Rearrangements and Insertions in the Moloney Murine Leukemia Virus Long Terminal Repeat Alter Biological Properties In Vivo and In Vitro

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The effects of rearrangement and insertion of sequences in the Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR) were investigated. The alterations were made by recombinant DNA manipulations on a plasmid subclone containing an M-MuLV LTR. Promoter activity of altered LTRs was measured by fusion to the bacterial chloramphenicol acetyltransferase gene, followed by transient expression assay in NIH 3T3 cells. M-MuLV proviral organizations containing the altered LTRs were also generated, and infectious virus was recovered by transfection. Infectivity of the resulting virus was quantified by XC plaque assay, and pathogenicity was determined by inoculating neonatal NIH Swiss mice. Inversion of sequences in the U3 region containing the tandemly repeated enhancer sequences (–150 to –353 base pairs [bp]) reduced promoter activity approximately fivefold in the transient-expression assays. Infectious virus containing the inverted sequences (Mo[–] M-MuLV) showed a 20-fold reduction in relative infectivity compared with wild-type M-MuLV, but the virus still induced thymus-derived lymphoblastic lymphoma or leukemia in mice, with essentially the same kinetics as for wild-type M-MuLV. We previously derived an M-MuLV which carried inserted enhancer sequences from the F101 strain of polyomavirus (Mo+PyF101 M-MuLV) and showed that this virus is nonleukemogenic. In Mo+PyF101 M-MuLV, the PyF101 sequences were inserted between the M-MuLV promoter and the M-MuLV enhancers (at –150 bp). A new LTR was generated in which the PyF101 sequences were inserted to the 5' side of the M-MuLV enhancers (at –353 bp, PyF101+Mo M-MuLV). The PyF101+Mo LTR exhibited promoter activity similar (40 to 50%) to that of wild-type M-MuLV, and infectious PyF101+Mo M-MuLV had high infectivity on NIH 3T3 cells (50% of wild type). In contrast to the nonleukemogenic Mo+PyF101 M-MuLV, PyF101+Mo M-MuLV induced leukemia with kinetics similar to that of wild-type M-MuLV. Thus, the position of the PyF101 sequences relative to the M-MuLV LTR affected the biological behavior of the molecular construct. Furthermore, PyF101+Mo M-MuLV induced a different spectrum of neoplastic disease. In comparison with wild-type M-MuLV, which induces a characteristic thymus-derived lymphoblastic lymphoma with extremely high frequency, PyF101+Mo M-MuLV was capable of inducing both acute myeloid leukemia or thymus-derived lymphoblastic lymphoma, or both. Tumor DNA from both the PyF101+Mo[–] and Mo[–] M-MuLV-inoculated animals contained recombinant proviruses with LTRs that differed from the initially inoculated virus.

Retroviruses synthesize viral DNA via reverse transcriptase during infection (40, 41). A linear double-stranded viral DNA copy of the virion RNA is made which contains terminally repeated sequences, the long terminal repeats (LTRs). Viral DNA integration into the host chromosomal DNA yields the provirus, and the LTRs provide the regulatory signals for initiation and termination of viral transcription. These signals, located in the U3 region of the LTR, include promoter elements such as TATA and CCAAT sequences and also transcriptional enhancers (in the form of 75-base-pair [bp] tandem repeats) (21, 38). The U3 sequences provide more than simple promoter function. In particular, enhancer sequences of several retroviruses have been shown to influence their biological activities and pathogenicity (2, 5, 22).

In these experiments, the effects of rearrangements and insertions into the Moloney murine leukemia virus (M-MuLV) LTR were investigated. M-MuLV is a replication-competent retrovirus lacking an oncogene, which induces T-cell lymphoma or leukemia in mice. Two questions were addressed. First, the effect of inverting the enhancer se-

quences was studied. In general, enhancers have been reported to function in an orientation- and position-independent fashion (19). However, these experiments generally have been performed on plasmid DNA in transient-expression systems, rather than on genes integrated into normal chromatin. Retroviruses are convenient for this purpose, since the proviral DNA integrates into host chromatin during infection. Moreover, some nondefective murine and avian retroviruses induce neoplasms by integrating in the vicinity of a specific cellular gene and by increasing the expression of the cellular gene (3, 17, 23, 32). In several of these cases, the enhancer sequences are responsible for this activation; nevertheless, proviral integration is both orientation and position specific with respect to the host genome (28, 32, 34).

The second question addressed was the position effect of two enhancers on a promoter. In both viral and cellular systems, multiple enhancers and promoters may exist in a localized region (12, 27). Nevertheless, the patterns of expression are specific. Other workers have reported that the promoters nearest an enhancer are those which show activation. However, the relationship of relative enhancer position to promoter activity has not been systematically

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investigated. In this report, polyomavirus enhancer sequences were inserted into an M-MuLV LTR which contained its own enhancer sequences.

In these experiments, the altered M-MuLV LTRs were investigated at three levels. First, effects on LTR promoter activity were investigated by transfection and transient-expression assays. Second, effects on the general replication ability of the M-MuLV were investigated by recovering infectious M-MuLV and by measuring relative infectivity in permissive cells. Finally, the effects on pathogenicity were investigated by inoculating the viruses into animals.

