gcatggaaaa	atacataact	gagaatagag	aagttcagat	caaggtcagg		
Mo+PyF101	GCATGGAAAA	ATACATAACT	GAGAATAGAG	AAGTTCAGAT	CAAGGTCAGG		
Consensus	GCATGGAAAA	ATACATAACT	GAGAATAGAG	AAGTTCAGAT	CAAGGTCAGG		
251						300	
2873S	aacagatgga	acagctgaat	atggggccaaa	caggatatct	gtggttaagca		
Mo+PyF101	AACAGATGGA	ACAGCTGAAT	ATGGGGCCAAA	CAGGATATCT	GTGGTAAGCA		
Consensus	AACAGATGGA	ACAGCTGAAT	ATGGGGCCAAA	CAGGATATCT	GTGGTAAGCA		
301						350	
2873S	gttcctgccc	cggtctcagg	ccaagaacag	atggaacagc	tgaatatggg		
Mo+PyF101	GTTCCTGCCC	CGGCTCAGGG	CCAAGAACAG	ATGGAACAGC	TGAATATGGG		
Consensus	GTTCCTGCCC	CGGCTCAGGG	CCAAGAACAG	ATGGAACAGC	TGAATATGGG		
351						400	
2873S	ccaaacagga	tatctgtggt	aagcagttcc	tgccccggct	cagggccaag		
Mo+PyF101	CCAAACAGGA	TATCTGTGGT	AAGCAGTTCC	TGCCCCGGCT	CAGGGCCAA		
Consensus	CCAAACAGGA	TATCTGTGGT	AAGCAGTTCC	TGCCCCGGCT	CAGGGCCAA.		
401						450	
2873S	aacagatgga	acagctgaat	atggggccaaa	caggatatct	gtggttaagca		
Mo+PyF101							
Consensus							

FIG. 3. (a) Sequencing of U3 region (*Cla*I-*Sac*I) of Mo+PyF101 M-MuLV-Mo+PyF101 MCF-induced tumor 2873S and comparison with that of input virus. Sequence of Mo+PyF101 M-MuLV is based on reported sequences of M-MuLV (25) and the polyomavirus F101 *Pvu*II-4 enhancer fragment (17). Relevant restriction sites, the beginning of U3 region, and direct repeats (dr) of M-MuLV enhancer are indicated. The polyomavirus F101 enhancers, indicated by an overline, lie between the two *Xba*I sites shown. Dots in the consensus sequence line indicate differences for 2873S from the expected sequence of Mo+PyF101 M-MuLV. (b) Comparison of sequence downstream from the M-MuLV enhancers is shown for PCR clones derived from three Mo+PyF101 M-MuLV-Mo+PyF101 MCF-induced tumors. 2873S and 2873T are spleen and thymus from the same animal, while 2977T was a thymic tumor from a second animal. Mo+PyF101 M-MuLV and Mo+PyF101 MCF sequences are identical in this region. Dashes indicate deleted nucleotides.

Cloning and sequencing of variant LTRs. We wished to characterize the nature of the altered LTRs in the tumors in more detail. In particular, the LTRs containing the addition of 70 to 100 bp were of interest. Therefore, PCR cloning from tumor DNAs was carried out. For this purpose, PCR primers spanning the *Cla*I and *Sac*I sites were used to amplify the regions containing the downstream LTRs from proviruses in tumors induced by Mo+PyF101 M-MuLV-Mo+PyF101 MCF. The amplified fragments were then digested with *Cla*I

plus *Sac*I and cloned, through two cloning steps, into pUC19 (see Materials and Methods). The resulting PCR clones were screened, and those corresponding to the LTRs containing the additional 70- to 100-bp insert were selected for further analysis. Sequence analysis of one such PCR clone from splenic tumor 2873S is shown in Fig. 3a. The sequence data confirmed the presence of exactly one additional copy of the M-MuLV 75-bp repeat, consistent with our predictions.

