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Recombinant Mink Cell Focus-Inducing Virus and Long Terminal Repeat Alterations Accompany the Increased Leukemogenicity of the Mo+PyF101 Variant of Moloney Murine Leukemia Virus after Intraperitoneal Inoculation

BARBARA BELLI, ANIL PATEL, AND HUNG FAN*

Department of Molecular Biology and Biochemistry and Cancer Research Institute, University of California, Irvine, California 92717

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We recently showed that different routes of inoculation affect the leukemogenicity of the Mo+PyF101 variant of Moloney murine leukemia virus (M-MuLV). Intraperitoneal (i.p.) inoculation of neonatal mice with Mo+PyF101 M-MuLV greatly enhanced its leukemogenicity compared with subcutaneous (s.c.) inoculation. We previously also suggested that the leukemogenic defect of Mo+PyF101 M-MuLV when inoculated s.c. may result from the inability of this virus to form *env* gene recombinant (mink cell focus-inducing [MCF]) virus. In this study, virus present in end-stage tumors and in preleukemic animals inoculated i.p. by Mo+PyF101 M-MuLV was characterized. In contrast to s.c. inoculation, all tumors from i.p.-inoculated mice contained high levels of recombinant MCF virus. Furthermore, Southern blot analyses demonstrated that the majority of the tumors contained altered Mo+PyF101 M-MuLV long terminal repeats. The U3 regions from several tumors with altered long terminal repeats were cloned by PCR amplification. Sequence analyses indicated that the M-MuLV 75-bp tandem repeat in the enhancer region was triplicated. This amplification was also previously observed in mice infected s.c. with a pseudotypic mixture of Mo+PyF101 M-MuLV and Mo+PyF101 MCF virus. The enhancer triplication was an early event, and it occurred within 2 weeks postinfection. Recombinant MCF viruses were not detected by Southern blot analyses until 4 weeks postinfection. Thus, the M-MuLV enhancer triplication event was initially important for efficient propagation of ecotropic Mo+PyF101 M-MuLV. The increased leukemogenicity following i.p. inoculation could be explained if the triplication enhances Mo+PyF101 M-MuLV replication in the bone marrow and bone marrow infection is required for recombinant MCF virus formation.

Moloney murine leukemia virus (M-MuLV) is a typical nonacute retrovirus that induces T-lymphoblastic lymphoma in mice, with a disease latency of 3 to 4 months. An important mechanism in nonacute retroviral leukemogenesis is proviral activation of cellular proto-oncogenes, either by promoter insertion or by enhancer activation (9). Experiments on MuLVs demonstrated that the enhancers in the viral long terminal repeat (LTR) are major determinants of the type of leukemia induced (4, 8, 14). We have demonstrated that various enhancer rearrangements and insertions in the M-MuLV LTR alter biologic properties in vivo and in vitro (11–13).

Another event associated with MuLV leukemogenesis is the appearance of recombinant mink cell focus-inducing (MCF) viruses. These are *env* gene recombinants between the infecting MuLV and endogenous polytropic provirus resident in the mouse genome. The importance of MCF viruses in leukemogenesis has been demonstrated (20). We have previously made extensive use of the poorly leukemogenic Mo+PyF101 M-MuLV variant to study preleukemic events in M-MuLV leukemogenesis (2, 5, 10, 15). This virus contains an LTR in which enhancer sequences from the F101 strain of murine polyomavirus (Py) have been inserted at position −150 in the LTR, between the M-MuLV enhancer sequences and the basal promoter elements (7). From comparative studies of wild-type and Mo+PyF101 M-MuLV, we have proposed that wild-type M-MuLV induces a preleukemic state consisting of general hematopoietic hyperplasia in the spleen, which secondarily results from a defect in bone marrow hematopoiesis. Moreover, the bone marrow hematopoietic defect results from growth-inhibitory effects on bone marrow stroma, which in turn result from combined infection by M-MuLV and an MCF recombinant virus (16). Analysis of Mo+PyF101 M-MuLV was instrumental in allowing us to reach these conclusions. In particular, the low leukemogenicity of Mo+PyF101 M-MuLV after subcutaneous (s.c.) inoculation was correlated with (i) a lack of preleukemic splenic hyperplasia (6), (ii) no in vitro bone marrow stromal defects (16), and (iii) an inability to generate (and/or propagate) MCF recombinant viruses in the animal (2).

The utility of Mo+PyF101 M-MuLV in leukemogenesis studies was recently extended by the observation that intraperitoneal (i.p.) inoculation greatly enhanced its leukemogenicity. In contrast, wild-type M-MuLV was equally leukemogenic when infected i.p. or s.c. (1). Virological studies on mice inoculated i.p. with Mo+PyF101 M-MuLV indicated that the enhanced leukemogenicity was highly correlated with establishment of early infection in the bone marrow. It was not correlated with establishment of early infection in either the spleen or the thymus. This finding pointed to a role for bone marrow infection in early stages of M-MuLV leukemogenesis. Apparently a cell type normally involved in delivery of wild-type M-MuLV from the site of s.c. inoculation to the bone marrow cannot replicate Mo+PyF101 M-MuLV, presumably because of incompatibility with the PyF101 sequences in the Mo+PyF101 M-MuLV LTR.

* Corresponding author. Mailing address: Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717. Phone: (714) 824-5554. Fax: (714) 824-4023.

In this study, we have characterized the proviruses present in mice infected i.p. by Mo+PyF101 M-MuLV in end-stage tumors and at preleukemic times. We report that i.p. inoculation of Mo+PyF101 M-MuLV leads to the generation of recombinant MCF virus. This result provides a molecular explanation for the enhanced leukemogenic potential of Mo+PyF101 M-MuLV when inoculated i.p. Further, the viruses in these mice contain changes within the LTR that are apparently important for in vivo propagation and leukemogenesis.

