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

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Moloney murine leukemia virus (M-MuLV) is a replication-competent retrovirus which induces T-cell lymphoma in mice. The enhancer sequences present within the M-MuLV long terminal repeat (LTR) region of the proviral genome have been shown to influence the disease specificity of the virus strongly. We examined the contribution of the M-MuLV enhancers to the transcriptional activity and pathogenesis of M-MuLV by constructing LTRs containing heterologous enhancer elements. The simian virus 40 enhancer region (72- and 21-base-pair repeats) was inserted into the U3 region (at -150 base pairs) of the M-MuLV LTR (Mo+SV) and also into a deleted form of the LTR which lacks the M-MuLV enhancer sequences (Δ Mo+SV). These chimeric LTRs were used to generate infectious M-MuLVs by transfection of corresponding proviral plasmids into mouse fibroblasts. The relative infectivities of Mo+SV and Δ Mo+SV recombinant viruses as determined by rat XC cell plaque assay and reverse transcriptase assay were 60 to 70% of wild-type M-MuLV levels. To study the pathogenicity of these two recombinant viruses, we inoculated newborn NIH Swiss mice with either Mo+SV or Δ Mo+SV M-MuLV. Both viruses induced disease more slowly than M-MuLV, which induces disease 2 to 4 months postinoculation. Mo+SV M-MuLV-inoculated animals became moribund at 3 to 13 months postinoculation, whereas Δ Mo+SV M-MuLV-inoculated animals became moribund at 6 to 24 months postinoculation. The tumors induced by the two viruses were characterized histologically and molecularly. Mo+SV M-MuLV-induced tumors were primarily T-cell-derived lymphoblastic lymphomas containing extensive rearrangements of the T-cell receptor beta gene. In contrast, Δ Mo+SV M-MuLV induced pre-B- and B-cell lymphoblastic lymphomas, B-cell-derived follicular-center cell lymphomas, and acute myeloid leukemia. The Δ Mo+SV tumor DNAs from B-lineage tumors were typically rearranged at the immunoglobulin gene loci and contained germ line configurations of the T-cell receptor beta gene. Southern blot hybridization confirmed that the tumor DNAs contained the predicted Mo+SV M-MuLV or Δ Mo+SV M-MuLV provirus.

Retroviruses are useful models for investigating the molecular bases for gene expression and lymphomagenesis-leukemogenesis. In particular, the long terminal repeats (LTRs) of the integrated retroviral DNA (provirus) are important, since they carry the regulatory elements for viral expression (41). The U3 region of the retroviral LTR contains characteristic promoter and promoter-proximal transcription signals, such as TATAA and CCAAT. In addition, the U3 region contains transcriptional enhancer sequences (41). In the Moloney murine leukemia virus (M-MuLV) LTR, the enhancers consist of a tandemly repeated 75-base-pair (bp) region, and they have been shown to be essential for LTR activity in transient-expression assays and for infectious virus production (27, 30, 34).

The enhancer sequences also play an important role in pathogenesis by retroviruses which lack oncogenes. Experiments with retroviral recombinants indicate that the disease specificity of various MuLVs is controlled by the nature of the enhancer sequences (4, 10, 42). Moreover, the disease specificity is correlated with high transcriptional activity of the retroviral LTR in the cell type targeted for disease (28, 45).

We have been investigating the role of enhancer se-

quences in gene expression and pathogenesis for M-MuLV. We have used molecular cloning to generate a number of recombinant M-MuLV LTRs containing insertions and substitutions of enhancer sequences from other viral and cellular genes (9, 14, 24, 30, 34). Moreover, these chimeric LTRs were also used to generate infectious M-MuLVs by transfection of proviral DNA constructs with the altered LTRs. Several of these recombinant viruses showed responses to viral or cellular *trans*-activator molecules (24, 34) or altered biological properties (9, 14).

In the experiments reported here, regulatory sequences from the simian papovavirus 40 (SV40) were inserted or substituted into the M-MuLV LTR. The SV40 genome has been extremely well studied in terms of regulatory elements, and important elucidation of enhancer and promoter-proximal elements has been achieved in this system. The SV40 enhancers are contained in tandemly repeated 72-bp sequences (2, 19), and adjacent 21-bp sequences also influence the transcriptional activity of the SV40 promoter (12, 16). The SV40 enhancers can transcriptionally activate a variety of heterologous promoters (11, 23, 40), and they are active in many different cell types (1, 3, 43). They do not share extensive sequence homology with the M-MuLV enhancers. In these experiments, we describe the effects of inserting or substituting the SV40 72- and 21-bp repeats into the M-

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MuLV LTR on the transcriptional activity of the LTR and on the biological activity of the resulting M-MuLV.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 and rat XC cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum. Virus stocks were prepared by harvesting 24-h supernatants from confluent infected NIH 3T3 cell cultures. The viral supernatants were centrifuged ($1,200 \times g$; 10 min) to remove cells, and aliquots were stored at -80°C . The virus stocks were titered by the UV-XC syncytial plaque assay as described by Rowe et al. (36). Briefly, NIH 3T3 cells were infected with serial dilutions of the viral supernatants. The cells were grown to confluency, irradiated with UV light to inhibit further growth, and overlaid with rat XC cells. Regional foci of multinucleate XC syncytia were counted to obtain the infectious titer of the virus. The amount of virus particles present was determined by the exogenous reverse transcriptase assay by using a poly(rA) · oligo(dT) template-primer complex as described previously (13).