The altered LTR from tumor 2873S showed four other

	451		500
2873S	gttcctgccc	cggctcaggg	ccaagaacag atgggtcccca gatgcggtcc
Mo+PyF101			GAACAG ATGGTCCCCA GATGCGGTCC
Consensus		GAACAG	ATGGTCCCCA GATGCGGTCC
	501		550
		XbaI Py→	
2873S	agccctcagc	agtttctaga	gctggccgtg cgacatcctc ttttaattag
Mo+PyF101	AGCCCTCAGC	AGTTTCTAGA	GCTGGCCGTG CGACATCCTC TTTTAATTAG
Consensus	AGCCCTCAGC	AGTTTCTAGA	GCTGGCCGTG CGACATCCTC TTTTAATTAG
	551		600
2873S	ttgctaggca	actgccctcc	agagggcagt gtgggttttgc aagaggaagc
Mo+PyF101	TTGCTAGGCA	ACTGCCCTCC	AGAGGGCAGT GTGGT TTTTGC AAGAGGAAGC
Consensus	TTGCTAGGCA	ACTGCCCTCC	AGAGGGCAGT GTGGT TTTTGC AAGAGGAAGC
	601		650
2873S	aaaaagcctc	tccacccagc	cctggaatgt cagtgtggtt ttgcaagagg
Mo+PyF101	AAAAAGCCTC	TCCACCCAGG	CCTGGAATGT CAGTGTGGTT TTGCAAGAGG
Consensus	AAAAAGCCTC	TCCACCCAGG	CCTGGAATGT CAGTGTGGTT TTGCAAGAGG
	651		700
2873S	aagcaaaaaag	cctctccacc	caggcctgga atgtatccac ccaatcatta
Mo+PyF101	AAGCAAAAAG	CCTCTCCACC	CAGGCCTGGA ATGTTTCCAC CCAATCATT
Consensus	AAGCAAAAAG	CCTCTCCACC	CAGGCCTGGA ATGT.TCCAC CCAATCATT
	701		750
		←Py XbaI	
2873S	ctatgacaac	agctctatag	aa atcaga tgtttccagg gtgccccaa
Mo+PyF101	CTATGACAAC	AGCTCTAGAG	AACCATCAGA TGTTTCCAGG GTGCCCAAG
Consensus	CTATGACAAC	AGCTCTA.AG	AA..ATCAGA TGTTTCCAGG GTGCCCAAG
	751		800
2873S	gacctgaaat	gaccctgtgc	ctcatttgaa ctaaccaatc agttcgcttc
Mo+PyF101	GACCTGAAAT	GACCCTGTGC	CTTATTGAA CTAACCAATC AGTTCGCTTC
Consensus	GACCTGAAAT	GACCCTGTGC	CT.ATTGAA CTAACCAATC AGTTCGCTTC
	801		
		Sac I	
2873S	tcgcttctgt	tcgcgcgctt	ctgctccccg
Mo+PyF101	TCGCTTCTGT	TCGCGCGCTT	CTGCTCCCCG
Consensus	TCGCTTCTGT	TCGCGCGCTT	CTGCTCCCCG
b	681		
287#3S	a		t --
287#3T	t		g cc
297#7T	a		t cc
Mo+PyF101	atgtttccac	ccaatcatta	ctatgacaac agctctagag aaccatcaga
	NF-1 Site		Xba I
			780
287#3S			c
287#3T			t
297#7T			c
Mo+PyF101	tggttccagg	gtgccccaa	gacctgaaat gaccctgtgc cttatttgaa

FIG. 3—Continued.

minor changes. One was a T→A transversion within the polyomavirus enhancer region at an NF-1 site (nucleotide 685), and a second transversion (G→T) was present at the downstream *Xba*I site (the junction between polyomavirus and M-MuLV sequences). Immediately downstream of the *Xba*I site a CC dinucleotide was absent, and a T→C transition was present at nucleotide 773. There were two possible explana-

tions for these alterations. First, they could have been selected for in the animal because they increased the ability of the altered LTRs to propagate MCF. Alternatively, they could have arisen during the PCR amplification, although this would probably not explain the loss of the CC dinucleotide. To address this, two additional PCR clones containing the enlarged LTRs were cloned and sequenced. One clone (2873T)

