

Importance of a c-Myb Binding Site for Lymphomagenesis by the Retrovirus SL3-3

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All murine leukemia viruses (MuLVs) and related type C retroviruses contain a highly conserved binding site for the Ets family of transcription factors within the enhancer sequences in the viral long terminal repeats (LTRs). The T-cell lymphomagenic MuLV SL3-3 (SL3-3) also contains a c-Myb binding site adjacent to the Ets site. The presence of this Myb site distinguishes SL3 from most other MuLVs. We tested the importance of these two sites for the lymphomagenicity of SL3-3. Mutation of the Ets site had little effect on viral pathogenicity, as it only slightly extended the latency period to disease onset. In contrast, mutation of the Myb site strongly inhibited pathogenicity, as only a minority of the inoculated mice developed tumors in the two mouse strains that were tested. All tumors that were induced by either mutant appeared to be lymphomas, and no evidence for reversion of either mutation was detected. The effects of the Ets and Myb site mutations on transcriptional activity of the SL3 LTR were tested by inserting the viral enhancer sequences into a plasmid containing the promoter region of the *c-myc* gene linked to a reporter gene. Mutation the Myb site almost eliminated enhancer activity in T lymphocytes, while mutation of the Ets site had smaller effects. Thus, the effects of the enhancer mutations on transcriptional activity in T cells paralleled their effects on viral lymphomagenicity. The absence of the c-Myb site in the LTR enhancer of the weakly lymphomagenic MuLV, Akv, likely contributes to the low pathogenicity of this virus relative to SL3-3. However, Moloney MuLV also lacks the Myb site in its LTR, although it induces T-cell lymphomas with a potency similar to that of SL3-3. Thus, it appears that SL3-3 and Moloney MuLV evolved genetic determinants of T-cell lymphomagenicity that are, at least in part, distinct.

Murine leukemia viruses (MuLVs) are retroviruses that induce various types of hematopoietic neoplasias in mice, including T- and B-cell lymphomas, erythroleukemia, and myeloid leukemia. Recombination studies using molecular clones of MuLVs established that the viral long terminal repeats (LTRs) contain major genetic determinants of viral leukemogenicity (6, 7, 10, 13, 17, 20, 21). Specifically, a portion of the U3 region of the LTR that functions as a transcriptional enhancer plays a major role in determining the type of leukemia that the virus causes and the potency of the virus. Often these viral LTR enhancers are present as tandem repeats about 50 to 100 nucleotides in length (14). They evolved to be recognized by a set of transcription factors in the target cell for disease and appear to play multiple roles in the process of leukemogenesis (4, 11, 27, 32). One key step that the viral LTR enhancers affect is the transcriptional activation of proto-oncogenes when a provirus is integrated adjacent to such gene (27). For example, the *c-myc* gene is utilized as a common integration site in 15 to 20% of T-cell lymphomas induced by MuLVs (22, 27, 29, 31, 34). Since many of these proviruses are found in the opposite transcriptional orientation relative to *c-myc*, it appears that the mechanism of its deregulation or activation is by enhancer insertion. Genetic studies indicated that the ability to activate *c-myc* depended on the nature of the viral LTR enhancer (27). Specifically, in lymphomas induced by a variant MuLV with a single base pair mutation in the LTR, the tumor cell genomes contained LTRs with the original mutations, reversions, or putative second-site suppressor mutations. However, the pro-

viruses adjacent to *c-myc* always contained reversions or putative suppressor mutations. Thus, viral LTR enhancers in the provirus adjacent to *c-myc* apparently must have an appropriate structure to be capable of activating the proto-oncogene.

SL3 is an MuLV that causes T-cell lymphomas. Multiple transcription factor binding sites have been identified in the SL3 enhancer (Fig. 1). Within each of the 72-bp tandem repeats that comprise the SL3 enhancer, there are adjacent binding sites for c-Myb and Ets-1. The Ets site, also called the LVb site, is conserved in all MuLVs and can bind multiple members of the Ets family of transcription factors (14, 25, 36, 38). However, the Myb site was identified only in the LTRs of SL3 and the very similar Gross passage A virus (20, 23, 43). On either side of these two sites, there is a binding site for core binding factor (CBF), which is also called AML1, polyomavirus enhancer-binding protein 2, and SL3 enhancer factor 1 (SEF1) (2, 18, 30, 39). The CBF site adjacent to the Ets site is called core I, while the one next to the Myb site is termed core II. The core I element of SL3 is similar to the core elements of other MuLVs (14). The core II element, like the c-Myb site, is unique to SL3 and the Gross passage A virus (20, 40).