MATERIALS AND METHODS

Cells and viruses. All cells were grown in Dulbecco-modified Eagle medium supplemented with 10% calf serum. NIH 3T3 cells have been described previously (36). Virus stocks were obtained by harvesting culture medium from confluent infected cultures, followed by removal of cells and cell debris by centrifugation at $1,200 \times g$ for 10 min. Aliquots of the clarified supernatant were stored at -80°C . Each aliquot was used only once after thawing.

M-MuLV infections were performed as described previously (8). Infectious M-MuLV was measured by the UV-XC syncytial plaque assay, as described by Rowe et al. (33). Assay for virus particles was done by reverse transcriptase enzymatic assay, using a poly(rA) · oligo(dT) template-primer complex (8).

Molecular cloning and transfections. Molecular cloning procedures, plasmid preparations, high-molecular-weight DNA isolation, and Southern blotting were done according to standard protocols (26). Detailed descriptions of similar clonings were reported by Overhauser and Fan (30).

Transfection of NIH 3T3 cells to recover infectious virus was performed as described previously (24). Transfections for transient assays and chloramphenicol acetyltransferase (CAT) enzyme assays were also performed essentially as described previously (24, 30).

Leukemogenicity experiments. Neonatal NIH Swiss mice were inoculated intraperitoneally with 2.5×10^4 XC PFU per animal. Animals were monitored for signs of disease, and moribund animals were killed. Fixed tissue specimens were appropriately sectioned and mounted for histopathological analysis (9, 31). High-molecular-weight DNA was extracted from other portions of tumor tissues.

Immunoperoxidase staining. The rat monoclonal antibody Mac-2 (10), kindly provided by Timothy Springer, was used at a dilution of 1:60 on deparaffinized, deparaffinized slides of tissue sections. After incubation, biotinylated rabbit anti-rat immunoglobulin G at a 1:30 dilution was added, followed by the addition of avidin-biotin peroxidase complex (Vector Laboratories, Inc., Burlingame, Calif.). Amino-ethyl carbonyl was used as the chromogen, giving a permanent brown reaction product that contrasted well with the hematoxylin counterstain.

RESULTS

Construction of altered M-MuLV LTRs. The altered M-MuLV LTRs are diagramed in Fig. 1A. The first alterations were designed to test if inversion of the enhancer sequences (present as tandem 75-bp duplications) resulted in a functional LTR or virus or both. To achieve this, a *Sau3A*-to-*XbaI* DNA fragment (from -353 to -150 bp) from a plasmid clone of the wild-type M-MuLV LTR was converted to an

XbaI fragment by ligation of an *XbaI* linker to the *Sau3A* site. This *XbaI* fragment then was introduced into a clone containing a deleted M-MuLV LTR, ΔMo . ΔMo lacks sequences from -357 to -150 bp and has been described previously (24). Introduction of the *XbaI* fragment in either orientation resulted in the Mo^+ and Mo^- LTRs. The Mo^+ LTR was actually very similar to the wild-type M-MuLV LTR, except that 4 bp (-357 to -353) to the 5' side of the 75-bp duplications was deleted and an *XbaI* linker was inserted at that site.

The second alteration was designed to test the influence of insertion position in M-MuLV LTRs containing polyomavirus regulatory sequences. The inserted polyomavirus sequences were derived from *PvuII* fragment 4 of the F101 host range mutant of polyomavirus (11). This fragment contains the "B" enhancer elements of polyomavirus (18, 27). The Mo^+ PyF101 LTR, which has been described previously (4), is also shown in Fig. 1A. In this LTR, the PyF101 sequences were inserted at the *XbaI* site between the M-MuLV enhancers and the M-MuLV promoter. The new LTR, PyF101+Mo, was constructed for these experiments; its PyF101 sequences were inserted into the Mo^+ LTR at the *XbaI* site 5' to the M-MuLV 75-bp repeats. Thus, the PyF101+Mo LTR had the polyomavirus and M-MuLV enhancer elements in the opposite order as in the Mo^+ PyF101 LTR.

These alterations (Fig. 1A) were generated from a plasmid subclone of M-MuLV, pMLV C/R/B (Fig. 1B) (24). Appropriate portions of the altered pMLV C/R/Bs were used to generate CAT expression plasmids or infectious virus as described below.

Promoter activity. To test promoter activity of the altered LTRs, LTR fusions to the bacterial CAT gene were generated. This was achieved by cloning the altered portions of the LTRs (as *ClaI*-to-*SacI* fragments) from the pMLV C/R/B derivatives into the corresponding position of an M-MuLV LTR-CAT expression plasmid, pM1CAT (30) (Fig. 1B). The CAT plasmids were then tested by transfection of NIH 3T3 cells and by transient assay for the CAT enzyme (13, 24, 30). To improve the quantification, different amounts of each CAT plasmid were transfected, and the assays were performed under conditions in which CAT enzyme activity was linear with respect to input DNA. The results of these assays are shown in Fig. 2 and Table 1.

In the transient-expression assays, the Mo^+ LTR showed almost the same promoter activity as did the wild-type M-MuLV LTR (67% of wild type). In contrast, the Mo^- LTR showed reduced although significant promoter activity (20%). Investigators in other systems have also observed that inversion of enhancer sequences may affect the magnitude of the enhancement in other systems (25). It should also be noted that the sequences between the 75-bp repeats and the *XbaI* site at -150 bp were also inverted and that their position with respect to the promoter in the Mo^- LTR also changed (see below). The PyF101+Mo LTR also showed high promoter activity (65%). We previously showed that the Mo^+ PyF101 LTR has promoter activity essentially equal to that of the wild type (24).