was derived in an independent PCR amplification from the tumor-containing thymus of the same animal as shown in Fig. 3a. The other clone (2977T) was obtained from a thymic tumor from a completely different animal. Sequence analysis of these two clones confirmed that they both came from LTRs with three M-MuLV tandem repeats (not shown). The downstream sequences are compared with those of the first clone in Fig. 3b. The clone from the 2873T thymic tumor did not show any of the base changes in comparison to the Mo+PyF101 LTR, so its only alteration was the triplication of the M-MuLV tandem repeats. On the other hand, the clone from 2977T showed exactly the same single-base changes as the 2873S clone, but not the deletion of the CC dinucleotide. Moreover, no other changes were observed. While the limited number of PCR clones sequenced made it difficult to assess the relative frequencies of these variations, the data from the 2873T clone suggest that the additional nucleotide changes were not essential for propagation of the LTRs with the three M-MuLV tandem repeats. On the other hand, exact conservation of the nucleotide changes in the other two clones was striking, particularly since they were derived from different animals. One explanation could be that the Mo+PyF101 M-MuLV-Mo+PyF101 MCF stock contained an LTR with these changes and that it gave rise to the LTR with the three M-MuLV tandem repeats. Alternatively, it is theoretically possible that there was a strong selection for these changes during propagation in the animals.

Pathogenicity of altered viruses. It seemed possible that the altered viruses might be more leukemogenic than the original Mo+PyF101 M-MuLV-Mo+PyF101 MCF mixture. Therefore, virus was recovered from one moribund animal (2836) by cocultivation of tumor thymocytes or splenocytes with NIH 3T3 cells to give the lines 28T and 28S, respectively. When virus from 28T or 28S cells was used to infect newborn mice, they showed mortality curves essentially equivalent to those of the original Mo+PyF101 M-MuLV-Mo+PyF101 MCF inoculum (Fig. 4). Given that Mo+PyF101 M-MuLV-Mo+PyF101 MCF gave disease with kinetics nearly equivalent to those of wild-type M-MuLV, this may not have been surprising.

Southern blot hybridization on the tumors from the 28T- and 28S-inoculated animals was also carried out. When the *Sau3a* plus *SacI* digestion coupled with hybridization with the polyomavirus probe was carried out, the tumors did not show detectable hybridization (Fig. 5b). Thus, these secondary tumors appeared to contain proviruses with little or no polyomavirus sequences in them.

In order to investigate this more thoroughly, a different digestion scheme was used: *HpaI* plus *SacI*, with a polyomavirus probe. As shown in Fig. 5a, *HpaI* plus *SacI* digestion of a Mo+PyF101 MCF provirus (two direct repeats) will give fragments of 1.1 and 0.65 kb that hybridize with an *env* probe. The same analysis of a Mo+PyF101 M-MuLV ecotropic provirus will give the 1.1-kb fragment, as well as a 1.4-kb fragment. (Lack of the 1.4-kb band in the Mo+PyF101 M-MuLV-Mo+PyF101 MCF producer line may be due to methylation of a *HpaI* site, since digests shown in Fig. 2 indicate that the expected ecotropic virus is present.) Alterations in the LTRs would be evident from changes in the 1.1-kb fragment. Analysis of second-round 28T and 28S producer cell line DNAs, as well as the resulting tumors, is shown in Fig. 5c. By comparison to DNA from the first-round Mo+PyF101 M-MuLV-Mo+PyF101 MCF producer cell line (two M-MuLV tandem repeats) and the 2873S first-round tumor (three repeats), the 28T and 28S second-round producer cell lines had altered Mo+PyF101 LTRs that contained three and perhaps four copies of the M-MuLV tandem repeats. In addition, the

FIG. 4. Pathogenesis of altered Mo+PyF101 M-MuLV-Mo+PyF101 MCF. Neonatal mice were inoculated with 28T or 28S cell line viral supernatant containing altered Mo+PyF101 M-MuLV-Mo+PyF101 MCF ($\diamond$) as described in the text. Pathogenicity of the viral stock was similar to that seen for the original Mo+PyF101 M-MuLV-Mo+PyF101 MCF stock ($\bullet$). The number of mice inoculated is in parentheses.