Enhancer elements differ in their importance for viral pathogenicity. Mutations in the conserved core I element of SL3 significantly reduced viral lymphomagenicity (15, 27). However, alteration of the unique core II element had little effect on pathogenicity unless core I was also mutated, in which case the virus was even less lymphomagenic than the core I mutant (15). Mutation of the core element of another T-cell lymphomagenic virus, Moloney MuLV (Mo-MuLV), also significantly reduced viral lymphomagenicity (37). Moreover, in the case of Mo-MuLV, mutations in the enhancer core element also altered the tissue specificity of disease from T-cell lymphomas to erythroleukemias (37). Thus, the conserved core

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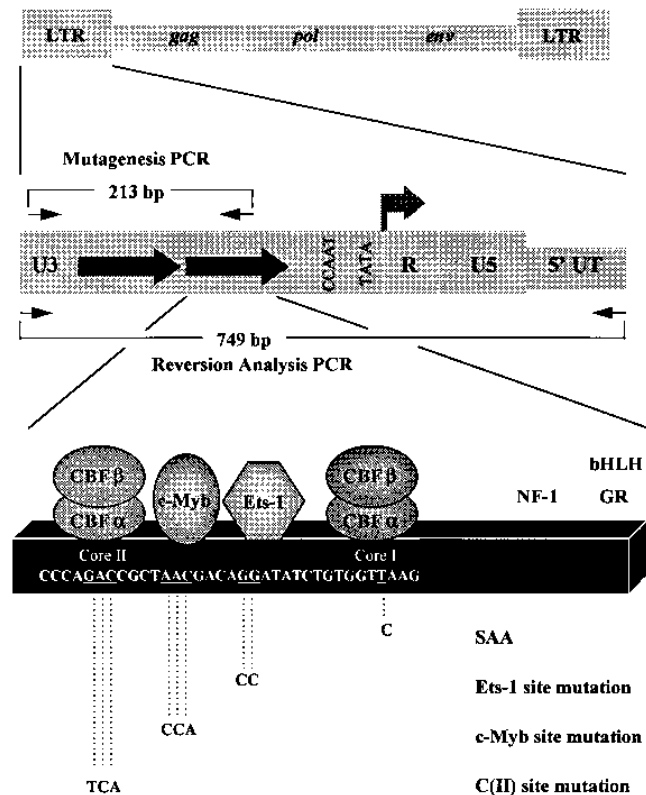


FIG. 1. Organization of the SL3 LTR and positions of mutations. The top diagram shows the positions of the LTRs in the viral genome. The middle diagram shows the organization of the 5' LTR. The large arrows represent the tandem 72-bp repeats in the U3 region. The arrow at the U3-R boundary indicates the initiation site and direction of transcription. The small arrows show the positions of the primers used for PCR; those above the diagram were used in the mutagenesis procedure, and those below the line were used to amplify the 5' LTR from proviruses in infected cells and tumors. The bottom diagram shows the organization of one 72-bp enhancer repeat of SL3. Binding sites for various transcription factors are indicated, and the core elements where CBF binds are marked. Geometrical figures are used to indicate the factors that are relevant to this study. Positions of sites for other factors are also shown. These are a site for NF-1, a site for the glucocorticoid receptor (GR), and an E box that binds basic helix-loop-helix (bHLH) transcription factors (5, 9, 28, 36). Part of the sequence of the enhancer is shown within the box, and the positions of the mutations are underlined. Sequences of the mutated sites are shown below the line. The mutations were introduced simultaneously into both of the 72-bp repeats in the viral LTRs. The single base pair mutation of the core I site to the sequence of Akv virus is indicated as SAA, the name previously given to a mutated SL3 virus that contained this mutation in both 72 bp repeats (27). C(II) indicates core II. UT, untranslated region.

element is an important determinant of viral tumorigenicity. Similarly, mutation of the Ets site of Mo-MuLV also reduced the lymphomagenic potential of the virus and resulted in a fraction of the resulting tumors being erythroleukemias (37).

In this report, we tested the importance of the c-Myb and Ets sites for the lymphomagenicity of SL3. It was of interest to determine whether the Ets site, which is highly conserved in MuLVs (14), was crucial for pathogenicity. It was also of interest to determine whether the relatively unique c-Myb site was important or whether, like the unique core II element, it had only modest effects on virally induced disease.