Generation of infectious M-MuLV containing the altered LTRs. Since all of the altered LTRs showed significant promoter activity, recovery of infectious M-MuLV containing the altered LTRs was carried out. NIH 3T3 cells were transfected with M-MuLV proviral DNA containing organizations with altered 3' LTRs. During subsequent rounds of viral infection in the transfected cultures, the alterations would be conferred to both 5' and 3' LTRs as a consequence

FIG. 1. Altered LTRs and recombinant cloning. (A) The organization of the altered LTRs is shown. Wild-type (wt) M-MuLV LTR is shown at the top. The R and U5 regions of the LTR are indicated; all other sequences are in the U3 region. Nucleotide numbering is from the transcriptional start site at the 5' end of the R region. The standard promoter elements (TATA and CCAAT homologies) are located between -80 and -30 bp. The tandemly repeated 75-bp enhancer sequences are indicated (▨). Abbreviations: Sm, *SmaI*; S, *SacI*; Xba, *XbaI*; S3a, *Sau3A*. The Δ Mo LTR has been described previously (Linney et al. [24]) and has a deletion from -357 to -150 bp. The deletion is accessible via an added *XbaI* site. The Mo⁺ LTR represents cloning of the *Sau3A*-to-*XbaI* fragment (via an *XbaI* linker at the *Sau3A* site) back into the Δ Mo LTR. The Mo⁻ LTR is similar to the Mo⁺ LTR, except that the *Sau3A*-to-*XbaI* fragment was inserted into the Δ Mo LTR in the opposite orientation. The Mo + PyF101 LTR represents insertion of *PvuII* fragment 4 of the F101 mutant of polyomavirus into the *XbaI* site of the wild-type M-MuLV LTR via *XbaI* linkers (4). The PyF101 + Mo LTR represents insertion of the same PyF101 *PvuII* fragment 4 into the Mo⁺ LTR at the 5' *XbaI* site. (B) The molecular cloning strategy is shown. Alterations were first generated in a plasmid subclone containing a single M-MuLV LTR, from a circularly permuted clone of M-MuLV DNA (pMLV-1A [1]), pMLV C/R/B (24). To generate infectious virus, we cloned the altered LTR as a *Clal*-to-*EcoRI* fragment into the 3' position of a molecular clone of integrated M-MuLV proviral DNA, p63-2 (24). Transfection of this clone yields proviruses with the altered LTR at both 5' and 3' sides in secondarily infected cells as a consequence of reverse transcription (24). This is because the alterations were in the U3 region of the LTR. For transient-expression assays, the altered portion of the LTR was transferred (as a *Clal*-to-*SacI* fragment) into pM1CAT. This plasmid consists of the M-MuLV LTR promoter sequences fused to the bacterial CAT gene (24). The pM1CAT plasmid also contained a synthetic polyadenylation site (TATAAA) to enhance efficiency of mRNA expression (25).

of retroviral reverse transcription (24). The general scheme for the transfections is also shown in Fig. 1B. For the Mo⁺ and Mo⁻ LTR transfections, the M-MuLV proviruses containing the altered 3' LTR were generated by in vitro ligation. For PyF101+Mo, a proviral plasmid containing a provirus with a wild-type 5' LTR and PyF101+Mo 3' LTR was generated and used in the transfection. In all three cases, transfection gave rise to infectious virus (measured by the XC syncytial plaque assay), and confluent infected cultures were obtained after serial transfer of the original transfections. Virus was then harvested from the infected cultures, and the relative infectivity was determined by measuring the amount of infectious virus (by XC plaque assay) as well as the amount of virus particles (by reverse transcriptase assay) in a standard volume. The results are shown in Table 2. Mo⁺ and PyF101+Mo M-MuLVs both had virtually normal infectivities (60 and 49% of the wild type, respectively). However, Mo⁻ M-MuLV had considerably reduced infectivity (6% of wild type). This general trend was consistent with the relative promoter activities of these

LTRs as measured by CAT assays, but the magnitude of the difference was significantly greater for Mo⁻ M-MuLV.

One potential problem could be the appearance of M-MuLV with wild-type LTRs through recombination during transfection (see below). However, restriction digestion indicated that the transfected NIH 3T3 cells only contained the desired viruses (see Fig. 5B and C, and 6B).

Leukemogenicity of the Mo⁺ and Mo⁻ M-MuLVs. The leukemogenicity of the altered M-MuLVs was tested by inoculating neonatal NIH Swiss mice intraperitoneally with 2×10^4 XC PFU per animal. Animals were monitored for appearance of disease, and moribund animals were subjected to necropsy. Histopathology was performed on fixed tumor tissue sections, and high-molecular-weight DNA was extracted from other portions of the same tumors. Mo⁺ and Mo⁻ M-MuLVs both induced disease, with time courses similar to that previously observed for induction by equivalent titers of wild-type M-MuLV (mean time of death, 10 to 12 weeks; Fig. 3a). Moribund animals showed typically enlarged spleens and thymuses. Interestingly, even though

FIG. 2. Transient-expression assays. CAT expression plasmids containing the different altered LTRs were transfected into NIH 3T3 cells, and CAT enzyme activity was measured as previously described (13, 24). In all cases, 20 μ g of calf thymus DNA was added as a coprecipitant during the transfection. Amounts of cell extracts were adjusted for equal A_{260} , and incubations were carried out for the same length of time (30 min at 37°C). The extent of chloramphenicol acetylation was determined by thin-layer chromatography, and the amount of acetylated and unacetylated chloramphenicol was quantified by scintillation counting. Incubation conditions were such that incorporation was linear with respect to time. Furthermore, the enzymatic activity was linear with respect to amount of transfecting DNA. Symbols: ●, wild-type (wt) LTR; ○, PyF101+Mo LTR; ▲, Mo⁺ LTR; △, Mo⁻ LTR.