Plasmid construction and transfections. Molecular cloning procedures were performed by standard protocols (31). Construction of LTR–chloramphenicol acetyltransferase-encoding gene fusion plasmids and proviral plasmids with altered LTRs has been previously described (34). NIH 3T3 cells were transfected for transient assays and for production of infectious virus as previously described (34).

Inoculation of mice and histopathological analysis of tissues. Two-day-old NIH Swiss mice were inoculated intraperitoneally with 10^5 XC PFU per animal. At least three separate litters of mice were inoculated for each experimental virus. Animals were monitored for signs of disease, moribund animals were sacrificed, and tissue sections were fixed and mounted for pathology (15, 35). High-molecular-weight DNA was also extracted from the same tissues.

Extraction and analysis of genomic DNAs. High-molecular-weight DNA was extracted from concentrated suspensions of tumor cells by sodium dodecyl sulfate (SDS) lysis of cells, proteinase K treatment of cellular extracts, and phenol-chloroform extraction (31). Restriction enzyme digests, agarose gel electrophoresis in Tris-borate or Tris-acetate buffer, and Southern blot hybridization procedures were performed as described previously (31).

Hybridization probes. The T-cell receptor beta gene (TCR_β) probe was a 600-bp *EcoRI* DNA fragment from the thymus-derived cDNA clone 86T5 (21), which primarily hybridizes with the constant regions of TCR_β receptor genes. The JH probe was an 800-bp *XbaI-EcoRI* DNA fragment which maps 400 bp 3' to the immunoglobulin G heavy-chain mu [$\text{Ig}_H(\mu)$] J segments (44). The kappa light-chain ($\text{Ig}_L(\kappa)$) probe was a 2.8-kilobase (kb) *HindIII* fragment and contained all of the J_K region (29). The SV40-specific probe was a 1.1-kb *HindIII* fragment from pES131 (kindly provided by Bert Semler [38]). The *HindIII* fragment spans the SV40 early promoter, including the 72- and 21-bp repeats. The M-MuLV envelope probe used in our studies has been described previously (20). The integration probes *c-myc*, *c-pim*, *pvt*, and *c-myb* have been described before (5-7, 18, 32, 39).

RESULTS

Construction of recombinant LTRs and proviruses. We constructed M-MuLV LTR recombinants carrying the SV40

FIG. 1. SV40 early promoter, altered LTRs, and proviral plasmids. (A) The SV40 early promoter region, consisting of the origin of viral replication at 5243/0, the TATA element, and the 21- and 72-bp repeat elements. For the M-MuLV LTR, the R and U5 regions are indicated. Nucleotides are denoted by numbers and are relative to the start site for M-MuLV viral transcription at no. 0. The M-MuLV enhancer element, consisting of two 75-bp tandem repeats, is shown. The ΔMo LTR is identical to the M-MuLV LTR except that nucleotides -150 to -357 have been deleted (30). The SV40 *NcoI-PvuII* (233-bp) DNA fragment obtained from the plasmid pSVA13 (25) was inserted via *XbaI* linkers into the M-MuLV and ΔMo LTRs at the unique *XbaI* site located at -150 bp. The resulting chimeric LTRs (Mo+SV and ΔMo +SV) are shown. Orientation of the SV40 fragment was such that the 21-bp repeats were proximal to the M-MuLV cap site. (B) A proviral plasmid containing altered 5' and 3' LTRs.

regulatory region (Fig. 1A). A 233-bp DNA fragment containing the SV40 regulatory sequences (72- and 21-bp repeats) was inserted via *XbaI* linkers into the M-MuLV LTR at the unique *XbaI* site at -150 bp. The insertion was into a subclone of circular M-MuLV DNA containing one LTR, pMLVC/R/B (30). The resulting chimeric LTR was designated Mo+SV. The same SV40 sequences were also inserted into a deleted version of the M-MuLV LTR (ΔMo), which lacks the tandemly repeated M-MuLV enhancer sequences as well as adjacent GC-rich sequences, to yield the ΔMo +SV LTR.

TABLE 1. Infectivity of Mo+SV and Δ Mo+SV MuLVs

Virus	Titer of infectious virus (XC PFU/ml)	No. of virus particles (reverse transcriptase assay) ^a	Infectivity/particle no. ratio ^b	Normalized infectivity ^c
M-MuLV	3.7×10^6	8.0×10^5	4.63	1
Mo+SV MuLV	6.4×10^5	1.97×10^5	3.25	0.70
Δ Mo+SV MuLV	5.0×10^5	1.87×10^5	2.67	0.58

^a The number of virus particles was determined by reverse transcriptase assay of each of the culture supernatants used for the infectivity measurements. The values shown represent [³H]dTTP counts per minute incorporated per milliliter of supernatant from 10^6 cells in 24 h. Supernatant from uninfected cells gave less than 1,000 cpm.

^b Obtained by dividing the virus titer by the number of virus particles.

^c Infectivity/particle ratio relative to that of wild-type M-MuLV, which was given a value of 1.