MATERIALS AND METHODS

Virus and inoculation of mice. The generation of Mo+PyF101 M-MuLV has been previously described (7). Viral stocks were cell culture supernatants derived from productively infected NIH 3T3 fibroblasts. Virus titers were determined by the UV/XC plaque assay (18). NIH Swiss mice were inoculated i.p. or s.c. with 0.15 ml of virus stock (10^5 XC PFU) within 48 h of birth.

Southern blot analysis. DNA was obtained from tumors as previously described (12). Southern blot analyses were performed as previously described (12), using GeneScreen Plus (New England Nuclear). Hybridization probes for c-*myc*, *pim-1*, and *pvt-1* were as described previously (11). The c-*myb* probe was a gift from Linda Wolff (17). The Py enhancer probe was prepared from the PyF101 *Pvu*II-4 enhancer fragment isolated from a pGEM construct containing the complete genome of PyF101. The probe for the 3′ *env* region of M-MuLV was prepared from the 1.1-kb *Bam*HI-*Cla*I fragment of M-MuLV. Radioactive probes were prepared by the random primer method according to standard procedures (21).

PCR cloning and sequencing. Primers and amplification were as previously reported (3). Amplified fragments from four independent tumor DNAs were isolated on low-melting-temperature agarose gels, purified with Gene Clean (Bio 101), and ligated to the TA cloning vector (Stratagene). Recombinant clones were selected by blue/white colony screening on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. Colonies containing recombinant plasmid were screened for inserts by PCR. One plasmid clone from each transformation reaction was amplified and subsequently isolated by using Qiagen maxiprep columns (Qiagen Inc.). Sequencing was performed with an automated laser fluorescence sequencer, using fluoresceinated PCR primers.

RESULTS

Proviral insertion near cellular proto-oncogenes. T-lymphoblastic lymphomas induced by wild-type M-MuLV typically show proviral insertions adjacent to at least one of three cellular loci, *c-myc*, *pim-1*, and *pvt-1* (19). Because Mo+PyF101 M-MuLV inoculated i.p. efficiently induced the same disease as M-MuLV, we tested if proviral insertions were occurring adjacent to the same cellular proto-oncogenes. Tumor DNAs from mice infected i.p. with Mo+PyF101 M-MuLV were tested by Southern blot hybridization using radioactive probes for each of these three loci as described previously (11). Mo+PyF101 M-MuLV-induced tumors showed proviral integrations near these proto-oncogenes in less than 25% of the cases examined (2 of 17 for c-*myc*, 2 of 17 for *pim-1*, and none for *pvt-1*), which was significantly less than the 60 to 75% frequency of insertion next to c-*myc*, *pim-1*, and *pvt-1* observed in wild-type M-MuLV-induced tumors (11). We also examined these tumors for integration in the c-*myb* locus, frequently observed in M-MuLV-induced promyelomonocytic tumors (17). Again, no integrations (0 of 17) were observed. These results suggested that other cellular proto-oncogenes were activated in the majority of Mo+PyF101 M-MuLV-induced tumors or that leukemogenesis did not require proto-oncogene activation for these virus-induced tumors.

Detection of MCF proviruses in mice inoculated i.p. with Mo+PyF101 M-MuLV. We previously reported that the leukemogenic defect of Mo+PyF101 M-MuLV when inoculated s.c. into neonatal mice was correlated with a failure to generate recombinant MCF viruses (2). Because i.p. inoculation resulted in a significant increase in leukemogenicity, we tested the possibility that Mo+PyF101 M-MuLV could generate recombinant MCF virus when inoculated i.p. We tested DNAs from 17 independently derived tumors induced in mice infected i.p. by Mo+PyF101 M-MuLV for recombinant MCF viruses by Southern blot analysis as done previously (3) (Fig. 1). Digestion with *Bam*HI plus *Cla*I would yield a 1.1-kb fragment specific for the ecotropic envelope (electrophoresed off the bottom of the gel) and a diagnostic 1.9-kb fragment specific for a MCF recombinant virus detectable by a labeled DNA fragment from the 3′ portion of the ecotropic M-MuLV envelope gene (Fig. 1A). As expected, the MCF virus-specific band was not present in control uninoculated mouse DNA (Fig. 1B, lane a). Furthermore, tumor DNA from an animal inoculated s.c. with Mo+PyF101 M-MuLV showed no evidence for MCF recombinant viruses, consistent with our previous studies (2) (Fig. 1B, lane k). The 1.9-kb MCF recombinant virus-specific band was detected in all of the tumors induced by i.p. inoculation of Mo+PyF101 M-MuLV examined (Fig. 1B, lanes b to j, and eight additional tumors not shown). These results indicated that the efficient leukemogenesis associated with i.p.

