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An enhancer variant of Moloney murine leukemia virus defective in leukemogenesis does not generate detectable mink cell focus-inducing virus *in vivo*

(preleukemia)

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ABSTRACT **Moloney murine leukemia virus (Mo-MuLV) induces T-cell lymphoma when inoculated into neonatal mice. This is a multistep process. Early events observed in infected mice include generalized hematopoietic hyperplasia in the spleen and appearance of mink cell focus-inducing (MCF) recombinants; end-stage tumors are characterized by insertional proviral activation of protooncogenes. We previously showed that an Mo-MuLV enhancer variant, Mo+PyF101 Mo-MuLV, has greatly reduced leukemogenicity and is deficient in induction of preleukemic hyperplasia. In this report, we have examined Mo+PyF101 Mo-MuLV-inoculated mice for the presence of MCF recombinants. In contrast to wild-type Mo-MuLV-inoculated mice, Mo+PyF101 Mo-MuLV-inoculated mice did not generate detectable MCF recombinants. This failure was at least partly due to an inability of the MCF virus to propagate *in vivo*, since a molecularly cloned infectious Mo+PyF101 MCF virus did not replicate, even when inoculated as a Mo+PyF101 Mo-MuLV pseudotype. These results show that the leukemogenic defect of Mo+PyF101 Mo-MuLV is associated with its inability to generate MCF recombinants capable of replication *in vivo*. This, in turn, is consistent with the view that MCF recombinants play a significant role in Mo-MuLV-induced disease and, in particular, may play a role early in the disease process.**

Moloney murine leukemia virus (Mo-MuLV) induces T-cell lymphoma in mice with a typical latency of 3–4 months. We previously described a preleukemic state in Mo-MuLV-inoculated mice characterized by generalized hemotopoietic hyperplasia, predominantly in the spleen (1). We also reported that a variant of Mo-MuLV, Mo+PyF101 Mo-MuLV, which contains polyoma F101 enhancer sequences inserted into the *U3* region of the Mo-MuLV long terminal repeat (LTR), was defective both for induction of preleukemic hyperplasia and for leukemogenesis (1, 2). Thus, Mo+PyF101 Mo-MuLV is a useful tool for investigating preleukemic events in Mo-MuLV-induced leukemogenesis, since it is apparently unable to perform some function necessary for disease induction. In view of the fact that Mo+PyF101 Mo-MuLV is an enhancer variant of Mo-MuLV, it seems possible that it is defective in its ability to infect some cell type in the animal required by wild-type Mo-MuLV for efficient induction of leukemia.

A previously reported feature of MuLV-induced disease is the appearance of *env* gene recombinant (mink cell focus-inducing or MCF) viruses at preleukemic times and in end-stage tumors (3–5). MCF viruses infect cells via a different receptor from that used by Mo-MuLV and are generated by recombination *in vivo* between the inoculated ecotropic MuLV and endogenous MuLV proviruses (3). The high concentration of MCF viruses in end-stage tumors has led to the suggestion that they are important late in leukemogenesis (6). Recent results from our laboratory have suggested a role for MCF recombinants early in leukemogenesis as well—i.e., in induction of preleukemic hyperplasia (7).

In the experiments reported here, the basis for the leukemogenic defect of Mo+PyF101 Mo-MuLV was investigated further. We report here that Mo+PyF101-inoculated animals do not contain detectable MCF recombinants and that an artificially constructed Mo+PyF101 MCF does not propagate when inoculated into newborn mice.

MATERIALS AND METHODS

Viruses and Inoculation of Mice. Wild-type and Mo+PyF101 Mo-MuLV have been described (2). All stocks were culture supernatants from productively infected NIH 3T3 cells. In some cases, productively infected NIH 3T3 cells were obtained by cocultivation of the NIH 3T3 cells with tumor thymocytes from inoculated mice for 24 hr in the presence of 2 μg of Polybrene per ml. Cultures were then washed extensively to remove thymocytes. Upon reaching confluence, they were passed twice (in the presence of 20 μg of Polybrene per ml for 1 hr after each transfer) to obtain confluently infected cells. Infectivity titrations of ecotropic Mo-MuLV were performed by the UV-XC plaque-forming assay (8) as follows: the infected cells were irradiated with ultraviolet light to inhibit further growth and overlaid with rat XC cells. Foci of multinucleate XC syncytia were counted to obtain the infectious titer of the virus. Titer of the Mo+PyF101 MCF producer cell line prior to superinfection with Mo+PyF101 ecotropic virus was $\approx 10^6$ reverse transcriptase-inducing units/ml. Viral stocks were inoculated subcutaneously at 2×10^4–2×10^5 XC plaque-forming units per animal.