We initially assayed the promoter activities of the Mo+SV and Δ Mo+SV LTRs by transient expression of plasmids containing the chimeric LTRs fused to the bacterial chloramphenicol acetyltransferase-encoding gene (30). The LTR-chloramphenicol acetyltransferase-encoding gene fusion plasmids were transfected into NIH 3T3 cells, and cell extracts were assayed for chloramphenicol acetyltransferase enzyme activity. The Mo+SV LTR showed levels of promoter activity almost identical to that of the wild-type M-MuLV LTR, and the Δ Mo+SV LTR exhibited approximately 50% of the promoter activity of the wild-type M-MuLV LTR (data not shown). Since the Δ Mo LTR is transcriptionally inactive in NIH 3T3 cells (20, 30), the results indicated that the SV40 72- and 21-bp repeats could functionally replace the M-MuLV enhancers in the M-MuLV LTR.

Generation of M-MuLVs containing the chimeric LTRs. Since the transient-expression assays indicated that the Mo+SV and Δ Mo+SV LTRs were functional, we attempted to generate infectious M-MuLVs containing these LTRs. This was accomplished by first generating recombinant plasmids containing proviral organizations with the chimeric LTRs at both the 5' and 3' ends, as described previously (20, 34). The organization of the 2 $\times$ Δ Mo+SV plasmid is shown in Fig. 1B. It should be noted that the substitutions or deletions were only in the U3 region of the LTR, which does not code for viral protein; the remainder of the genome was identical to M-MuLV.

Proviral plasmids with altered 5' and 3' LTRs were transfected into NIH 3T3 cells. The transfected cells were serially transferred and assayed at each transfer for virus expression by the UV-XC syncytial plaque assay. Cells transfected with a wild-type M-MuLV, 2 $\times$ Mo+SV, or 2 $\times$ Δ Mo+SV proviral construct all became XC positive. Both wild-type M-MuLV-transfected cells and 2 $\times$ Mo+SV-transfected cells produced XC syncytia upon the first transfer, whereas transfection with the 2 $\times$ Δ Mo+SV proviral organization yielded virus on the second transfer. The results with the 2 $\times$ Δ Mo+SV proviral plasmid provided further evidence that the SV40 72- and 21-bp repeats could functionally substitute for the M-MuLV enhancers and GC-rich sequences.

The transfected cultures were serially transferred until they were confluent, and virus stocks were prepared as described previously (14, 20, 34). High-molecular-weight DNAs from the cultures were also examined by Southern blot analysis, and the results confirmed that the cells contained the predicted chimeric viruses (see, e.g., Fig. 6). The relative infectivities of the Mo+SV and Δ Mo+SV M-MuLVs were determined by infectivity titration of virus stocks on NIH 3T3 cells, and particle determinations were by reverse transcriptase assays of the same virus stocks. The

results are shown in Table 1. Mo+SV and Δ Mo+SV M-MuLVs had specific infectivities essentially equal (60 to 70%) to that of wild-type M-MuLV.

Leukemogenicity of Mo+SV and Δ Mo+SV M-MuLVs. The pathogenicity of SV40 enhancer-containing M-MuLVs was tested by intraperitoneal inoculation of neonatal NIH Swiss mice. The animals were monitored for the appearance of disease, and moribund animals were subjected to necropsy. Both viruses induced lymphoma-leukemia, and time courses for disease are shown in Fig. 2. Under the conditions used, animals inoculated with wild-type M-MuLV develop leukemia in 2 to 4 months, and both Mo+SV and Δ Mo+SV M-MuLVs induced disease with markedly longer latency. For animals inoculated with Mo+SV M-MuLV, mean time to death was 7 months, whereas for Δ Mo+SV M-MuLV-inoculated animals the mean latency was extremely long—17 months. Nevertheless, the resulting leukemias were virus induced in both cases, since the tumor cells contained viral DNA (see below).

Gross pathological changes in moribund Mo+SV and Δ Mo+SV M-MuLV-inoculated animals included enlarged spleens and lymph nodes in both cases. In addition, Mo+SV M-MuLV-inoculated animals generally showed greatly enlarged thymuses, whereas Δ Mo+SV M-MuLV-inoculated animals either lacked a visible thymus or showed only slight thymic enlargement. Histopathological analyses are summarized in Table 2. Mo+SV M-MuLV primarily induced thymic cell (T-cell)-derived lymphoblastic lymphoma (Fig. 3A), typical of that induced by wild-type M-MuLV, in 21 of 22 animals examined. In contrast, Δ Mo+SV M-MuLV induced lymphoblastic lymphomas (9 of 15 animals), which did not characteristically involve the thymus. In addition, 5 of 15 animals developed B-cell-derived follicular-center cell (FCC) lymphomas (Fig. 3B), and 3 of 15 animals developed acute myeloid leukemia (AML). Thus, Δ Mo+SV M-MuLV

FIG. 2. Pathogenicity of Mo+SV M-MuLV (○) and Δ Mo+SV M-MuLV (●). Two-day-old NIH Swiss mice were inoculated intraperitoneally with 10^5 XC PFU of virus per animal. The rates of appearance of moribund animals are shown.