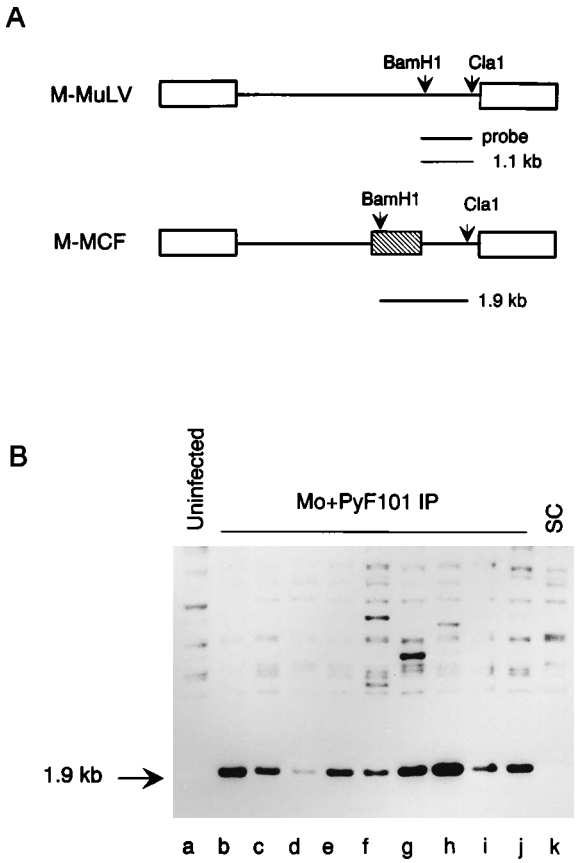

FIG. 1. Detection of MCF provirus by Southern blot hybridization. (A) Restriction maps of M-MuLV (top) and the *env* gene MCF recombinant virus (bottom). Probe is indicated by the heavy bar. The portion of the *env* gene involved in recombination with endogenous polytropic sequences is shown by the hatched box. Digestion of high-molecular-weight DNA with *Bam*HI and *Cla*I yields a diagnostic 1.9-kb fragment hybridizable with an ecotropic 3′ *env* probe from tumors containing MCF recombinant provirus. (B) High-molecular-weight DNA (5 μg) was digested with *Bam*HI plus *Cla*I and analyzed by gel electrophoresis and Southern blot hybridization using a probe specific for the envelope region (shown as the bold line in panel A). Lanes: a, uninfected control thymus; lanes b to j, Mo+PyF101 M-MuLV-induced tumor DNAs from i.p.-inoculated mice; k, tumor DNA obtained from an animal inoculated s.c. with Mo+PyF101 M-MuLV. The s.c.-infected animal's tumor was confirmed to be virus infected by the presence of the 1.1-kb band (not shown). The more slowly migrating bands that were also detected in uninfected DNA represented cross-hybridizing M-MuLV-related endogenous proviruses.

inoculation of Mo+PyF101 M-MuLV was correlated with the generation of MCF recombinant viruses. This finding was also consistent with our previous conclusion that the leukemogenic defect associated with Mo+PyF101 M-MuLV after s.c. inoculation was related to an inability to generate recombinant MCF viruses (2).

Alterations in the viral LTR in tumors induced by i.p. inoculation of Mo+PyF101 M-MuLV. We previously investigated the MCF virus defect of Mo+PyF101 M-MuLV when it is infected s.c. Using an Mo+PyF101 MCF virus generated by molecular cloning, we found that although Mo+PyF101 MCF virus was infectious in NIH 3T3 fibroblasts in vitro, it failed to propagate efficiently in NIH Swiss mice (3). On the other hand, a pseudotypic mixture of Mo+PyF101 M-MuLV and Mo+PyF101 MCF virus allowed in vivo propagation and efficient leukemogenesis (1). This was accompanied by two kinds of alterations in the Mo+PyF101 M-MuLV LTR: triplication of the M-MuLV enhancer element and loss of the Py sequences. Thus, the restriction on the Mo+PyF101 M-MuLV LTR in MCF virus propagation and/or leukemogenesis could be circumvented by these two alterations. These results suggested that similar changes might occur in animals inoculated i.p. with Mo+PyF101 M-MuLV, allowing for the generation and/or propagation of recombinant MCF viruses. To test this possibility, tumor DNAs were digested with *Sau*3a plus *Sst*I, which encompasses the M-MuLV enhancer region and includes the inserted Py sequences. The molecular organization of the Mo+PyF101 LTR is diagrammed in Fig. 2A. Hybridization with a probe specific for the inserted Py enhancer sequences would yield a 500-bp fragment which allowed for more accurate determination of size differences. Analysis of representative tumors is shown in Fig. 2B. All tumor DNAs yielded the 500-bp fragment diagnostic of input virus (Fig. 2B, lane a), and they therefore retained the inserted Py sequences. Additionally, most tumors yielded a hybridizing species larger than the 500-bp input virus. In more than 80% of the tumors examined, the larger fragment had an apparent size of approximately 600 bp. This increase in LTR size would be consistent with the triplication of the 75-bp M-MuLV enhancer element (originally present in two copies), as we had observed previously (3).

Molecular cloning and sequencing of the variant LTRs. To characterize the LTR changes observed in Fig. 2, DNA sequence analyses were performed. The strategy used to molecularly clone and sequence the altered LTRs is diagrammed in Fig. 3. Briefly, oligonucleotide primers flanking the LTR region were used in PCR of four independently derived tumor DNAs. Three of the four tumor DNAs chosen contained almost exclusively an LTR fragment of the altered size as detected by Southern blot analyses. One of the tumors chosen for analysis contained hybridizing fragments similar in size to both input virus and the altered-size LTR. The amplified fragments were ligated and cloned into the TA cloning vector (Stratagene) as described in Materials and Methods. Each of the four PCRs yielded clones, which were sequenced. The sequence data confirmed the presence of Mo+PyF101 M-MuLV LTRs with three copies of the M-MuLV 75-bp repeat in all clones (diagrammed in Fig. 3B). This was the same specific LTR alteration previously observed in animals inoculated with the pseudotypic mixture of Mo+PyF101 MCF virus plus Mo+PyF101 M-MuLV (1). Tumor 151, which contained both the increased-size LTR fragment plus a fragment similar in size to input virus, was particularly interesting. Sequence analysis of a single PCR-derived clone showed a deletion of one of the 54-bp tandem repeats of the B and C_1 cores found within the Py enhancer sequences. This was interesting in light of the