Mo⁻ M-MuLV had a considerably lower relative infectivity than Mo⁺ M-MuLV in vitro (Table 2), it did not show reduced leukemogenicity. In fact, the animals inoculated with Mo⁻ M-MuLV actually developed disease somewhat more rapidly than those inoculated with Mo⁺ M-MuLV (72 versus 92 days), but the significance of this difference must be investigated further. Both viruses induced lymphoblastic lymphoma (Table 3 and Fig. 4A). Mice inoculated with Mo⁺ M-MuLV developed a characteristic thymus-derived lymphoblastic lymphoma (TLL) typical of wild-type M-MuLV. Figure 4A illustrates the histologic appearance of these TLLs. They were composed of uniform, monomorphic, medium-size malignant lymphoblasts with round-to-oval nuclei and scant cytoplasm (31). Mo⁻ M-MuLV also induced a similar type of lymphoblastic lymphoma in all animals examined, although there were suggestive minor differences (see below). These results suggested that inversion of the region containing the enhancer sequences did not

TABLE 1. Promoter activity of altered LTRs by transient-expression assay

Expt	Plasmid ^a	% Acetylation ^b at following amt (μg):			% Wild-type ^c activity
		1	2	5	
1	pM1CAT	8	20	55	100
	pMo ⁺ CAT	6	15	40	74
	pMo ⁻ CAT	2	4	13	23
	pPyF101+MoCAT	4		45	81
2	pM1CAT	12	25	75	100
	pMo ⁺ CAT	4	13	62	60
	pMo ⁻ CAT	2	4	12	16
	pPyF101+MoCAT	4	11	42	50

^a The organization of the CAT expression plasmids is shown in Fig. 1. pM1CAT contains the wild-type M-MuLV LTR fused to the bacterial CAT gene.

^b Enzymatic assays for CAT activity were performed as described in the text and in the legend to Fig. 2. Background levels of chloramphenicol acetylation in the absence of cell extract were less than 0.1%.

^c CAT enzyme expression for each construct relative to wild-type M-MuLV (pM1CAT) is shown. Values represent averages of the relative values at the three different transfecting DNA concentrations.

alter the biological activity of the Mo⁻ M-MuLV construct, either with respect to the ability to induce disease or with respect to the nature of the induced neoplasm.

To study the nature of the M-MuLV infecting the tumor cells, DNA from thymic tumors induced by the two viruses also was investigated by Southern blotting. For these experiments, DNA samples were digested with *Xba*I endonuclease, and blots were probed with a nick-translated DNA fragment corresponding to the M-MuLV *env* region (Fig. 5A). *Xba*I cleaves M-MuLV proviral DNA in the LTRs, as well as internally, and generates a 2.35-kilobase-pair (kb) *env*-containing fragment from each integrated provirus (37). In Mo⁺ or Mo⁻ M-MuLV proviral DNA, the corresponding *Xba*I fragment would be 2.15 kb because of the presence of the new *Xba*I site at -357 bp in the LTR. Thus, *Xba*I digestion could be used to test rapidly for the presence of the

TABLE 2. Biological activity of M-MuLVs containing altered LTRs

LTR	Titer of infectious virus ^a	No. of virus particles (reverse transcriptase assay) ^b	Infectivity/particle ratio ^c	Normalized infectivity ^d
WT	7.2×10^6	3.7×10^5	19.4	1
Mo ⁺	4.2×10^6	3.6×10^5	11.6	0.60
Mo ⁻	3.2×10^5	2.8×10^5	1.14	0.06
PyF101+Mo	1.4×10^6	1.4×10^5	10.0	0.52
Mo+PyF101				0.20 ^e

^a Tissue culture supernatant was harvested from infected cells in standard conditions (10 ml of medium incubated with 10^6 infected cells for 24 h). The concentration of infectious virus was determined by titration of NIH 3T3 cells, using the UV-XC assay (33). WT, Wild type.

^b Portions of the same culture supernatants used for infectivity measurements were concentrated by centrifugation, and the amount of virus particles was quantified by reverse transcriptase assay (8). Values represent dTTP counts per minute incorporated in a 1-h reaction per milliliter of supernatant. Typical incorporation values ranged from 10^4 to 10^5 cpm. Assay of supernatant from uninfected cells was less than 2,000 cpm.

^c Ratios were determined by dividing the values for infectious titer by the reverse transcriptase values for the same supernatant.

^d Relative infectivities with respect to the infectivity of wild-type M-MuLV (assigned a value of 1) are shown.

^e The relative infectivity of Mo+PyF101 was not determined in this experiment, but the value reported previously (4) is shown.