TABLE 2. Pathologic features of tumors in Mo+SV- and Δ Mo+SV-inoculated animals

Virus	No. of animals with tumors/no. examined	Mean time (months) to death	No. of animals with neoplasm/no. examined for histopathology
Mo+SV	21/22 ^a	7	21/21 Lymphoblastic lymphoma
Δ Mo+SV	16/16 ^b	17	9/15 Lymphoblastic lymphoma 5/15 FCC lymphoma 3/15 AML

^a One animal had no apparent pathologic changes upon histological examination.

^b One tumor-bearing animal was not evaluated microscopically because of severe autolysis. Two animals demonstrated two separate and distinct neoplasms. Of these two animals, one had lymphoblastic lymphoma and AML, and the second had FCC lymphoma and AML. Therefore, 17 lymphohematopoietic neoplasms were observed in 15 mice.

induced a broader spectrum of tumors than did Mo+SV or wild-type M-MuLV.

Molecular characterization of tumors. Since almost all of the tumors induced by Mo+SV and Δ Mo+SV M-MuLVs were lymphoid, molecular studies were used to identify lineage-specific lymphoid tumor types. Genomic tumor DNAs were therefore tested for rearrangements of B- or T-lymphoid cell-specific genes. The TCR genes are rearranged during thymocyte and T-lymphoid cell differentiation and maturation, and immunoglobulin genes are sequentially rearranged in B-lymphoid cells during differentiation and maturation. Thus, these genes can be used as definitive markers for lineage, as well as for determining the extent of differentiation within these lineages.

Since wild-type M-MuLV induces T-lymphoid tumors, the possibility that the tumors induced by the SV40 enhancer-containing viruses were T lymphoid was first tested by assaying for rearrangements of the TCR β gene. DNAs from Mo+SV M-MuLV- and Δ Mo+SV M-MuLV-induced tumors were digested with *Hpa*I and analyzed by gel electrophoresis and Southern blot hybridization with a probe specific for the TCR β constant regions (21; Fig. 4). Any rearrangement of either of the two tandem TCR β genes would alter the size of the germ line 11- to 12-kb *Hpa*I fragment. A large proportion of the Mo+SV M-MuLV-induced tumors (13 [76%] of 17 tested) showed rearrangements at the TCR β locus (Fig. 4A; Table 3), suggesting that they were T-lymphoid tumors, since TCR β gene rearrangements are generally detectable only in T-cell neoplasms. Therefore, insertion of the SV40 enhancer sequences into the M-MuLV LTR did not appear to alter the nature of the resulting tumors significantly. Of 17 Mo+SV M-MuLV-induced tumors, 2 showed a germ line configuration in the TCR β region and germ line configurations of the immunoglobulin heavy-chain gene. These probably represent non-T-, non-B-lymphoid tumors which have not undergone lineage-specific rearrangements. The remaining 2 of 17 Mo+SV M-MuLV-induced tumors also showed germ line configurations of the TCR β locus but demonstrated rearrangements of the immunoglobulin heavy-chain locus. These probably represent either pre-B- or B-lymphoid types.

In contrast to the Mo+SV M-MuLV-induced tumors, most of the tumors induced by Δ Mo+SV M-MuLV (12 [80%] of 15) showed germ line configurations in the TCR β region (Fig. 4B; Table 3). This strongly indicated that these tumors were not T lymphoid in origin. Two of the Δ Mo+SV M-MuLV-induced tumors did contain faint hybridization patterns which probably represented minor populations of cells carrying a rearranged TCR β gene (Fig. 4B, lanes 3 and

FIG. 3. Histopathologic changes in Mo+SV M-MuLV- and Δ Mo+SV M-MuLV-induced malignant disease. (A) Characteristic lymphoma of T lymphoblasts (T-cell type lymphoblastic lymphoma) from a Mo+SV M-MuLV-inoculated animal. Note the uniform, monomorphic population of intermediate-size lymphoid cells with round to slightly irregular nuclei and delicate, finely dispersed and stippled immature chromatin. Hematoxylin and eosin staining of the spleen from Mo+SV M-MuLV-inoculated animal 1 (Table 3) is shown (magnification, $\times 1,000$). (B) A characteristic FCC (B-cell) lymphoma induced in mice inoculated with Δ Mo+SV M-MuLV. B-cell-derived large FCC lymphoma is shown. Note the predominance of intermediate-to-large cohesive lymphoid cells with irregularly shaped, notched (cleaved) nuclei and moderate amounts of cytoplasm. Also note that the chromatin is often condensed and margined on the nuclear membrane. Hematoxylin and eosin staining of the spleen from animal 6(k) (Table 3) is shown (magnification, $\times 1,000$).

5). Three tumors showed extensive TCR β gene rearrangement and were judged to be T cell derived. However, two of these latter tumors appeared to be infected with an M-MuLV lacking the SV40 sequences (probably arising by recombination), and one tumor was not infected with M-MuLV (data not shown). Thus, essentially all of the Δ Mo+SV M-MuLV-induced tumors were not T-lymphoid cell derived.