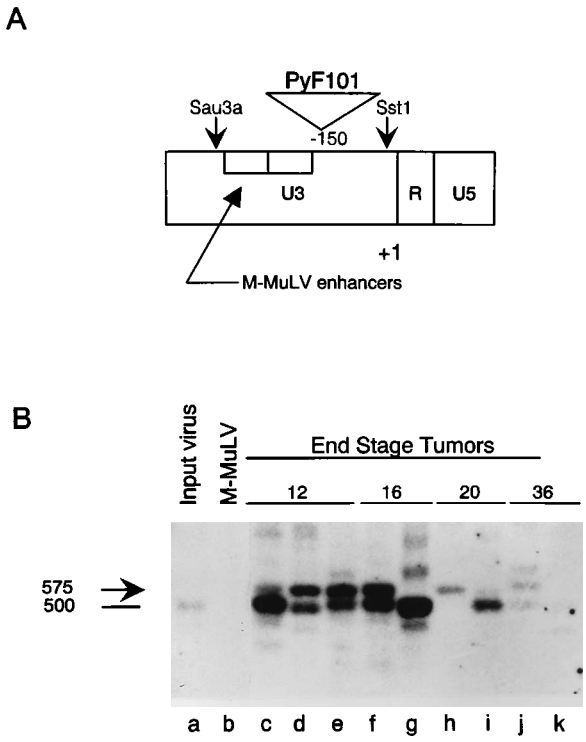

FIG. 2. Analysis of proviral LTRs in end-stage tumors. (A) Organization of the Mo+PyF101 M-MuLV LTR. The relative locations of the *Sau*3a and *Sst*I restriction sites are indicated. (B) High-molecular-weight DNA (5 μg) from tumors was digested with *Sau*3a plus *Sst*I and analyzed by gel electrophoresis and Southern blot hybridization using a probe specific for the Py enhancers. Lanes: a, Mo+PyF101 M-MuLV producer cell line DNA (10 μg); b, uninfected control thymus DNA; c to k, Mo+PyF101 M-MuLV-induced tumor DNAs from i.p.-inoculated mice. The age of the animal when moribund is indicated in weeks. Lanes under the same number represent tumorous tissues from different animals. All lanes were from the same Southern blot, but the photographic exposures were adjusted to account for different numbers of viral integration events. Sizes are indicated in base pairs.

observation that a different Py enhancer variant of M-MuLV, Mo+PyF441 M-MuLV, is as leukemogenic as wild-type M-MuLV (11). The PyF441 enhancer element contains only one copy of the B and C_1 cores. Thus, the deletion in clone 151 of one of the 54-bp tandem duplications in the Py sequences of Mo+PyF101 M-MuLV was likely important in the increased leukemogenicy (11).

Presence of the altered LTR in preleukemic animals. The preceding results showing LTR alterations in the Mo+PyF101 M-MuLV-induced tumors after i.p. inoculation suggested that the M-MuLV enhancer triplication was important for relieving a defect of the Mo+PyF101 LTR in leukemogenesis. However, the restrictive step for Mo+PyF101 M-MuLV could be either an early or a late step in this process. The presence of virus with the altered LTR only in end-stage tumors, not in preleukemic animals, would suggest that the LTR change was a late event in leukemogenesis. Alternatively, the detection of virus with the altered LTR in preleukemic animals would suggest that the block for Mo+PyF101 M-MuLV that could be corrected by triplication of the M-MuLV 75-bp repeat was at an early step (2, 10). Therefore, DNA was prepared from the hematopoietic organs of preleukemic animals infected i.p. with Mo+PyF101 M-MuLV and analyzed for LTR alterations by Southern blot analysis as described above. Our initial studies showed that at 4 weeks postinfection, virus with altered LTRs could be found in the spleen, thymus, and bone marrow (data