28S cells also had a population of provirus with an LTR that appeared to have a deletion. Prolonged exposure of the gel lane indicated that 28T cells also had very low but detectable levels of a similar deleted provirus. Comparison of the relative intensities of the 0.65- and 1.4-kb bands also indicated that these cultures contained relatively low (but detectable) concentrations of MCF proviruses in comparison to ecotropic viruses. This in part might explain the fact that this virus mixture was no more efficient at inducing leukemia than the original Mo+PyF101 M-MuLV-Mo+PyF101 MCF mixture.

Examination of tumors 1 to 5 induced by 28T and 28S virus (Fig. 5c) was revealing. Most of the tumors showed relatively low levels of the 1.1-kb fragment corresponding to the predominant LTRs in the inoculum. Instead, more rapidly migrating fragments close to M-MuLV size (having two direct repeats and no polyomavirus enhancer sequences) were present, which is indicative of deletions. For any given tumor, these fragments had slightly different mobilities, suggesting that the deletions took place after inoculation of the virus, with elimination of the polyomavirus sequences. One potential alternative explanation was that the deletions represented outgrowth of proviruses with LTR deletions present in the injected viral stock. However, for at least two of the tumors (tumors 2 and 4), the deletion fragments were somewhat larger than the low-level deletion fragments in the original 28T or 28S stocks.

The blot shown in Fig. 5c also indicated that in some of the tumors (tumors 2 to 4), there was a selection for MCF proviruses relative to ecotropic proviruses (compare the intensities of hybridization for the 0.65- and 1.4-kb bands), even though the 28T and 28S stocks had relatively little MCF. This confirms the importance of MCF proviruses in M-MuLV leukemogenicity and the ability of MCF proviruses with an altered Mo+PyF101 LTR to propagate.

DISCUSSION

In these experiments, we have studied the LTRs of Mo+PyF101 MCF proviruses present in tumors induced by the Mo+PyF101 M-MuLV-Mo+PyF101 MCF mixture. As we

FIG. 5. Southern blot analysis of tumor DNAs in the second round of inoculations. (a) Restriction maps of the Mo+PyF101 M-MuLV and Mo+PyF101 MCF showing *Hpa*I (Hp), *Sac*I (Sc), and *Sau*3a (Sa) sites. (b) DNAs were digested with *Sau*3a plus *Sac*I and hybridized with the polyomavirus enhancer probe, as for Fig. 2c. While DNA from producer cell lines 28T and 2836T tumor giving rise to 28T cell line yielded a 575-bp fragment (~75 bp larger than the original Mo+PyF101 M-MuLV-Mo+PyF101 MCF proviruses), second-round tumor DNAs (lanes 1 to 5) did not hybridize with this probe. (c) Tumor DNAs were digested with *Hpa*I plus *Sac*I and hybridized with the 3' *env* probe as discussed in the text to determine the number of direct repeats of the M-MuLV enhancer in second-round tumors lacking polyomavirus enhancer sequences. The 0.65-kb fragment (+) specific for Mo+PyF101 MCF, the 1.4-kb fragment (•) specific for Mo+PyF101 M-MuLV, and the 1.1-kb (*) fragment expected for both viruses are indicated. *Hpa*I-*Sac*I *env* LTR fragments are shown for 2873S tumor (having three direct repeats plus ~200-bp polyomavirus enhancer sequences), Mo+PyF101 M-MuLV-Mo+PyF101 MCF (having two direct repeats plus the polyomavirus sequences), and M-MuLV (having two direct repeats but lacking polyomavirus enhancer sequences). Lanes 28T and 28S, second-round producer cell lines. Some, but not all, polyomavirus enhancer-negative provirus bands were of slower mobility than that of M-MuLV, indicating retention of three direct repeats of the M-MuLV enhancer (e.g., tumors 2 and 4). Tumors 1, 2, and 3 are the same DNAs as for Fig. 5b, tumors 2, 5, and 4, respectively. One *Hpa*I site of ecotropic provirus in Mo+PyF101 M-MuLV-Mo+PyF101 MCF producer cell line may be methylated, thus masking the expected 1.4-kb band. Ecotropic provirus is intact, however, as indicated by Fig. 2b and d. Tumors 1, 3, and 5 were induced by virus from 28T cells, and tumors 2 and 4 were induced by 28S cell virus.