Assay for Infectious MCFs. $S^+ L^-$ cells are transformed by a replication-defective murine sarcoma virus (MSV); when they are superinfected by a replication-competent MuLV, they exhibit a morphological response and also release infectious MSV particles with the host range of MuLV. The morphological response has been exploited in an infectivity assay for replication-competent MuLV (9).

Tissue culture supernatants were used to infect mouse $S^+ L^-$ cells by standard procedures (9). The titer of replication-competent MuLVs (Mo-MuLV and MCF virus) was determined from the number of foci after 5 days. To enumerate the foci specifically induced by MCF particles, we

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Abbreviations: Mo-MuLV, Moloney murine leukemia virus; MCF, mink cell focus-inducing; LTR, long terminal repeat.

UV-irradiated the assay plates and overlaid them with CCL64 mink cells. The plates were rescored for MCF-transformed mink cell foci after an additional 5 days. These foci, induced by murine sarcoma virus particles able to infect mink cells, arise from those original S^+ L^- foci that were induced by MCF virus particles.

DNA Analysis. DNA was extracted from single-cell suspensions of splenocytes and thymocytes as described (10). Briefly, cells were lysed in 1% sodium dodecyl sulfate (SDS), treated with 50 μg of proteinase K per ml overnight, and then extracted with phenol followed by chloroform. RNA was digested with 50 μg of RNase A per ml followed by a second round of phenol/chloroform extraction. DNA was then precipitated in 100% ethanol and resuspended in 10 mM Tris, pH 7.4/1 mM EDTA. Restriction enzyme digests, electrophoresis, and Southern blotting were performed as described in ref. 10 and in figure legends.

RESULTS

Limited Pathogenenicity of Mo+PyF101 Mo-MuLV. We have described (1, 2) an enhancer variant of Mo-MuLV, Mo+PyF101 Mo-MuLV, that was defective for induction of preleukemic hyperplasia and for leukemogenesis when inoculated into neonatal mice. Genomic organization of Mo+PyF101 Mo-MuLV compared with wild-type Mo-MuLV is shown in Fig. 1*a*. Mo+PyF101 Mo-MuLV was generated by molecular cloning and transfection, and it contains enhancers from the F101 variant of polyoma virus inserted at the *Xba* I site (base pair −150) in the *U3* region of the Mo-MuLV LTR, downstream from the Mo-MuLV enhancers (11). The structural genes of this virus are identical to wild-type Mo-MuLV.

We have reported (2) that Mo+PyF101 Mo-MuLV did not induce disease in NIH Swiss or NFS/N mice when inoculated subcutaneously, and no disease was observed over 17 months. Recently, in more extensive experiments in the same strains of mice, a low incidence of T-cell lymphomas was observed in Mo+PyF101 Mo-MuLV-inoculated animals after an extended latent period (Fig. 1*b*). Whereas 50% of wild-type Mo-MuLV-inoculated mice were moribund with leukemia at 14 weeks and 100% had died by 20 weeks, only 7% of Mo+PyF101 Mo-MuLV-inoculated mice developed leukemia at 14 weeks, and at 50 weeks only 62% had died. We are unable to account for the somewhat higher leukemogenicity of Mo+PyF101 Mo-MuLV in these later experiments in comparison with the original study, except that more animals were tested. Nevertheless, Mo+PyF101 Mo-MuLV should be viewed as having markedly decreased leukemogenicity compared with wild-type Mo-MuLV. Agar colony assays for hematopoiesis confirmed that the great majority of Mo+PyF101 Mo-MuLV-inoculated mice in the present study did not show splenic hyperplasia, consistent with our previous observations (1).