Since most of the Δ Mo+SV M-MuLV-induced tumors did not contain TCR β gene rearrangements, they were next analyzed for IgH(μ) or IgL(κ) gene rearrangements. IgH(μ) genes are consistently rearranged early during B-cell differentiation, and occasionally they can be rearranged in some T-lymphoid tumors which, in addition, have TCR β rear-

FIG. 4. TCR β gene configuration in tumor DNAs from Mo+SV M-MuLV- and Δ Mo+SV M-MuLV-inoculated mice. *HpaI* digests of primary tumor DNAs from Mo+SV M-MuLV- and Δ Mo+SV M-MuLV-inoculated mice were hybridized with a TCR β -specific probe (86T5). The 86T5 probe hybridizes to the CB1 and CB2 loci indicated by hatched boxes. The probe also carries sequences which hybridize to the D region and to a segment of the J β 1 region. The diagram depicts two distinct forms of the germ line TCR β gene in the NIH Swiss mice used in our studies (B. K. Brightman and H. Fan, unpublished data). Lanes: T, normal thymus; S, normal spleen; 1 to 8 (panel A), *HpaI* digests of Mo+SV tumor DNAs; 1 to 9 (panel B), Δ Mo+SV tumor DNAs.

rangements. Genomic DNA from Δ Mo+SV M-MuLV-induced tumors was analyzed by digestion with *EcoRI* and Southern blot hybridization with a probe which hybridizes 3' to the Ig μ J regions (Fig. 5A and B; Table 3) (44). All but one of the Δ Mo+SV M-MuLV-induced tumors (14 [93%] of 15) had distinct rearrangements at the Ig μ locus. The absence of a TCR β gene rearrangement, combined with the Ig μ gene rearrangements in these cases, demonstrated that most of the Δ Mo+SV M-MuLV-induced tumors were derived from the B-cell lineage. Although a portion of the Mo+SV M-MuLV-induced tumors (4 of 17) were also positive for Ig μ gene rearrangement, these tumors also contained rearranged TCR β genes. Therefore, these tumors were classified as T lymphoid. Other investigators have also described the rearrangement of Ig μ genes in T-lymphoid tumors (22, 26).

To determine further whether the Δ Mo+SV M-MuLV-induced lymphoid tumors were derived from pre-B or B cells, we tested them for rearrangement of the Ig κ locus. During B-cell differentiation, rearrangement of an Ig μ gene (as seen in pre-B cells) is followed in ontogeny by rearrangement of an immunoglobulin light-chain gene (primarily kappa in the mouse). Therefore, kappa light-chain gene rearrangement is characteristic of more differentiated B cells. Tumor DNAs were digested with *BamHI* plus *EcoRI*, followed by Southern blot hybridization with an Ig κ probe (Fig. 5C). Of the nine lymphoid tumors examined, two (no. 1 and 10) showed a 6.8-kb germ line Ig κ fragment and were therefore classified as lymphoblastic lymphomas of the pre-B type. In contrast, seven lymphoid cell neoplasms showed distinct rearrangements at the Ig κ locus. Three tumors (no. 2, 4, and 9) were classified as B-cell lymphoblastic lymphomas (Burkitt's type), and the remaining four (no.

TABLE 3. Rearrangement of TCR β and immunoglobulin genes in tumors from Mo+SV and Δ Mo+SV-inoculated mice

Tumor type and no. (lane) ^a	Histologic diagnosis	TCR β ^b	J μ	J κ ^c	SV40 ^d
ΔMo+SV					
1 (b)	Lymphoblastic lymphoma, pre-B-cell type	G	R	G	+
10 (h)	Lymphoblastic lymphoma, pre-B-cell type + AML ^e	G	R	G	+
13 (i)	Lymphoblastic lymphoma, pre-B- vs B-cell type	G	R	ND ^f	+
2 (c), 4 (e)	Lymphoblastic lymphoma, B-cell (Burkitt) type	G	R	G/R	+
9 (g)	Lymphoblastic lymphoma, B-cell (Burkitt) type	G	R	R	+
7, 14	Lymphoblastic lymphoma, T-cell type	R	G	ND	-
15	Lymphoblastic lymphoma, T-cell type	R	R	ND	-
3 (d)	FCC lymphoma (B-cell)	G/R	R	G/R	+
5 (j)	FCC lymphoma (B-cell)	G	R	ND	+
6 (k)	FCC lymphoma (B-cell)	G/R	R	G/R	+
11 (l)	FCC lymphoma (B-cell)	G	R	G/R	+
12 (m)	FCC lymphoma (B-cell) + AML ^e	G	R	G/R	+
8 (f)	AML	G	G	G	+
Mo+SV					
1, 14, 15, 17	Lymphoblastic lymphoma, T-cell type	R	R	ND	+
2, 3, 4, 5, 16	Lymphoblastic lymphoma, T-cell type	R	G	ND	+
9, 11, 12, 13	Lymphoblastic lymphoma, T-cell type	R	ND	ND	+
6, 10	Lymphoblastic lymphoma, non-T, non-B type	G	G	ND	+
7, 8	Lymphoblastic lymphoma, pre-B- vs B-cell type	G	R	ND	+

^a The 15 Δ Mo+SV and 17 Mo+SV tumors molecularly analyzed came from 16 and 22 animals, respectively. The letters in parentheses next to Δ Mo+SV tumor DNA samples correspond to lanes in Fig. 5B and C, 6B, and 7B.

^b Represents results of Southern analysis as follows: R, rearranged at either the CB1 or CB2 locus or at both loci; G, germ line; G/R, primarily germ line configuration but showing a subpopulation of cells in the tumor carrying rearranged loci (Fig. 4B, lanes 3 and 5).