FIG. 3. Pathogenicity of variant M-MuLVs. Neonatal NIH Swiss mice were inoculated intraperitoneally with 2×10^4 XC PFU of the variant viruses. Animals were monitored for evidence of disease, and moribund animals were killed and were subjected to necropsy. In all cases, at least two litters of animals were inoculated. Symbols: (a) ●, Mo⁺ M-MuLV; ○, Mo⁻ M-MuLV; (b) ●, PyF101+Mo M-MuLV. Abbreviation: d, days.

originally inoculated virus in the tumors. All eight animals inoculated with Mo⁺ M-MuLV which were examined showed the *Xba*I fragment which is diagnostic for Mo⁺ M-MuLV in the tumors. In contrast, all of the animals inoculated with Mo⁻ M-MuLV (Fig. 5C) showed altered forms of M-MuLV in the final thymic tumors, as evidenced by novel-size *Xba*I fragments. Only one animal out of eight showed any evidence for the original Mo⁻ M-MuLV provirus in the tumor. It is likely that the novel derivatives of M-MuLV in the tumors arose by recombination in the animal

TABLE 3. Pathology of inoculated mice

Virus	No. of animals with tumors/no. of animals examined	Mean time of death (day) ^a	Tumor type
Mo ⁺ M-MuLV	13/16 ^b	92	13/13 Lymphoblastic lymphoma
Mo ⁻ M-MuLV	10/10	74	10/10 Lymphoblastic lymphoma
PyF101+Mo M-MuLV	9/9 ^c	102	6/11 Lymphoblastic lymphoma 5/11 AMMoL

^a Mean time of death was taken from the data in Fig. 4. Measurements included animals which were not subjected to the histopathologic evaluation shown.

^b Three animals had no apparent pathology at time of sacrifice.

^c Two animals each had both lymphoblastic lymphoma and acute myelomonocytic leukemia (AMMoL). Therefore, 11 tumors were found in the nine animals: Six animals developed lymphoblastic lymphoma, and five animals developed AMMoL.

FIG. 4. Histopathology of M-MuLV-induced malignant disease. (A) Characteristic TLL from a Mo⁺ M-MuLV-inoculated animal. Note the relatively uniform monomorphic population of intermediate-size malignant lymphoblasts with round nuclei, primitive chromatin, and scant cytoplasm. Lymphoblastic lymphomas from Mo⁻ M-MuLV-inoculated mice were similar in appearance but had occasional areas of lymphoblasts with irregular nuclei and detectable cytoplasm (not shown). Hematoxylin and eosin staining of an involved lymph node is shown. Bars in all three panels represent 20 μm. (B) Characteristic appearance of AMMoL in mice inoculated with PyF101+Mo M-MuLV. Note predominance of a nonuniform, pleomorphic large myeloid cell population, often with oval-to-irregular nuclei and conspicuous cytoplasm. The chromatin, although immature, is characteristically clumped, and there are prominent nuclei. Hematoxylin and eosin staining of an involved lymph node is shown. (C) Immunoperoxidase staining of AMMoL (B) for the presence of Mac-2 antigen. Note the positive (black) intracytoplasmic staining in a significant proportion of malignant myeloid cells. Hematoxylin and eosin counterstaining of an involved lymph node is shown.

FIG. 5. Proviral DNA in tumors induced by Mo⁺ and Mo⁻ M-MuLVs. (A) Restriction map of wild-type, Mo⁺, and Mo⁻ M-MuLV viral DNAs. Because of the *Xba*I site at -357 bp in the Mo⁺ and Mo⁻ LTRs, the 2.35-kb internal 3' *Xba*I fragment is reduced to 2.15 kb. The region from which the *env*-specific hybridization probe was obtained (the internal *Hpa*I fragment from 5.82 to 7.20 kb is indicated (▨)). (B) Mo⁺ M-MuLV tumors. High-molecular-weight DNA from thymic tumors of Mo⁺ M-MuLV-inoculated animals was digested with *Xba*I, and was analyzed by agarose gel electrophoresis and Southern blotting with the *env*-specific probes. Lanes: a, uninfected NIH 3T3 cell DNA; b to i, tumor DNAs from eight different animals; j, DNA from NIH 3T3 cells infected with Mo⁺ M-MuLV; k, DNA from NIH 3T3 cells infected with wild-type M-MuLV. The position of the wild-type 2.35-kb and Mo⁺ 2.15-kb fragments is indicated. Hybridization in lane a results from endogenous M-MuLV-related sequences present in uninfected mouse cells (6). Although the autoradiographic exposure was light, the 2.15-kb diagnostic fragment was present in all tumor DNAs. (C) Mo⁻ M-MuLV tumors. Lanes: a, uninfected NIH 3T3 cell DNA; b to i, similar analysis of thymic tumor DNA from eight different Mo⁻ M-MuLV-inoculated animals; j, Mo⁻ M-MuLV-infected NIH 3T3 DNA; k, DNA from NIH 3T3 cells infected with wild-type M-MuLV.

between the input Mo⁻ M-MuLV and endogenous MuLVs. Interestingly, two specific recombinations with endogenous virus apparently took place, since independent tumors from different animals showed the same sizes of *Xba*I fragments. It should also be noted that the novel M-MuLV proviruses in

the Mo⁻ M-MuLV-induced tumors did not simply reflect contamination with a low level of wild-type M-MuLV in the original inoculum. Both novel *Xba*I fragments were distinguishable in electrophoretic mobility from the 2.5-kb fragment of wild-type M-MuLV.