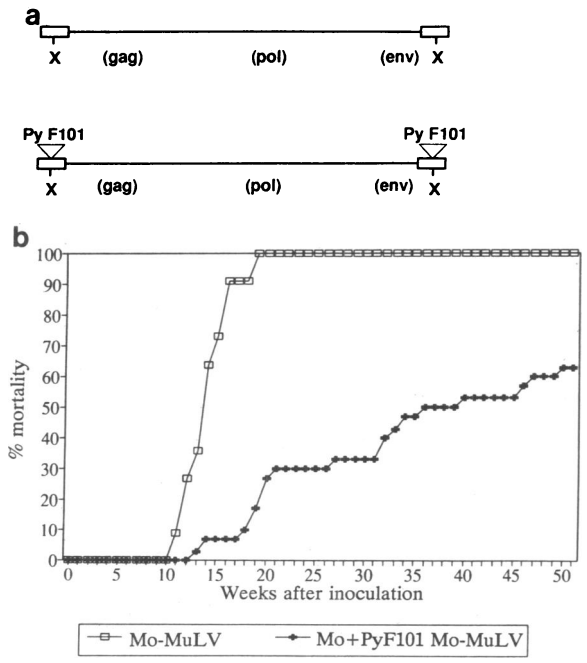

FIG. 1. Pathogenicity of Mo+PyF101 Mo-MuLV. (*a*) Proviral maps of Mo-MuLV (upper map) and Mo+PyF101 Mo-MuLV (lower map). Mo+PyF101 Mo-MuLV contains the *Pvu* II-4 enhancer fragment of polyoma virus F101 inserted in the *Xba* I site (X) of the Mo-MuLV LTR *U3* region (11). (*b*) Pathogenicity of Mo+PyF101 Mo-MuLV in NIH Swiss mice in comparison with wild-type Mo-MuLV after subcutaneous inoculation as neomates.

Absence of Detectable MCF Proviruses in Mo+PyF101 Mo-MuLV-Inoculated Mice. Since Mo+PyF101 Mo-MuLV-inoculated mice are defective for induction of preleukemic splenic hyperplasia (1), and since MCF recombinants have been implicated in induction of hyperplasia (7), we examined preleukemic mice inoculated with wild-type or Mo+PyF101 Mo-MuLV for the presence of MCF proviruses. Fig. 2 shows Southern blot analysis of DNAs taken at preleukemic times (4–10 weeks) from wild-type and Mo+PyF101 Mo-MuLV-inoculated mice. MCF recombinants from either wild-type or Mo+PyF101 Mo-MuLV would yield a unique 2.3-kilobase (kb) fragment after digestion with *Xba* I/*Bam*HI and hybridization with an MCF recombinant-specific probe (Fig. 2a). This MCF recombinant-specific band could be detected in 77% of wild-type Mo-MuLV-inoculated mice at preleukemic times (Fig. 2*b* and Table 1). In contrast, no MCF provirus was detected in 20 Mo+PyF101 Mo-MuLV-inoculated mice examined at these times. Since Mo+PyF101 and wild-type Mo-MuLV-infected mice develop similar levels of viral infection at preleukemic times (2, 12), the results suggested that Mo+PyF101 Mo-MuLV may be defective in generation or propagation of MCF recombinants.

Thymocytes from Mo+PyF101 Mo-MuLV-inoculated animals at 4–9 weeks were also cocultivated with NIH 3T3 fibroblasts, followed by serial passage to allow spread of low levels of virus. DNA from the cocultured and passaged NIH 3T3 cells also failed to reveal the presence of MCF recombinants by Southern blot analysis (not shown). Thus, by this more sensitive criterion, Mo+PyF101 Mo-MuLV did not generate MCF recombinants at preleukemic times.

Whereas MCF recombinants are often present at relatively low levels in preleukemic mice, end-stage Mo-MuLV-induced tumors generally have high levels of MCF proviruses (5, 13). As shown in Table 1 and Fig. 2*c*, five of five tumor DNAs from wild-type Mo-MuLV-inoculated mice showed the 2.3-kb MCF-specific proviral fragment at high concentrations. We also examined tumors arising at lower frequency and longer latency in Mo+PyF101 Mo-MuLV-inoculated mice (Fig. 2*c* and Table 1). Even in these tumors, no MCF recombinants could be detected.