^c Mo+SV tumor DNA samples were not examined for Ig κ gene rearrangements.

^d Tumor DNAs were analyzed for the presence of the Mo+SV or Δ Mo+SV LTR by Southern blot analysis of *SmaI*-digested tumor DNAs and hybridization with an SV40-specific probe (Fig. 6A and B). +, Positive for SV40 hybridization; -, no detectable SV40 sequences present in the tumor.

^e Animals 10 and 12 were mice which had two distinct neoplasms (see Table 2, footnote b).

^f ND, Tumor DNA not molecularly analyzed.

FIG. 5. Immunoglobulin gene rearrangements in Δ Mo+SV tumor DNAs. (A) The structures of the IgG(μ) (top diagram) and IgG(κ) (lower diagram) regions are shown. J_H and J_K segments are indicated by vertical solid bars. Hatched boxes in each line drawing illustrate sequences hybridizing to the 3' J_H or HJ_K probe. The 3' J_H probe detects a 6.4-kb germ line *Eco*RI (RI) fragment, and the HJ_K probe detects a 6.8-kb germ line *Bam*HI-*Eco*RI fragment. X, *Xba*I; H, *Hind*III; C_K , constant region. (B) High-molecular-weight DNAs prepared from Δ Mo+SV-induced tumors were digested with the enzyme *Eco*RI and examined by Southern blot hybridization with the 3' J_H probe. The germ line 6.4-kb DNA fragment is indicated. Lanes: T, DNA from a normal thymus; S, DNA from a normal spleen; a, DNA from a tumor induced by wild-type M-MuLV; b through m, DNAs from Δ Mo+SV-induced tumors; c', DNA extracted from the cell line (85-2M) derived from the Δ Mo+SV tumor shown in lane c. (C) Several of the same Δ Mo+SV tumor DNA samples analyzed for B were digested with *Eco*RI and *Bam*HI. Hybridization with the HJ_K probe is shown. The germ line *Eco*RI-*Bam*HI fragment of 6.8 kb is indicated. The lanes shown correspond to those in B. Lane T, DNA from a normal thymus.

3, 6, 11, and 12) were classified as B-cell-derived FCC lymphomas. The one nonlymphoid tumor examined, from an animal with AML (no. 8), in addition to germ line configurations for the TCR β and IgH(μ) loci, also showed germ line configurations for the IgL(κ) locus.

Presence of virus in the tumors. Given the long latencies of disease (particularly for Δ Mo+SV M-MuLV), it was important to determine whether the tumors resulted from viral infection. High-molecular-weight DNA from the thymus or spleen (or both) of each moribund animal was digested with

*Sma*I and analyzed by Southern blot hybridization with an SV40 enhancer-specific probe. A 2.06-kb fragment spanning most of *env* and all of the LTR U3 region would result from wild-type M-MuLV proviruses. The corresponding fragments from Mo+SV M-MuLV and Δ Mo+SV M-MuLV proviruses would be 2.29 and 2.09 kb, respectively (Fig. 6A and B). Most of the tumors induced by either Mo+SV M-MuLV or Δ Mo+SV M-MuLV gave SV40-specific *Sma*I fragments, indicating the presence of the appropriate proviruses. As mentioned above, three of the Δ Mo+SV M-

FIG. 6. Proviral DNA in Mo+SV M-MuLV- and Δ Mo+SV M-MuLV-induced tumors. Tumor DNAs were digested with the enzyme *Sma*I (Sm) and analyzed by Southern blot hybridization. The SV40-specific hybridization probe was a 1.1-kb *Hind*III fragment containing the SV40 regulatory region. Digestion of Mo+SV M-MuLV with *Sma*I yields a 2.29-kb DNA fragment which hybridizes to the SV40 enhancer probe, whereas Δ Mo+SV M-MuLV yields a corresponding fragment of 2.09 kb. (A) Mo+SV tumor DNAs digested with *Sma*I are shown in lanes c through j. Lane a shows *Sma*I-digested tumor DNA obtained from an M-MuLV-inoculated animal. (B) Δ Mo+SV tumor DNAs digested with *Sma*I are shown in lanes b through i and in lane l. Lanes k show *Sma*I-digested DNA from the thymus of an uninoculated mouse. Lane v in A shows Mo+SV-infected NIH 3T3 DNA, and lane v in B shows Δ Mo+SV-infected NIH 3T3 DNA. The positions of *Hind*III-digested lambda DNA markers are denoted by black bars at the side of each panel. Xb, *Xba*I.

FIG. 7. Proviral DNA in Mo+SV M-MuLV and ΔMo+SV M-MuLV end-stage tumors. Tumor DNAs were digested with the enzyme *Xba*I (Xb) and analyzed by Southern blot hybridization. The probe used in the analysis was a 1.4-kb *Hpa*I (envelope region) DNA fragment which is shown as a hatched box in the line drawings. Digestion of wild-type M-MuLV with *Xba*I yields a 2.35-kb DNA fragment which hybridizes to the *Hpa*I probe. A DNA fragment of the same size is released from Mo+SV M-MuLV, since the SV40 DNA insert is liberated. *Xba*I digestion of ΔMo+SV M-MuLV yields a 2.14-kb fragment. (A) Mo+SV M-MuLV tumor DNAs digested with *Xba*I are shown in lanes b through j; (B) ΔMo+SV M-MuLV tumor DNAs digested with *Xba*I are shown in lanes b through j. Lanes a represent *Xba*I-digested tumor DNA from an M-MuLV-inoculated animal. Lanes k show *Xba*I-digested DNA obtained from a normal mouse thymus. The hybridization pattern observed in lanes k is the result of hybridization of the envelope probe with endogenous M-MuLVs present in the mouse genome. The tumor DNAs analyzed in lanes c through j of panels A and B correspond to the DNA samples shown in Fig. 6A and B. Sm, *Sma*I.