FIG. 6. Proviral DNA in PyF101+Mo M-MuLV-induced tumors. (A) Restriction map of wild-type and PyF101+Mo M-MuLV DNAs. Insertion of the PyF101 DNA sequences results in a larger *Smal* fragment from PyF101+Mo M-MuLV (2.28 versus 2.06 kb). *XbaI* digestion of PyF101+Mo M-MuLV DNA yields the same 2.15-kb 3' fragment as for Mo⁺ M-MuLV. The hybridization probe was the same as for Fig. 5. (B) Tumor DNAs were digested with *Smal* and analyzed by Southern blotting with the *env*-specific probe. Lanes: a, uninfected NIH 3T3 DNA; b to i, tumor DNAs; j, PyF101+Mo M-MuLV-infected NIH 3T3 DNA; k, wild-type M-MuLV-infected NIH 3T3 DNA. Positions of the 2.28- and 2.06-kb bands are indicated. Most tumors show the diagnostic 2.28-kb fragment, although high concentrations of a fragment with mobility similar to that of the wild-type M-MuLV 2.06-kb fragment were generally observed. (C) The same DNAs as in panel B were digested with *XbaI* and were analyzed in the same fashion. The great majority of tumors showed diagnostic 2.15-kb *XbaI* fragments, although larger fragments were also observed. Several tumors (e.g., lanes b to d) showed high concentrations of larger 3' *XbaI* fragments. Some of these might result from MCF-type proviruses.

Leukemogenicity of PyF101+Mo M-MuLV. PyF101+Mo M-MuLV also induced leukemia in inoculated animals, with a time course within the range observed for wild-type M-MuLV (102 days; Fig. 3b). This was in striking contrast to Mo+PyF101 M-MuLV, which is nonleukemogenic (4). Thus, the relative positions of the M-MuLV and PyF101

enhancers in these viruses were important in determining their biological activity. Furthermore, PyF101+Mo M-MuLV had an altered disease spectrum, in that acute myelomonocytic leukemia (AMMoL) or lymphoblastic lymphoma or both were induced (Table 3 and Fig. 4). Of nine animals examined, six showed characteristic TLL (Fig. 4A),

FIG. 7. Provirus in preleukemic animals inoculated with PyF101+Mo M-MuLV. Infectious virus was recovered from two PyF101+Mo M-MuLV-inoculated animals 3 weeks after neonatal inoculation. This was done by preparing a thymocyte cell suspension and cocultivating the cells with NIH 3T3 cells. After a 24-h incubation, the thymocytes were removed and the NIH 3T3 cells were transferred (two passages) until they showed uniform infection by XC cell assay. High-molecular-weight DNA then was prepared from the infected NIH 3T3 cells and was analyzed by *Sma*I digestion and Southern blotting as described earlier. Lanes: a, uninfected NIH 3T3 DNA; b and c, DNA from cells infected by the recovered virus; d, PyF101+Mo M-MuLV-infected NIH 3T3 DNA; e, wild-type M-MuLV-infected cell DNA. Virus recovered from the preleukemic animal only showed the *Sma*I fragment diagnostic for PyF101+Mo M-MuLV DNA at this autoradiographic exposure. Prolonged exposure indicated very low levels of provirus with recombinant LTRs.

and five showed AMMoL (Fig. 4B and C). Strikingly, two animals each had both a TLL as well as an AMMoL. In contrast to the TLLs (Fig. 4A), the AMMoLs were composed of nonuniform pleomorphic populations of primitive cells with moderate amounts of cytoplasm and irregularly shaped nuclei (Fig. 4B). Occasionally, more differentiated (less primitive) myeloid cells (e.g., myelocytes and metamyelocytes) were observed. While Fig. 4B shows routine histologic staining of an AMMoL, Fig. 4C shows immunoperoxidase staining of an AMMoL with a monoclonal antibody to the Mac-2 antigen. Mac-2 is present on normal monocytes, macrophages, and some granulocytes (10), and there was positive staining of granulocytic and monocytic cells within the tumor. Thus, the Mac-2-positive staining confirmed the routine morphological identification of the tumors as myeloid. Another characteristic difference between the TLLs and the AMMoLs was evident. TLLs typically involved the white pulp of the spleen and uniformly replaced the entire lymph node architecture in a diffuse manner. In contrast, AMMoL primarily involved the red pulp of the spleen and usually replaced the medullary and paracortical (inner) regions of the lymph nodes.

Southern blotting of tumor DNA from PyF101+Mo M-MuLV-inoculated animals was also performed. In this case, digestion was done first with *Sma*I, and hybridization was done with the *env*-specific probe. *Sma*I digestion yielded a

2.06-kb *env*-specific fragment from wild-type M-MuLV provirus, while it yielded a 2.28-kb fragment from PyF101+Mo M-MuLV provirus due to the inserted PyF101 sequences (Fig. 6A). The results of Southern blotting with *Sma*I are shown in Fig. 6B. Most of the tumors studied showed several proviruses with *Sma*I fragments different from those in the inoculated PyF101+Mo M-MuLV; most appeared to have eliminated sequences. However, detectable levels of the diagnostic 2.35-kb *Sma*I fragment for input virus were present in most of the tumors. The tumor DNAs also were digested with *Xba*I and were analyzed (Fig. 6C). The same diagnostic *Xba*I fragment typical of Mo⁺ and Mo⁻ M-MuLVs should also result from PyF101+Mo proviruses. The *Xba*I analysis also indicated the presence of new recombinant M-MuLV proviruses in the tumors (Fig. 6C). In contrast to the results for the Mo⁻ M-MuLV tumors, all tumors still contained detectable amounts of the diagnostic 2.15-kb *Xba*I fragment.