In addition to the molecular detection of MCF recombinants, we also assayed Mo+PyF101 Mo-MuLV-induced tumors for infectious MCF recombinants. Tumor cells were cocultivated with NIH 3T3 cells to amplify putative MCF recombinants, and supernatants from these cultures were assayed for both infectious Mo-MuLVs and MCF viruses in a two-step mouse S^+ L^- cell assay. Two tumors induced by Mo+PyF101 Mo-MuLV produced no detectable infectious MCF recombinants in this assay, while one tumor induced by wild-type Mo-MuLV released an easily detectable level (Table 2). Thus, by both molecular and biological assays, we were unable to detect any MCF recombinants in Mo+PyF101 Mo-MuLV-inoculated mice.

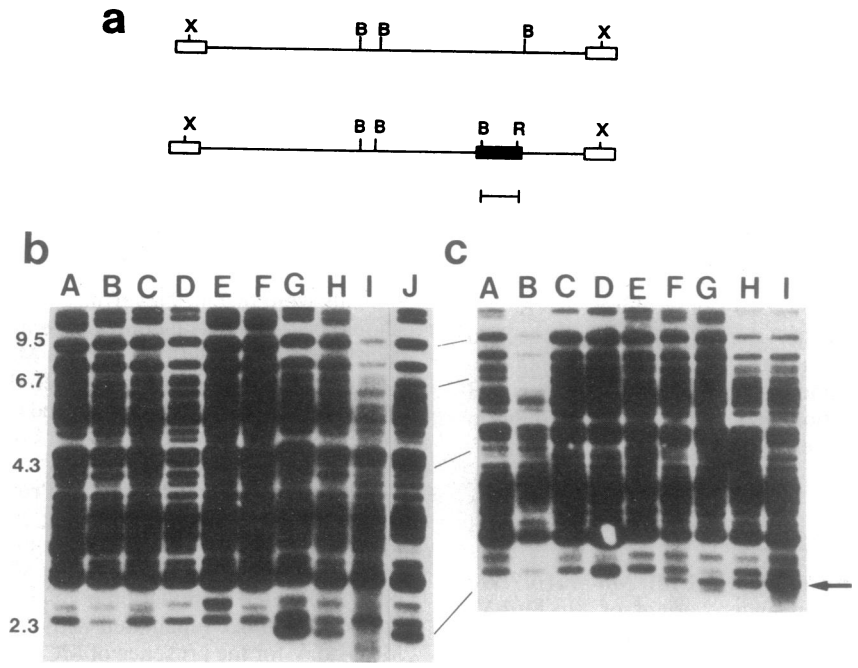

FIG. 2. Detection of MCF provirus by Southern blot analysis. (*a*) Restriction maps of wild-type Mo-MuLV (upper map) and expected Mo-MuLV MCF recombinant (lower map) derived from recombination between the inoculated ecotropic virus and endogenous polytropic sequences. MCF recombinants are detected by the diagnostic 2.3-kb *Bam*HI/*Xba* I fragment of the *env* region that hybridizes with the *Bam*HI–*Eco*RI MCF proviral probe indicated below the black box ( ). X, *Xba* I; B, *Bam*HI; R, *Eco*RI. (*b*) DNAs from preleukemic mice. DNA (10 μg) from preleukemic splenocytes or thymocytes was digested with *Xba* I/*Bam*HI and analyzed by gel electrophoresis and Southern blot hybridization using the *Bam*HI–*Eco*RI MCF provirus-specific probe. This probe also hybridizes with endogenous polytropic but not exogeneous ecotropic sequences. Lanes: A, uninoculated NIH Swiss mouse splenocyte DNA control; B–F, DNA from Mo+PyF101 Mo-MuLV-inoculated mice; G–J, DNA from wild-type Mo-MuLV-inoculated mice. Each lane represents a different animal. The MCF-specific *Xba* I–*Bam*HI band of 2.3 kb is indicated by an arrow in *c*. This band was detectable in DNA of lane I in other experiments. (*c*) DNAs from tumors. Cells from tumor-containing spleens or thymuses were analyzed as in *b*. Lanes: A, uninoculated NIH Swiss mouse DNA control; B–D, Mo+PyF101 Mo-MuLV-inoculated tumor DNAs; E, producer cell line for Mo-MuLV viral stock in which the MCF provirus-specific band is not detectable; F–I, wild-type Mo-MuLV-induced tumor DNAs showing the 2.3-kb MCF provirus-specific band (arrow).