MuLV-induced tumors did not contain proviruses with SV40 sequences (data not shown), and these were the only tumors induced by this virus which showed rearrangements of TCR β genes. These results strongly supported the belief that the tumors were induced by the SV40 enhancer-containing viruses.

Whereas the *Sma*I digests of Fig. 6 confirmed that the tumors contained Mo+SV M-MuLV or ΔMo+SV M-MuLV, it was desirable to carry out an additional analysis. (i) Since hybridizations used an SV40-specific probe, other viruses in the tumors which lacked SV40 sequences would not have been detected. (ii) As evident in Fig. 6, (particularly for the Mo+SV M-MuLV-induced tumors), novel *Sma*I fragments which did not conform to the predicted sizes were also present in some tumors. These novel fragments could have resulted from recombinant viruses with altered *env* regions (e.g., mink cell focus-forming virus derivatives) or altered LTRs or from partial methylation of *Sma*I sites in the proviral DNA. Therefore, tumor DNAs were digested with *Xba*I and analyzed by Southern blot hybridization with an M-MuLV *env*-specific probe (Fig. 7). Wild-type M-MuLV provirus would yield a 2.35-kb *Xba*I fragment encompassing *env* and most of the LTR U3 sequences. An identical DNA fragment should be released from Mo+SV M-MuLV proviruses, whereas ΔMo+SV M-MuLV proviruses would yield a smaller 2.14-kb fragment. None of these fragments would contain SV40 sequences. All of the Mo+SV M-MuLV- and ΔMo+SV M-MuLV-induced tumors showed the diagnostic DNA fragments for the expected viruses. For ΔMo+SV M-MuLV-induced tumors, ΔMo+SV M-MuLV was the predominant virus in the tumors. For the Mo+SV M-MuLV-induced tumors, other M-MuLVs giving different sizes of

*Xba*I fragments were frequently detected. These presumably represented recombinant M-MuLVs with altered *env* or LTR regions. These results are consistent with those of Zijlstra et al. (46), who found that virus-induced leukemia that developed rapidly in C57BL mice contained mink cell focus-forming proviruses, whereas virus-induced leukemias that developed more slowly in the same strain contained ecotropic MuLVs.

Proviral insertion near proto-oncogenes. For retroviruses which lack oncogenes (e.g., M-MuLV), proviral integration in the vicinity of a cellular proto-oncogene is frequently an important event in the leukemogenic process (5, 6, 33, 37, 39). We were particularly interested in testing ΔMo+SV M-MuLV-induced tumors for proto-oncogene activation since, in contrast to M-MuLV- or Mo+SV M-MuLV-induced lymphomas or leukemias, the tumors occurred with extremely long latency and were predominantly of pre-B or B type. Therefore, several tumors induced by ΔMo+SV M-MuLV were examined for proviral integrations near four proto-oncogene loci: *c-myc*, *pim-1*, *pvt-1*, and *c-myb*. These loci have been activated by wild-type M-MuLV during leukemogenesis in mice. High-molecular-weight tumor DNA was digested with *Eco*RI (or *Kpn*I for *c-myc* analysis) and analyzed by agarose gel electrophoresis and Southern blot hybridization with the different proto-oncogene probes. None of the ΔMo+SV M-MuLV-induced tumors showed insertions near *c-myb*, *pvt-1*, or *c-myc* (Table 4). However, two of eight tumors contained insertions adjacent to *pim-1*, as indicated by novel-size *pim-1* restriction fragments (Table 4). In the conditions used here, most (ca. 75%) of the tumors induced by wild-type M-MuLV show proviral integrations near *c-myc*, *pim-1*, or *pvt-1* (H. Fan, H. Chute, E. Chao, and

TABLE 4. Integration of Δ Mo+SV M-MuLV near proto-oncogene loci^a

Tumor no.	Histologic diagnosis	<i>c-myc</i>	<i>c-pim</i>	<i>c-myb</i>	<i>pvt</i>
1	Lymphoblastic lymphoma	—	+	—	—
2	Lymphoblastic lymphoma	—	+	—	—
3	FCC lymphoma	—	—	—	—
4	Lymphoblastic lymphoma	—	—	—	—
8	AML	—	—	—	—
9	Lymphoblastic lymphoma	—	—	—	—
10	Lymphoblastic lymphoma	—	—	—	—
	+ AML	—	—	—	—
13	Lymphoblastic lymphoma	—	—	—	—

^a —, Denotes a germ line configuration of the locus; +, indicates the presence of an additional DNA band (other than the germ line) which hybridized with the probe.