Since the end-stage tumors in the PyF101+Mo M-MuLV-inoculated animals demonstrated high concentrations of recombinant virus, it was important to investigate the nature of virus in preleukemic animals. Infectious virus was recovered from an animal 3 weeks after inoculation by cocultivating thymocytes (which released infectious virus) with NIH 3T3 cells, and by serially passaging the NIH 3T3 cells until they were uniformly infected. Southern blotting of the infected NIH 3T3 cells is shown in Fig. 7. Only the 2.28-kb *Sma*I fragment which is diagnostic for the inoculated PyF101+Mo M-MuLV was evident. Thus, the recombinant derivatives present in the final tumors were apparently not present at high levels 3 weeks after inoculation.

DISCUSSION

In these experiments, the effects of rearrangement and insertion of sequences within the U3 region of the M-MuLV LTR were investigated. Two altered viruses, Mo⁺ and Mo⁻ M-MuLVs, were used to test inversion of the enhancer sequences. The Mo⁻ LTR demonstrated promoter activity, and yielded infectious virus. In comparison with the Mo⁺ LTR, the Mo⁻ LTR showed lower promoter activity as measured by transient-expression assay, and the effect was even more pronounced when the specific infectivity of the resulting virus was examined. Nevertheless, these sequences could function in the inverted orientation, as would be expected for enhancers.

The quantitative difference between the efficiency of the Mo⁺ and Mo⁻ LTRs was not initially expected, given the orientation-independent nature of enhancers. As mentioned above, in other model systems enhancers also give different levels of enhancement, depending on orientation and position with respect to a given promoter. The important result is that these sequences showed significant enhancement even when their orientation was inverted. Another fact also could have affected the relative levels of promoter activity for the Mo⁺ and Mo⁻ LTRs. The *Sau*3A-to-*Xba*I fragment that was inverted also contained sequences between the 75-bp repeats and the *Xba*I site at -150 bp. Laimins et al. (20) and Graves et al. (15) reported that these sequences, which are rich in deoxyguanosine and deoxycytidine residues (G + C rich), are important for function in the Moloney murine sarcoma virus LTR in transient assays. Furthermore, the Moloney murine sarcoma virus G + C-rich sequences show nucleotide sequence homology to the G + C-rich region of the simian virus 40 early promoter which binds transcription factor Sp-1 (7). We studied internal deletions within the

M-MuLV LTR encompassing this region and found that such deletions showed relatively little effect on promoter activity in transient-expression assays, at most a fivefold reduction (R. Hanecak, S. Mittal, B. Davis, and H. Fan, manuscript in preparation). As viruses, however, these deletions showed a large reduction in infectivity (20- to 100-fold). In the Mo⁻ LTR, the G + C-rich sequences were both inverted and were moved to the other side of the 75-bp repeats, approximately 150 bp away from the promoter.

Inversion of the enhancer sequences in Mo⁻ M-MuLV did not decrease the frequency or rate at which inoculated animals developed leukemia. Animals inoculated with Mo⁻ M-MuLV developed TLL, which was morphologically similar to the classical lymphoblastic lymphoma typical of wild-type or Mo⁺ M-MuLV. However, it should be noted that subtle morphological differences were observed for the Mo⁻ M-MuLV-induced tumors (more irregular nuclei and somewhat more cytoplasm [scant to moderate] than wild-type or Mo⁺ M-MuLV-induced tumors [data not shown]). This raises the possibility that the Mo⁻ M-MuLV-induced tumors represent a different stage of T-lymphoid cell differentiation. Further molecular and antigenic studies may shed light on this possibility. It also should be noted that the great majority of Mo⁻-inoculated animals showed evidence for two recombinant viruses in end-stage thymic tumors. The fact that different independent tumors appeared to show the same recombinant viruses suggests that they arose by relatively specific recombination with one or a few endogenous retroviral sequences expressed in NIH Swiss mice. It should be noted that each of the recombinant viruses was present exclusively in the tumor tissue of one animal (Fig. 5). It will be very interesting to isolate and further examine the recombinant viruses present in these tumors.

The other virus studied here was PyF101+Mo M-MuLV, which contained regulatory sequences from the F101 variant of polyomavirus. The PyF101+Mo LTR showed high levels of promoter activity and yielded M-MuLV with infectivity essentially equal to that of the wild-type virus. PyF101+Mo M-MuLV was generated for comparison with Mo+PyF101 M-MuLV, which is completely nonleukemogenic (4). In contrast, PyF101+Mo M-MuLV induced leukemia at a rate characteristic of wild-type M-MuLV. Thus, the relative position of the regulatory elements in the M-MuLV LTR strongly influenced the biological activity. The results are consistent with the enhancer sequences closer to the promoter having the dominant effect: in PyF101+Mo M-MuLV, the M-MuLV enhancer sequences were closer to the promoter than were the PyF101 sequences; in Mo+PyF101 M-MuLV (and also in a virus which contained only the PyF101 sequences, Δ Mo+PyF101 M-MuLV), the PyF101 enhancers were proximal to the M-MuLV promoter. Presumably, the PyF101 enhancers were nonfunctional in some critical cell type for which infection is necessary to develop leukemia. It is also of considerable interest that PyF101+Mo M-MuLV-inoculated animals developed both AMMoLs or TLL or both.