Correlation Between MCF Virus Production and Pathogenesis of Mo-MuLV Enhancer Variants. The results of Fig. 2 show that the low pathogenicity of Mo+PyF101 Mo-MuLV is correlated with the absence of MCF recombinants because of the PyF101 enhancers in the Mo-MuLV LTR. To investigate this further, other Mo-MuLV LTR variants were studied. Mo+Pywt, Mo+Py441, Mo+SV, and Mo+HTLV-II$^+$ Mo-MuLVs contain heterologous enhancers from wild-type polyoma, polyoma F441, simian virus 40 (SV40), and human T-cell lymphotropic virus type II (HTLV-II) viruses, respectively, inserted into the Mo-MuLV LTR at the same site as Mo+PyF101 Mo-MuLV (see Fig. 1*a*) (14–16). These viruses induce disease in 100% of inoculated animals; the latent period for Mo+SV and Mo+HTLV-II$^+$ Mo-MuLVs is approximately equivalent to wild-type Mo-MuLV (15) (H. Kitado and H.F., unpublished data), whereas Mo+Py441 and Mo+Pywt Mo-MuLVs have latencies intermediate between Mo+PyF101 and wild-type Mo-MuLVs (14). The incidence of MCF recombinants in preleukemic and leukemic mice appeared to correlate with pathogenicities of the different viruses (Table 1). MCF recombinants were not detected in preleukemic Mo+Pywt and Mo+Py441 Mo-MuLV-inoculated mice. However, MCF recombinants were detected in three of seven Mo+Py441 Mo-MuLV-induced tumors. Six of

Table 1. Detection of MCF recombinants in mouse splenocytes and thymocytes by Southern blot analysis

Virus inoculated	Stage of disease	MCF recombinants detected*
Wild-type	Preleukemic	10/13
	Moribund	5/5
Mo+PyF101	Preleukemic	0/20
	Moribund	0/3
Mo+Py441	Preleukemic	0/2
	Moribund	3/7
Mo+Pywt	Preleukemic	0/3
	Moribund	6/6†
Mo+SV	Moribund	4/4
Mo+HTLV-II$^+$	Moribund	3/3

*MCF recombinants were detected by Southern blot analysis as described in the text. The number of mice in which MCF recombinants were detected out of the total number examined is indicated.
†Intensity of the MCF provirus-specific band was very faint for three of six Mo+Pywt Mo-MuLV-inoculated moribund animals, indicating a low level of MCF provirus.

Table 2. Detection of infectious virus in tumors from Mo-MuLV- and Mo+PyF101 Mo-MuLV-inoculated mice

Viral supernatant*	FIU × 10^{-5} per ml†	MCF virus, ‡ no. per ml
3T3/Mo-MuLV tumor no. 1	6	800
3T3/Mo+PyF101 Mo-MuLV tumor		
No. 1	1	<10
No. 2	2	<100

*Virus-containing supernatants were obtained from NIH 3T3 cells cocultivated with tumor thymocytes from Mo-MuLV- or Mo+PyF101 Mo-MuLV-inoculated mice.
†Focus-inducing units (FIU) (Mo-MuLV plus MCF virus) were determined in an S^+L^- focus assay as described in *Materials and Methods*.
‡Infectious MCF virus was determined by overlaying S^+L^- cultures with mink cells and assaying for secondary foci.

six Mo+Pywt Mo-MuLV-induced tumors were positive for MCF recombinants, although the intensities of the diagnostic band corresponded to considerably less than one copy per cell for three of the animals. In contrast, for Mo+SV and Mo+HTLV-II$^+$ Mo-MuLV-induced tumors, high levels of MCF virus were present in 100% of the cases. Thus, a correlation was observed for different viruses between pathogenicity and the efficiency of MCF virus generation, with Mo+PyF101 Mo-MuLV being at one extreme. Therefore, these data support the hypothesis that generation of MCF recombinants promotes rapid and efficient leukemogenesis.