MATERIALS AND METHODS

Cell lines. SL3H is a T-lymphoma cell line derived from a spontaneous thymic lymphoma of an AKR/J mouse. L691-6 is a T-lymphoma cell line from a radiation-induced thymic lymphoma of a C57L mouse. Jurkat is a human T-lymphoblast cell line from a tumor secondary to thymic lymphoblastic lymphoma. NIH

3T3 is a mouse embryo fibroblast cell line. SL3H and Jurkat cell lines were maintained in RPMI 1640 supplemented with either 10% fetal calf serum or fetal bovine serum, 100 U of penicillin per ml, 10 mg of streptomycin per ml, and 2 mM glutamine. The L691 cell line was grown in Dulbecco's modified Eagle medium with the same supplements mentioned above. NIH 3T3 cells were also grown in Dulbecco's modified Eagle medium and the same supplements, with the difference that 10% calf serum was added to supplement the medium. All cells were grown at 37°C in 100% humidity and 7.5% CO₂.

Mutagenesis of LTR plasmids. Reporter plasmids containing viral LTRs linked to the chloramphenicol acetyltransferase (CAT) gene have been previously described (3, 24, 27, 35). These plasmids contain the U3 and R sequences of the viral LTRs that extend from the *Pst*I site near the 5' end of the viral LTR to a *Sma*I site in the middle of the R region. These sequences include the viral promoter and the tandem 72-bp repeats that comprise the viral enhancer. Mutations were generated in the Ets-1 and c-Myb binding sites in the SL3 enhancer by using a PCR-based strategy (Fig. 1). The plus-strand PCR primer, 5'-AGTA TCCTCGAGGCTGCAGTAACGCCATTTTGCAGGCAT-3', was homologous to sequences near the 5' end of the U3 region of the SL3 LTR. The minus-strand primers contained the corresponding mutated bases in the Ets-1 or c-Myb site. The sequences of the minus-strand primers used were 5'-ATGGTG TCGTTAGCGGTCTGGGGACCATC-3' (Ets-1) and 5'-ATCCTGTCACCAG CCGTCTGGGGACCATCTGT-3' (c-Myb), where the underlined nucleotides identify the mutations. Since the minus-strand primers hybridized to sequences within the 72-bp tandem repeats, this approach yielded PCR products containing either one or two 72-bp units. Those with two 72-bp repeats were a mixture of products with the mutation either in both 72-bp units or only in the downstream repeat unit. The optimal PCR conditions to increase the percentage of products with mutations on both 72-bp repeats were empirically determined to be an initial incubation at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C and 2 min at 72°C. After PCR, the products were digested with *Xho*I, which cuts at the 5' end. The 3' ends of these products were flush ends corresponding to an *Eco*RV site within the SL3 enhancer. The 213-bp pieces (*Xho*I to *Eco*RV) were then isolated by electrophoresis in 2% agarose gels and used to replace the corresponding wild-type sequences in a plasmid containing the SL3 LTR. Complete viral genomes containing a single viral LTR linked to the viral *gag*, *pol*, and *env* sequences were generated by the insertion of a 7.7-kbp *Bss*HII-to-*Eco*RI fragment as previously described (27). All of the final products were sequenced to verify the presence of mutations on both repeats and that no other mutations were generated during the PCR. Mutagenesis of the core elements, core I and core II, was previously described (44).

Transcriptional activity of each of the mutations was analyzed by inserting the viral enhancer sequences into a plasmid that contained the *c-myc* promoters linked to the CAT gene. Construction of a plasmid (Myc-CAT) that contained 534 bp of 5' flanking sequences, exon 1, and 8 bp of intron 1 from *c-myc* linked to the CAT gene was previously described (8). Generation of Myc-CAT plasmids that contained the LTR enhancer sequences of the viruses, SL3, Akv, and the core I mutant, SAA, 534 bp upstream of exon 1 was previously described (8). Plasmids containing the Ets-1, c-Myb, and core II mutations were constructed by substituting a 325-bp *Pst*I-to-*Dra*I fragment that contained each of the enhancer mutations for the wild-type sequences in the SL3-Myc-CAT plasmid.

Tumorigenicity assays. Infectious virus particles were generated from the single-LTR plasmid clones as previously described (27). Briefly, the 8.2-kbp viral genomes containing the mutations were excised from the plasmid vector by digestion with *Pst*I. These were ligated with T4 DNA ligase to form concatemers that contained two LTRs, each with the identical mutation. Ten micrograms of ligated DNA was transfected by using Lipofectin (GIBCO BRL) into NIH 3T3 mouse fibroblasts that were plated at a density of 10⁶ cells per 60-mm² plate 24 h before transfection. Cells were passaged 1:10 every third day. Supernatants from transfected cells were collected and tested for the presence of reverse transcriptase activity (12). Virus stocks were harvested after reverse transcriptase activity reached a maximum level, 2 to 3 weeks after transfection. Viral titers were determined by XC plaque assay on fresh NIH 3T3 cells (33). The presence of the mutations in the virus stocks was confirmed by direct sequencing of PCR products from NIH 3T3 cells infected with each virus, using the approach described below.