reported previously, MCF proviruses driven by the Mo+PyF101 LTR were not detected in 6- to 8-week-old NIH Swiss mice inoculated with Mo+PyF101 M-MuLV-Mo+PyF101 MCF (4); they apparently propagate inefficiently. Thus, the variant LTRs present in the tumors provided insight into changes that could relieve the in vivo restriction of the Mo+PyF101 LTR. We hypothesize that the LTR alterations in the Mo+PyF101 M-MuLV-Mo+PyF101 MCF-induced tumors reflect changes that allow for more efficient Mo+PyF101 MCF expression in MCF propagator cells (4), although this was not formally tested here. For the purposes of discussion, it will be assumed that these LTR alterations enhanced in vivo Mo+PyF101 MCF propagation, although effects on other steps in the pathogenic process must also be considered. The results indicated that there are two general ways to relieve restriction: acquisition of a third copy of the M-MuLV tandem repeats or deletion of the polyomavirus enhancer sequences. The LTRs with three M-MuLV tandem repeats indicate that a strong enough M-MuLV enhancer region can apparently dominate the behavior of the LTR even in the presence of polyomavirus enhancers.

TriPLICATION of the M-MuLV tandem repeats during reverse transcription is easy to envision, for instance, by template switching of reverse transcriptase after it has completed transcription of both tandem repeats back to the first tandem repeat (6). Alternatively, unequal crossing over at the DNA level could occur between reverse-transcribed DNAs or proviruses. For this reason, in the first-round tumors, Mo+PyF101 MCF LTRs with an added M-MuLV tandem repeat were

generally more common than those containing deletion of the polyomavirus sequences.

The results also suggested that while triplication of the M-MuLV tandem repeats may be the most common LTR alteration allowing for presumptive propagation of Mo+PyF101 MCF, those MCF proviruses that have deleted the polyomavirus sequences altogether may replicate more efficiently. Thus, upon infection of virus recovered from a Mo+PyF101 M-MuLV-Mo+PyF101 MCF-induced tumor, most mice developed tumors with MCF proviruses containing deletions of the polyomavirus enhancers (Fig. 5). The data (Fig. 5c) further suggested that in some cases Mo+PyF101 MCF escaped restriction by sequentially adding a copy of the M-MuLV tandem repeats and then deleting the polyomavirus sequences. In other cases, it is possible that deletion of the polyomavirus sequences occurred first.

As shown in Fig. 3, sequencing of the altered LTRs also identified single-nucleotide changes in the polyomavirus and downstream M-MuLV sequences in some of the variant Mo+PyF101 MCF proviruses. While the prevalence of these changes in the variant proviruses was not addressed, it is possible that these changes also contributed to escape from restriction. However, the sequence of clone 2873T suggested that none of these changes is required for propagation if three copies of the M-MuLV tandem repeats are present.

It should be noted that the accelerated leukemogenicity induced by Mo+PyF101 MCF only occurred in mice that were infected with the Mo+PyF101 M-MuLV-Mo+PyF101 MCF mixture. In particular, mice inoculated with Mo+PyF101 MCF

alone did not propagate the virus measurably, and no tumors resulted. The presumptive ability of the Mo+PyF101 M-MuLV-Mo+PyF101 MCF to eventually propagate an altered Mo+PyF101 MCF and thus accelerate leukemogenesis may have been facilitated by ecotropic pseudotypes of Mo+PyF101 MCF. Such pseudotypes would allow introduction of Mo+PyF101 MCF into cells that normally propagate ecotropic M-MuLV. We have previously shown that Mo+PyF101 M-MuLV efficiently replicates in mice (9, 11), which indicates that the Mo+PyF101 LTR is active in at least some cells that are ecotropic M-MuLV propagators. Thus, introduction of the Mo+PyF101 MCF genome into these cells as ecotropic M-MuLV pseudotypes would result in efficient expression of Mo+PyF101 MCF. Continued production of Mo+PyF101 MCF in the animal was probably necessary for the eventual appearance of variants that have escaped the restriction.

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