Examination of proviral DNA in tumors induced by PyF101+Mo M-MuLV by *Sma*I digestion indicated that most of the virus present in the final tumors was different from the initially infecting virus. The altered spectrum of induced disease might be related to the derivatives of the initially infecting PyF101+Mo M-MuLV. It will be interesting to isolate and compare the recombinant viruses from the PyF101+Mo M-MuLV-induced lymphoid and myeloid tumors and also to compare them with the recombinant viruses in thymic tumors induced by Mo⁻ M-MuLV.

The biological behavior of these variant viruses provides some insight into the process of M-MuLV-induced leukemogenesis. Previously, others have identified two events involved in MuLV-induced leukemogenesis by analyzing end-stage tumors: (i) generation of viruses recombinant in the envelope glycoprotein region in mink cell focus-forming (MCF) viruses (16, 39, 42), and (ii) chromosomal insertion of proviruses adjacent to specific cellular loci (e.g., *c-myc* and *c-pim*), which results in increased expression of these host genes (3, 23, 34). These events apparently occur at a relatively late stage of leukemogenesis (3, 29). Others have documented a virus-induced preleukemic stage in MuLV-inoculated animals (29, 35). Storch et al. (35) showed that M-MuLV induces a preleukemic state in the spleen, in which hyperplasia and increased numbers of hematopoietic cells (lymphoid, erythroid, and myeloid) of most lineages occur. Interestingly, the nonleukemogenic Mo+PyF101 M-MuLV fails to induce at least some of the preleukemic changes but can still infect mature thymocytes *in vivo* or *in vitro* (B. R. Davis, K. G. Chandy, B. K. Brightman, S. Gupta, and H. Fan, J. Virol., in press). Taken together, these results suggest two distinct stages for M-MuLV-induced leukemogenesis. The first stage (perhaps involving infection of hematopoietic stem cells) results in hematopoietic hyperplasia of several different lineages. The second stage involves reinfection (perhaps via MCF virus derivatives) and site-specific proviral insertion in hyperplastic lymphoid progenitors which have migrated to the thymus. Outgrowth of monoclonal T-lymphoid tumors would result from the second stage; T-lymphoid disease would reflect the specific ability of the M-MuLV LTR to function and to activate oncogenes in T-lymphoid cells (3, 5, 22).

The two-stage model for M-MuLV leukemogenesis suggests an explanation for the biological behavior of PyF101+Mo M-MuLV. It seems likely that this virus can induce the first stage of hematopoietic hyperplasia, since it causes leukemia. However, the altered pathogenic spectrum may reflect an inefficiency of the altered LTR in activating oncogenes in differentiated thymocytes. Rather, recombinant derivatives may be able to efficiently activate oncogenes in myeloid cells. Alternatively, the Mo+PyF101 itself may be more active in myeloid cells than the wild-type M-MuLV LTR.

An alternative explanation for the behavior of these viruses is that the recombinant derivatives formed first, and then induced both stages of the disease. In this case, a delay in disease appearance might be expected. However, the time course data of Fig. 4 and the results shown in Fig. 7 do not argue strongly in favor of this. It will be important to study in detail the viruses present in preleukemic mice.

The absence or low levels of input Mo⁻ and PyF101+Mo M-MuLV proviruses in the end-stage tumors (Fig. 5 and 6) was somewhat surprising, if they were directly involved in tumorigenesis. However, MuLV-induced tumors generally contain multiple copies of integrated provirus, and the analyses (Fig. 5 and 6) might have missed one or a few copies of input provirus. It will also be important to investigate the relationship of the recombinant viruses in the end-stage tumors to classical MCF viruses. Some MCF proviruses would be expected (39, 42), but preliminary experiments indicate that the recombinant viruses which constitute the majority in the Mo⁻ and PyF101+Mo M-MuLV-induced tumors are not classical Moloney MCF viruses. Low numbers of MCF proviruses in the tumors might also have escaped detection (Fig. 5 and 6).

An unusual phenomenon was observed during these ex-

periments. Recombinant DNA clones of M-MuLV provirus which contained the PyF101+Mo LTRs at both 5' and 3' ends were generated and were transfected in NIH 3T3 cells. This was done because transfection with recombinant proviruses carrying a wild-type M-MuLV LTR at the 5' end and an altered LTR at the 3' end can occasionally give rise to wild-type M-MuLV (30). In other experiments, this difficulty was circumvented by generating the proviral DNA clones with altered LTRs at both ends (30). However, when recombinant DNA clones containing two PyF101+Mo LTRs were transfected into NIH 3T3 cells, infectious virus was never recovered. In contrast, transfection with proviral DNA containing the wild-type M-MuLV LTR at the 5' end and the PyF101+Mo LTR at the 3' end yielded infectious virus with high efficiency, and the resulting virus was indeed PyF101+Mo M-MuLV. Perhaps cells which contain the high levels of PyF101+Mo LTR DNA resulting from DNA transfection are unable to express it, while infected cells (which generally contain only a few copies of the proviral DNA) can express the same LTR. One possible explanation could be the titration of factors which interact with the LTRs (14).

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