Failure of Mo+PyF101 MCF Recombinants to Propagate in Mice. The lack of detectable MCF recombinants in Mo+PyF101 Mo-MuLV-inoculated mice could result from a failure to generate MCF recombinants *in vivo*. Alternatively, Mo+PyF101 Mo-MuLV might generate MCF recombinants, but they might not propagate in the animal. To distinguish between these possibilities, we generated an MCF recombinant of Mo+PyF101 Mo-MuLV by molecular cloning and transfection into NIH 3T3 cells (Fig. 3*a*). When a Mo+-PyF101 MCF stock was injected into NIH Swiss mice, no virus was detected at later times, and no disease developed (not shown). Thus, Mo+PyF101 MCF virus did not propagate in the animal, while the same MCF virus driven by a wild-type Mo-MuLV LTR propagated and was leukemogenic (not shown). In case an ecotropic Mo-MuLV might promote spread of an MCF derivative in the animal, Mo+PyF101 MCF provirus producer NIH 3T3 cells were super-infected with Mo+PyF101 Mo-MuLV to yield a Mo+PyF101 Mo-MuLV/Mo+PyF101 MCF virus mixture. This might resemble the mixture of ecotropic and MCF viruses present in animals early during generation of MCF recombinants by wild-type Mo-MuLV. Mo+PyF101 Mo-MuLV/Mo+PyF101 MCF virus-inoculated mice were examined for the presence of propagating Mo+PyF101 MCF viruses at 6–8 weeks. Southern blot analysis indicated that none of eight mice examined contained detectable Mo+PyF101 MCF provirus (Fig. 3*b*), although they did show the presence of Mo+PyF101 Mo-MuLV (not shown). Lack of Mo+PyF101 MCF provirus in the mice was not due to a defect in the cloned virus, since it could be detected in NIH 3T3 cells infected with the same pseudotypic mixture (not shown). These results confirmed that MCF recombinants are not detected in Mo+PyF101 Mo-MuLV-inoculated mice because they do not propagate efficiently in the animal.