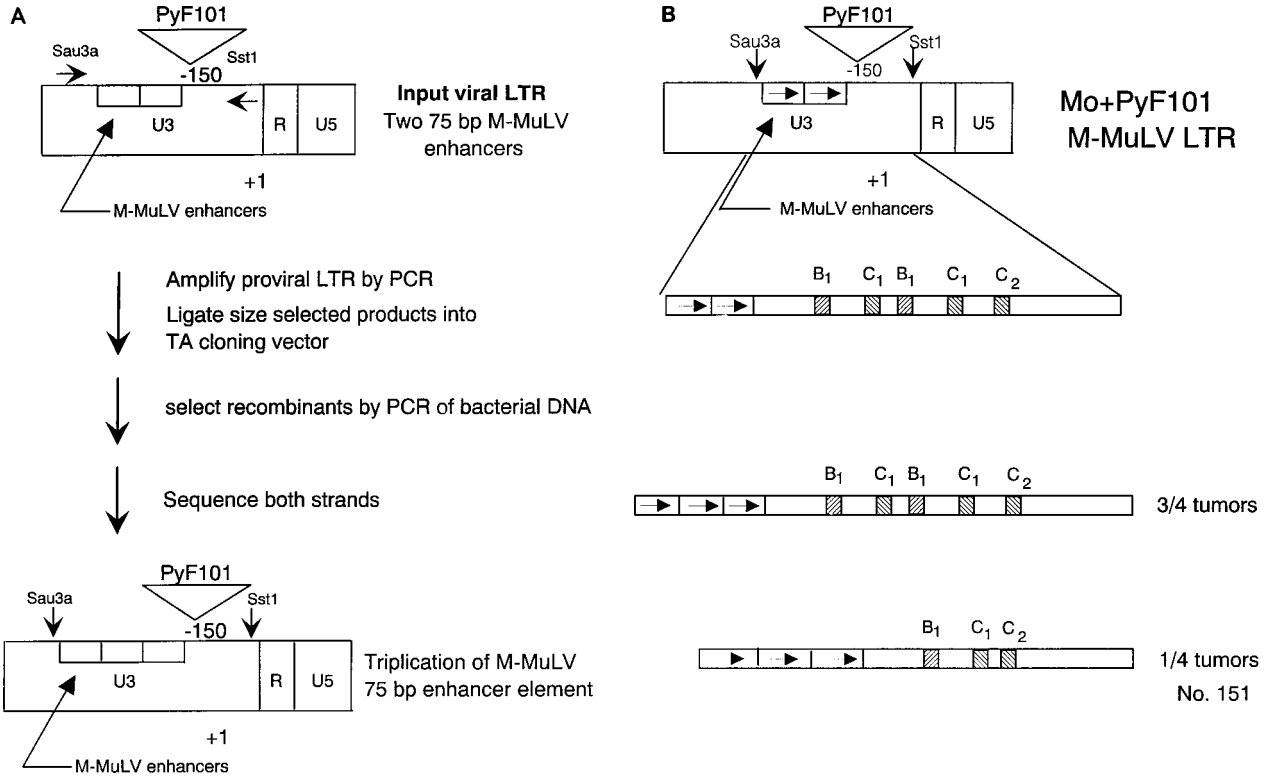

FIG. 3. (A) Schematic diagram of the strategy used to molecularly clone and sequence variant variant Mo+PyF101 M-MuLV LTRs. Details of the cloning and sequencing procedures are described in Materials and Methods. (B) Schematic diagram of the molecular organization of the altered LTRs as determined from sequence analyses. All sizes distances are drawn to scale. Three of the four tumors analyzed had the same structure, i.e., retention of the Py insertion and a triplication of the M-MuLV 75-bp enhancer. The corresponding Southern blot analyses of the parent tumors showed only one fragment hybridizable with the Py-specific probe, with an apparent mobility corresponding to 575 bp (data not shown). One tumor (tumor 151) had a deletion of one copy of the B-core sequence of the Py enhancer but also a triplication of the M-MuLV enhancer. The corresponding Southern blot analysis of tumor 151 showed a fragment with a mobility slightly faster than those of the other three tumors analyzed (data not shown).

not shown). Examination of progressively earlier time points demonstrated that virus with the altered LTR was present as early as 2 weeks postinfection in all organs examined. Figure 4 shows analyses of two animals at this time. It was interesting that the predominant virus detected at this time contained the altered LTR. We further attempted to define the time frame for triplication of the M-MuLV enhancer. Preleukemic DNA from animals analyzed at 10 days postinfection demonstrated extremely low or undetectable levels of proviral DNA. For those samples that had detectable proviral DNA, only input-size LTRs (two 75-bp repeats) were detected. It is possible that PCR analyses would have detected altered LTRs at earlier times.

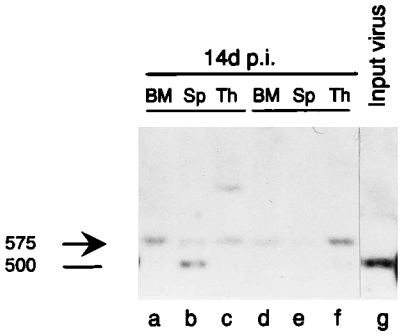

FIG. 4. Proviral LTRs in i.p.-inoculated mice at 2 weeks postinfection. High-molecular-weight DNAs (5 μg) from bone marrow (BM; lanes a and d), spleen (Sp; lanes b and e), and thymus (Th; lanes c and f) from two different mice neonatally inoculated i.p. with Mo+PyF101 M-MuLV were digested with *Sau*3a plus *Sst*I and analyzed by gel electrophoresis and Southern blot hybridization using a probe specific for the Py enhancers. Lane g contains DNA from the Mo+PyF101 producer cell line used as the virus source for the inoculations. 14d, 14 days. Sizes are indicated in base pairs.

Presence of the altered LTR on Mo+PyF101 MCF recombinant virus. These results indicated that LTR alteration of Mo+PyF101 M-MuLV occurred very early, between 10 and 14 days postinfection in i.p.-inoculated animals. We previously suggested that the M-MuLV enhancer triplication was important for in vivo propagation of an MCF recombinant virus driven by the LTR of Mo+PyF101 (3). Because the M-MuLV enhancer triplication occurred within 2 weeks postinfection in the mice inoculated i.p. with Mo+PyF101 M-MuLV, it seemed possible that MCF recombinant virus was forming at this time also. Therefore, several preleukemic animals (4 weeks of age) were assayed for MCF recombinant virus by the *Bam*HI-plus-*Cla*I analysis described above. As shown in Fig. 5, Mo+PyF101 MCF recombinant virus generally could not be detected in the spleen or thymus prior to 4 weeks postinfection, while ecotropic virus was uniformly detectable. Combined with the results of Fig. 4, this finding suggested that the enhancer triplication occurred first on ecotropic Mo+PyF101 M-MuLV, before MCF recombinant virus was generated.

The *Bam*HI-plus-*Cla*I digest used for Fig. 1 and 5 did not include LTR sequences in the diagnostic 1.9-kb fragment. Di-

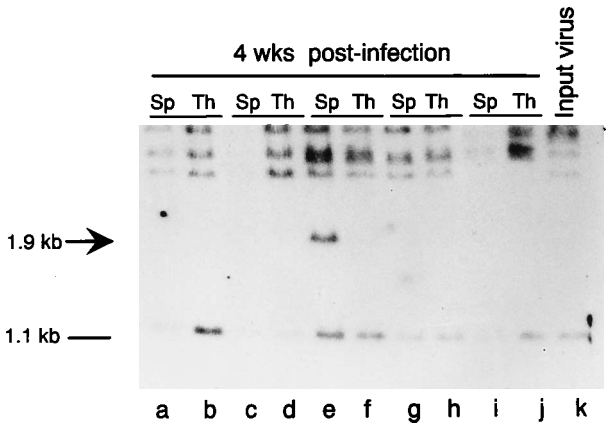

FIG. 5. Tests for MCF provirus early after infection. Restriction maps of ecotropic M-MuLV and MCF recombinant virus are shown in Fig. 1A. Restriction enzyme digestion of high-molecular-weight DNA with *Bam*HI plus *Cla*I yields a unique 1.9-kb fragment from recombinant provirus and a corresponding 1.1-kb fragment from M-MuLV. High-molecular-weight DNA was prepared from five animals inoculated i.p. with Mo+PyF101 M-MuLV at 4 weeks postinfection, and 5 μg from spleen (Sp) or thymus (Th) was digested with *Bam*HI plus *Cla*I and analyzed as for Fig. 1B. Lanes: a to j, Mo+PyF101 M-MuLV-infected tissue, induced tumor DNAs; k, Mo+PyF101 M-MuLV producer cell line DNA. Spleen and thymus from the same animal are indicated by continuous lines above the lanes. The 1.9-kb MCF diagnostic fragment is apparent only in the spleen of one infected animal.