P. K. Pattengale, submitted for publication). Thus, either leukemogenesis by Δ Mo+SV M-MuLV did not frequently involve these proto-oncogene activations or other proto-oncogenes were involved.

DISCUSSION

In these experiments, insertion of enhancer sequences from SV40 into the M-MuLV LTR was studied. Transcriptionally active LTRs were obtained when the SV40 sequences were inserted between the M-MuLV enhancers and the promoter (Mo+SV) or when they were substituted for the M-MuLV enhancers (Δ Mo+SV). Thus, the SV40 enhancers were compatible with M-MuLV promoter elements, consistent with previous results of Laimonis et al. (27). In these experiments, infectious M-MuLVs with the chimeric SV40 enhancer-containing LTRs were also generated. The specific infectivities of Mo+SV and Δ Mo+SV M-MuLVs in tissue culture cells were both quite high—within 50% of the level of wild-type M-MuLV, confirming the compatibility of the SV40 and M-MuLV sequences. Indeed, in comparison with other recombinant M-MuLVs with chimeric LTRs that we have generated (9, 13, 24, 34), the specific infectivities of these two viruses were the highest. The slight reductions in specific infectivities might be due to suboptimal spacing of the SV40 and M-MuLV regulatory elements or the fact that SV40 enhancers are actually most efficient in primate cells, whereas the infectivities were measured in mouse cells.

Both Mo+SV and Δ Mo+SV were pathogenic in NIH Swiss mice, inducing predominantly lymphoblastic lymphoma with long latency. Interestingly, the two viruses induced different types of lymphoid cell neoplasms, which may have reflected the tissue specificity of expression for the two chimeric LTRs. Δ Mo+SV M-MuLV induced B-lineage lymphomas, with an occasional AML, suggesting that the SV40 sequences conferred upon the Δ Mo+SV M-MuLV LTR preferential expression in these cell types.

Mo+SV M-MuLV primarily induced T-lineage lymphoid cell neoplasms, the same as those induced by the parental wild-type M-MuLV, although with somewhat longer latency. Thus, insertion of the SV40 sequences into the Mo+SV LTR apparently did not alter the specificity of expression for T-lymphoid cells. This suggests that the M-MuLV enhancers exerted a dominant effect over the SV40 enhancers, although the SV40 enhancers were closer to the M-MuLV promoter.

The pathogenic behaviors of these two SV40 enhancer-containing viruses provide an interesting contrast to M-

MuLVs carrying enhancer sequences from polyomavirus, which we described previously (9, 14). Insertion of enhancer sequences from the PyF101 strain of polyomavirus in positions analogous to those of Mo+SV and Δ Mo+SV M-MuLVs abolished leukemogenicity in subcutaneously inoculated mice, presumably because of an alteration in tissue specificity such that a critical target cell could not be infected (9). Moreover, for Mo+PyF101 M-MuLV, the position of insertion of the polyomavirus sequences was important; insertion of the PyF101 sequences upstream from the M-MuLV enhancers did not abolish leukemogenicity, although the types of leukemia that resulted were altered (14). In contrast, both of the SV40 enhancer-containing viruses described here were leukemogenic. Furthermore, as mentioned above, the M-MuLV enhancers in the Mo+SV LTR were apparently dominant over the SV40 enhancers, even though they were in the upstream position.

Given the long latency for disease induced by Δ Mo+SV M-MuLV, a primary concern was that the lymphomas were spontaneous and unrelated to the virus. Several observations argue against this. (i) NIH Swiss mice have a low reported incidence of spontaneous malignancies, whereas 75% of the Δ Mo+SV M-MuLV-inoculated animals developed lymphoma-leukemia. (ii) Spontaneous malignancies in NIH Swiss mice are generally thymic lymphocytic lymphoma-leukemia (17), whereas the Δ Mo+SV M-MuLV-induced tumors were mainly of the pre-B- or B-cell type. Most importantly, (iii) most of the Δ Mo+SV M-MuLV tumors examined contained the Δ Mo+SV M-MuLV provirus, even though general levels of viral infection in the animal at preleukemic times were extremely low. It was noteworthy that both viruses induced disease with a latency longer than that of the disease induced by wild-type M-MuLV, particularly Δ Mo+SV M-MuLV. We have studied the lymphoma-genic-leukemogenic process for wild-type M-MuLV and suggested that M-MuLV lymphomagenesis-leukemogenesis involves two independent infection events in the animal (8). The first infection leads to a prelymphoma-preleukemia state characterized by generalized hematopoietic hyperplasia; the second infection occurs in a hyperplastic progenitor cell, resulting in LTR activation of proto-oncogenes and outgrowth of the final tumor. The very long latency for leukemia induced by Δ Mo+SV M-MuLV suggests that it might be unable to induce the preleukemic hyperplastic state. Indeed, animals inoculated with Δ Mo+SV M-MuLV showed extremely low levels of infection in hematopoietic organs during preleukemic times (data not shown), although the resulting tumors all contained Δ Mo+SV M-MuLV provirus. The mechanisms by which these two viruses induce lymphoma-leukemia remain to be investigated. Perhaps other spontaneous events which occur infrequently can also induce the preleukemic events necessary for lymphomagenesis-leukemogenesis. These SV40 enhancer-containing M-MuLVs may provide useful comparative tools for studying M-MuLV-induced lymphoma-leukemia.

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