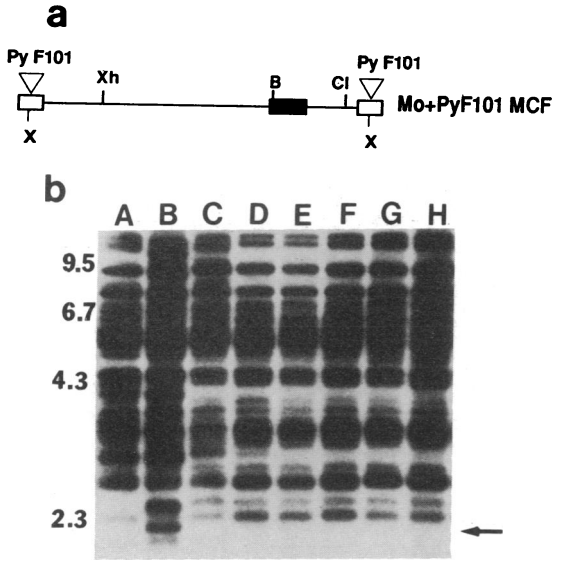

FIG. 3. Injection of Mo+PyF101 MCF recombinants into mice. (*a*) Map of molecularly cloned Mo+PyF101 MCF provirus. The *Xho* I–*Cla* I MCF proviral envelope-containing fragment of Mo-MCF$_I$-1 (17), a biologically active molecular Mo-MuLV MCF clone, was substituted into Mo+PyF101 Mo-MuLV plasmid DNA at corresponding restriction sites. X, *Xba* I; Xh, *Xho* I; B, *Bam*HI; Cl, *Cla* I. (*b*) Autoradiogram of Southern blots of DNAs from Mo+PyF101 Mo-MuLV/Mo+PyF101 MCF-inoculated 6- to 8-week-old mice, analyzed as in Fig. 2. Lanes: A, uninoculated NIH mouse DNA [An endogenous band migrating at ≈2.5 kb was missing (likely due to incomplete endonuclease digestion) but present when the same DNA was used in lane A of Fig. 2 *b* and *c*.]; B, Mo+PyF101 Mo-MuLV/Mo+PyF101 MCF virus producer cell line DNA showing the 2.3-kb MCF proviral-specific band (arrow); C–H, DNAs from thymus (lanes C, E, and G) and spleen (lanes D, F, and H) of three representative Mo+PyF101 Mo-MuLV/Mo+PyF101 MCF virus-inoculated mice.

DISCUSSION

We report here that inoculation of Mo+PyF101 Mo-MuLV into neonatal mice does not result in appearance of detectable MCF recombinants, either at preleukemic times or even in tumors that eventually develop. Presumably the addition of PyF101 enhancers to the Mo-MuLV LTR renders the virus incapable of productively infecting some cell type(s) that is an obligatory host of MCFs *in vivo*. These results suggest a basis for the leukemogenic defect of the Mo+PyF101 variant of Mo-MuLV.

We previously showed that Mo+PyF101 Mo-MuLV is defective in inducing preleukemic splenic hyperplasia (1). On the other hand, Mo+PyF101 Mo-MuLV is capable of establishing high-level infection in thymocytes, which suggests that it should be able to carry out later events in leukemogenesis such as insertional activation of protooncogenes (5, 18–20). The correlation between the inability of Mo+PyF101 Mo-MuLV to induce preleukemic hyperplasia and its failure to generate detectable MCF recombinants suggests a possible role for MCF recombinants early in the disease process. We recently proposed that preleukemic hyperplasia in the spleen may result from inhibitory effects on bone marrow hematopoiesis because of combined infection of bone marrow stroma with Mo-MuLV and MCF recombinant (7). The defect of Mo+PyF101 Mo-MuLV in generation of preleukemic hyperplasia and of MCF recombinants is completely consistent with this notion.

The importance of MCF viruses in efficient leukemogenesis was also supported by comparison of different Mo-MuLV enhancer variants (Table 1). Those variants that efficiently generated MCF recombinants were the most pathogenic, while those that could not were the least. With regard to the polyoma-containing variants, we previously showed that the exact organization of the PyF101 enhancers are important for the low leukemogenicity in that chimeric Mo-MuLV LTRs with related F441 and wild-type polyoma enhancers were not as tightly restricted for disease (14). These viruses showed intermediate levels of MCF recombinant generation, consistent with their pathogenicity.

The results (Fig. 3) also indicated that Mo+PyF101 Mo-MuLV-infected mice probably do not develop detectable MCF recombinants *in vivo* because MCF recombinants driven by a Mo+PyF101 LTR do not propagate efficiently in the animal. (However, they do not rule out the possibility that Mo+PyF101 Mo-MuLV may, in addition, be unable to generate MCF recombinants.) Initially this was somewhat surprising, particularly for the inoculated Mo+PyF101 Mo-MuLV/Mo+PyF101 MCF virus mixture. We have already shown that the Mo+PyF101 LTR is able to support virus expression in a number of cell types *in vivo*, including thymocytes, splenocytes, and hematopoietic progenitors (12, 21). Thus, if extensive pseudotype formation occurred in animals, Mo+PyF101 MCF should have been able to propagate as a pseudotypic mixture by infection of those cells. However, it is reasonable to suppose that the efficiency of

pseudotype formation is extremely low *in vivo* under the conditions of these experiments (since pseudotypes can only be produced by dually infected cells) and the effective multiplicity of infection in the animal is very low. A likely explanation for our results is that MCF viruses are normally targeted *in vivo* to certain cells with MCF virus receptors and that the Mo+PyF101 LTR is poorly active in those cells. Thus, a Mo+PyF101 MCF recombinant would not propagate efficiently in the animal, even if it were formed. It will be important to identify these MCF virus target cells, since they may be important in Mo-MuLV pathogenesis as well.

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