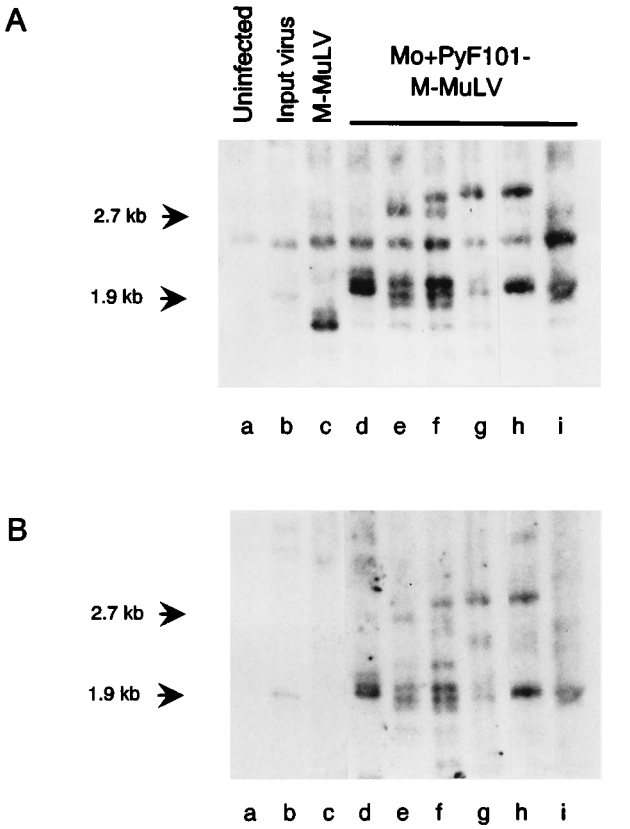

FIG. 6. Detection of altered LTRs on ecotropic and MCF provirus by Southern blot hybridization. Restriction maps of M-MuLV and M-MuLV–MCF virus are shown in Fig. 1A. The *Sst*I site is 3′ to the Py insertion, shown in Fig. 2A. Digestion of high-molecular-weight DNA with *Bam*HI and *Sst*I yields a fragment of 1.9 kb which is derived from ecotropic provirus and a fragment of 2.7 kb which is derived from the MCF recombinant virus. Both of these fragments should be hybridizable with the ecotropic *env* probe and the Py-specific probe. (A) High-molecular-weight DNA (5 μg) was digested with *Bam*HI plus *Sst*I and analyzed by gel electrophoresis and Southern blot hybridization using a probe specific for the envelope region (shown as the bold line in Fig. 1A). The relative mobilities of the parental ecotropic (1.9 kb) and the predicted MCF recombinant virus envelope fragment (2.7 kb) are indicated by arrows. Our molecular clone of Mo+PyF101 MCF virus happens to contain an additional *Sst*I site within the *env* region, unlike most M-MuLV MCF viruses. Therefore, this DNA was not used as a gel mobility standard. Lanes: a, uninfected control thymus DNA; b, Mo+PyF101 M-MuLV producer cell line DNA; c, M-MuLV producer cell line DNA; d to i, Mo+PyF101 M-MuLV-induced tumor DNAs from i.p.-inoculated mice. All lanes were from the same Southern blot, but the photographic exposures were adjusted to account for differential amounts of viral integrations. Note that there is an endogenous cross-hybridizing fragment of 2.5 kb that was detected in all lanes, including uninfected mouse DNA. (B) The blot shown in panel A was stripped of probe and rehybridized with a probe specific for the Py enhancers.

rect characterization of the LTR present on a recombinant MCF virus required a different approach. This was particularly important with regard to determining if the MCF recombinant viruses detected in Fig. 1 contained altered LTRs. Therefore, tumor DNAs were digested with *Bam*HI plus *Sst*I. *Sst*I digests 3′ to the inserted Py sequences in the Mo+PyF101 M-MuLV LTR. Ecotropic Mo+PyF101 M-MuLV would yield a fragment of 1.9 kb, while a recombinant Mo+PyF101 MCF virus would yield a fragment of 2.7 kb. M-MuLV enhancer triplication on either of these viruses would yield detectably larger fragments (2.0 and 2.8 kb, respectively). Compared with Mo+PyF101 M-MuLV producer cell line DNA and uninfected control DNA (Fig. 6, lanes a and b, respectively), tumor DNAs contained novel fragments in the 2.0- and 2.8-kb size ranges which hybridized to the ecotropic *env*-specific probe. These results showed that altered Mo+PyF101 M-MuLV LTRs were present on both the ecotropic and the recombinant MCF viruses. The minimal observed size increase could be accounted for by triplication of the M-MuLV enhancer. To determine if the size heterogeneities involved LTRs that contained Py sequences, the blot was rehybridized with a Py-specific probe (Fig. 6B). This analysis showed that all of the novel fragments in Fig. 6A retained the Py enhancer sequences. The mobility of the recombinant Mo+PyF101 MCF-specific fragments (e.g., lanes g and h) were slower than that predicted for a triplicated M-MuLV enhancer element. This finding suggested that further alterations may have occurred in the Mo+PyF101 MCF virus LTR. Additional changes beyond M-MuLV enhancer triplication might be necessary for efficient replication of the recombinant MCF virus.

DISCUSSION

In these experiments, molecular characterizations of T lymphomas arising after i.p. inoculation of Mo+PyF101 M-MuLV were carried out. Several points emerged. First, these tumors showed relatively low frequencies of proviral insertion near the proto-oncogenes that are commonly activated by wild-type M-MuLV (c-*myc*, *pim-1*, and *pvt-1*), even though the efficiencies of leukemogenesis and type of leukemia were the same. Second, tumors induced by i.p. inoculation with Mo+PyF101 M-MuLV showed the presence of recombinant MCF viruses in all cases. In contrast, tumors arising after s.c. inoculation with the same virus showed no evidence of MCF viruses. Third, in mice inoculated i.p. by Mo+PyF101 M-MuLV, triplication of the M-MuLV 75-bp enhancer sequences occurred at early times, apparently relieving an in vivo replication block for the virus.

These molecular studies confirm and extend our previous studies on M-MuLV leukemogenesis as described in the introduction. We were initially surprised to find recombinant MCF viruses in the tumors resulting from i.p. inoculation of

Mo+PyF101 M-MuLV, since s.c. inoculation with the same virus did not yield MCF viruses. However, the finding of recombinant MCF viruses after i.p. inoculation was actually very consistent with the enhanced leukemogenesis. Moreover, i.p. inoculation with Mo+PyF101 M-MuLV results in significantly enhanced splenic hyperplasia compared with s.c.-inoculated animals (1b), which is consistent with our previous conclusions that recombinant MCF viruses are involved in the establishment of this state. Thus, these results lend further support to our current model of M-MuLV leukemogenesis and the role of recombinant MCF viruses in preleukemic events.

We previously had shown that a pure Mo+PyF101 MCF virus is unable to propagate after s.c. inoculation into adult mice, from which we concluded that the in vivo block for Mo+PyF101 M-MuLV–MCF virus generation is in the propagation of MCF virus (3). Moreover, we also found that s.c. inoculation of a pseudotypic mixture of Mo+PyF101 M-MuLV and Mo+PyF101 MCF virus resulted in rapidly occurring tumors containing viral LTRs with the same triplication of the MuLV enhancers as observed in the i.p.-inoculated mice studied here (2). We suggested that the M-MuLV enhancer triplication may allow a recombinant MCF virus to escape a transcriptional block in MCF virus propagator cells. In light of these results, we initially expected that the selective pressure for the appearance of the altered LTRs in the mice inoculated i.p. with Mo+PyF101 M-MuLV would be for propagation of recombinant MCF viruses. However, this was not the case, as demonstrated in Fig. 4 and 5, since the altered LTRs appeared in the infected mice well before recombinant MCF virus did. Thus, the LTR alterations reflected selective pressure for replication of ecotropic Mo+PyF101 M-MuLV.

It was also noteworthy that in Fig. 4, several tissues at 14 days postinoculation contained only virus with the M-MuLV enhancer triplication. This finding emphasized the importance of this alteration for early Mo+PyF101 M-MuLV propagation in i.p.-inoculated animals. In contrast, end-stage tumors typically showed approximately equal concentrations of Mo+PyF101 M-MuLV LTRs with two and three copies of the M-MuLV enhancers (Fig. 2). Thus, at later times after i.p. infection, there may have been an opposite selection for the virus with the parental LTR. Alternatively, at later times, cell types that can efficiently propagate the parental LTR may have become infectable.

As indicated in the introduction, recent virological analyses of mice infected i.p. versus s.c. with Mo+PyF101 M-MuLV implicated early bone marrow infection as a critical event in leukemogenesis (1). It seems possible that the recombination event leading to triplication of the M-MuLV enhancers could take place in cells of the bone marrow. Animals infected i.p. with Mo+PyF101 M-MuLV showed the triplication, and they established early infection in the bone marrow, while those infected s.c. were deficient for both of these properties. The recent i.p.-versus-s.c. studies may also suggest an interpretation different from our previous conclusion that the primary defect for Mo+PyF101 M-MuLV relative to MCF viruses and leukemogenesis is at the level of MCF virus propagation. In our earlier experiments, the infections of cloned Mo+PyF101 MCF virus were done s.c. (3). It is now apparent that the cells that transport M-MuLV (and perhaps MCF viruses) from the site of s.c. inoculation to the bone marrow are restrictive for the Mo+PyF101 M-MuLV LTR as discussed above. Our previous experiments as well as those reported here could be explained by in vivo MCF virus formation requiring early infection of the bone marrow. Thus, s.c. infection by Mo+PyF101 M-MuLV would not result in appearance of MCF viruses as a result of lack of early infection in the bone marrow. In contrast, i.p. infection would yield MCF viruses as a result of early bone marrow infection. In support of this proposal, we have also found that delayed i.p. infection with Mo+PyF101 M-MuLV (12 days of age versus neonatal) results in reduced leukemogenesis and lack of early bone marrow infection (1); in parallel, MCF recombinants and LTRs with M-MuLV enhancer triplications were found at low frequency in the resulting tumors (1a).

LTR alterations have also been detected in tumors arising at lower efficiencies in mice inoculated s.c. by Mo+PyF101 M-MuLV (1a). However, in those tumors, the LTR alterations were generally deletions of the Py sequences from the Mo+PyF101 M-MuLV LTR rather than the M-MuLV enhancer triplications observed after i.p. inoculation. This mechanism for escape from the Mo+PyF101 M-MuLV LTR restriction has also been observed previously (3). This finding further supports that hypothesis that the M-MuLV enhancer triplication for Mo+PyF101 M-MuLV requires infection of the bone marrow, since s.c. inoculation does not result in efficient bone marrow infection or appearance of Mo+PyF101 M-MuLV LTRs with triplications. We have not systematically tested mice infected s.c. by Mo+PyF101 M-MuLV for the timing of appearance of the deleted LTRs.

As described above, less than 25% of the T lymphomas arising after i.p. inoculation of Mo+PyF101 M-MuLV contained proviral insertions in the vicinity of c-*myc*, *pim-1*, or *pvt-1*. One possible explanation for this could be that the PyF101 sequences in the Mo+PyF101 M-MuLV LTR altered its relative ability to activate these proto-oncogenes. Thus, it is possible that other proto-oncogenes known to be activated by M-MuLV in mice or rats might have been activated. Alternatively, other novel proto-oncogenes might have been involved. It is also theoretically possible that these tumors did not result from activation of proto-oncogenes, although the rapid time course of disease makes this unlikely.

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REFERENCES

1. **Belli, B., and H. Fan.** 1994. The leukemogenic potential of an enhancer variant of Moloney murine leukemia virus varies with the route of inoculation. J. Virol. **68:**6883–6889.
1a. **Belli, B., and H. Y. Fan.** Unpublished data.
1b. **Brightman, B. K., and H. Fan.** Unpublished data.
2. **Brightman, B. K., C. Farmer, and H. Fan.** 1993. Escape from in vivo restriction of Moloney mink cell focus-inducing viruses driven by the Mo+PyF101 long terminal repeat (LTR) by LTR alterations. J. Virol. **67:**7140–7144.
3. **Brightman, B. K., A. Rein, D. J. Trepp, and H. Fan.** 1991. An enhancer variant of Moloney murine leukemia virus defective in leukemogenesis does not generate detectable mink cell focus-inducing virus in vivo. Proc. Natl. Acad. Sci. USA **88:**2264–2268.
4. **Chatis, P. A., C. Z. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1983. Role for the 3′ end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. Proc. Natl. Acad. Sci. USA **80:**4408–4411.
5. **Davis, B., E. Linney, and H. Fan.** 1987. Characterization of a pre-leukemic state induced by Moloney murine leukemia virus: evidence for two infection events during leukemogenesis. Proc. Natl. Acad. Sci. USA **84:**4875–4879.
6. **Davis, B. R., K. G. Chandy, B. K. Brightman, S. Gupta, and H. Fan.** 1986. Effects of nonleukemogenic and wild-type Moloney murine leukemia virus on lymphoid cells in vivo: identification of a preleukemic shift in T-thymocyte subpopulations. J. Virol. **60:**423–430.
7. **Davis, B. R., E. Linney, and H. Fan.** 1985. Suppression of leukemia virus pathogenicity by polyoma virus enhancer. Nature (London) **314:**550–553.
8. **des Grosseillers, L. E. Rassart, and P. Jolicoeur.** 1983. Thymotropism of

murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. USA **80:**4193–4197.
9. **Fan, H. Y.** 1990. Influences of the long terminal repeats on retrovirus pathogenicity. Semin. Virol. **1:**165–174.
10. **Fan, H. Y., B. K. Brightman, B. R. Davis, and Q. X. Li.** 1991. Leukemogenesis by Moloney murine leukemia virus, p. 155–174. *In* H. Y. Fan et al. (ed.), Viruses that affect the immune system. ASM Press, Washington, D.C.
11. **Fan, H. Y., H. Chute, E. Chao, and P. K. Pattengale.** 1988. Leukemogenicity of Moloney murine leukemia viruses carrying polyoma enhancer sequences in the long terminal repeat is dependent on the nature of the inserted polyoma sequences. Virology **66:**58–65.
12. **Fan, H. Y., S. Mittal, H. Chute, E. Chao, and P. K. Pattengale.** 1986. Rearrangements and insertions in the Moloney virus long terminal repeat alter biological properties in vivo and in vitro. J. Virol. **60:**194–214.
13. **Hanecak, R., P. K. Pattengale, and H. Y. Fan.** 1988. Addition or substitution of simian virus 40 enhancer sequences into the Moloney murine leukemia virus (M-MuLV) long terminal repeat yields infectious M-MuLV with altered biological properties. J. Virol. **62:**2427–2436.
14. **Lenz, J., D. Celander, R. L. Crowther, R. Partarca, D. W. Perkins, and W. A. Haseltine.** 1984. Determination of the leukemogenicity of a murine retrovirus be sequences within the long terminal repeat. Nature (London) **308:**467–470.
15. **Li, Q. X., and H. Y. Fan.** 1991. Bone marrow depletion by ^{89}Sr complements a preleukemic defect in a long terminal repeat variant of Moloney murine leukemia virus. J. Virol. **65:**4442–4448.
16. **Li, Q. X., and H. Y. Fan.** 1991. Combined infection by Moloney murine leukemia virus a mink cell focus-forming virus recombinant induces cytopathic effects in fibroblasts or in long-term bone marrow cultures from preleukemic mice. J. Virol. **64:**3701–3711.
17. **Mukhopadhaya, R., and L. Wolff.** 1992. New sites of proviral integration associated with murine promonocytic leukemias and evidence for alternate modes of c-*myb* activation. J. Virol. **66:**6035–6044.
18. **Rowe, W. P., W. E. Pugh, and J. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42:**1136–1139.
19. **Selten, G., H. T. Cuypers, M. Zijlstra, C. Meleif, and A. Berns.** 1984. Involvement of c-myc in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. EMBO J. **3:**3215–3222.
20. **Storch, T. G., P. Arnstein, U. Manohar, W. M. Leiserson, and T. M. Chused.** 1985. Proliferation of infected lymphoid precursors before murine leukemia virus-induced T-cell lymphomas. J. Natl. Cancer Inst. **74:**137–143.
21. **Tabor, S., and K. Struhl.** 1988. Labelling of DNA by random oligonucleotide-primed synthesis, p. 3.5.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. I. John Wiley, New York.