Newborn NIH/Swiss and AKR/J mice (<1.5 days old) were injected intraperitoneally with 0.1 ml of virus (10³ to 10⁴ XC PFU). Diseased mice were sacrificed by CO₂ anesthesia and necropsied. Animals were examined for gross appearance of malignant lymphomas, which include enlargement of thymus, spleen, peripheral and/or mesenteric lymph node, and liver. Diagnosis of lymphoma was confirmed by the presence of T-cell receptor β -chain (TCR β) rearrangements upon Southern blot analysis of tumor DNA.

Reversion analysis of enhancer mutations. Proviral LTRs were amplified from tumor DNAs by PCR using a primer pair at positions -475 to -442 (5'-TTCA TAAGGCTTAGCCAGCTAACTGCAG-3') and 274 to 247 (5'-ACACACT CTCCC-3') relative to the viral transcriptional initiation site (Fig. 1) as previously described (27). PCR conditions were a 30-cycle program of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C. PCR products were resolved by electrophoresis through either a 2% agarose or a nondenaturing 5% polyacrylamide gel. Individual bands were excised, and DNA was isolated by using QIAGEN (Qiagen). The isolated fragments were then directly sequenced by using Sequenase (Amersham) with a minus-strand primer from positions -150 to -171 (5'-TTGAA

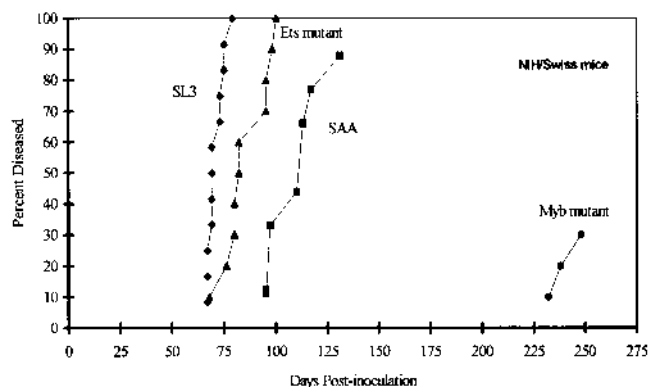


FIG. 2. Tumor induction by viral enhancer mutants in NIH/Swiss mice. Parallel controls were performed with SL3 and the core I mutant, SAA. Surviving mice that were inoculated with the Myb mutant were maintained for a total of 1.5 years, with no additional tumors detected.

ACTGTTGTTGTTTTCAGC-3') or a plus-strand primer from positions -382 to -364 (5'-ACAAGGAAGTACAGAGAGGC-3') relative to the transcription initiation site.

Analysis of TCR β and IgH gene rearrangements. Isolation of genomic DNA from tumors and Southern blot analysis of tumor DNA were performed as previously described (1). Organization of the TCR β locus was examined by using the 86T5 probe, a 600-bp *EcoRI* fragment of murine TCR β cDNA (16). Organization of the immunoglobulin heavy-chain (IgH) locus was examined by using the p2-1 probe, a 700-bp *XbaI-EcoRI* fragment containing sequences immediately 3' to the murine IgH J region (41).

CAT assays. The DEAE-dextran method was used for transfection of cell lines as described before (3, 35). A total of 5.0×10^6 cells were pelleted and resuspended in 1 ml of TD (25 mM Tris-HCl [pH 7.4], 0.7 mM Na₂HPO₄, 5.1 mM KCl, 137 mM NaCl) containing 250 μ g of DEAE-dextran per ml, 5 μ g of reporter plasmid DNA, and 1 μ g of a Rous sarcoma virus LTR-luciferase plasmid used as internal control. This mixture was incubated at room temperature for 15 min. Then 5 ml of medium supplemented with serum was added, and the cells were incubated for 20 min at 37°C. Cells were then pelleted, resuspended in 5 ml of medium supplemented with serum, and harvested 48 h later. Protein concentrations were determined, and aliquots of lysates were used to perform CAT assays and luciferase assays as previously described (44). CAT activity was quantified by calculating the percentage of chloramphenicol that was acetylated by using PhosphorImager analysis of thin-layer chromatography plates. CAT activities were normalized to luciferase activities for each sample. Multiple trials were performed for each experiment, and the means were calculated and plotted.

RESULTS

Generation of SL3 viruses mutated in the Myb and Ets sites.

To test the importance of the Myb and Ets binding sites for lymphomagenicity of SL3, point mutations were introduced simultaneously into these sites in each of the 72-bp repeats in the SL3 enhancer. Figure 1 shows the organization of the SL3 LTR and the sequences of the binding sites for three transcription factors, CBF, c-Myb, and Ets-1, within a portion of the 72-bp repeats. The mutations introduced at each site were previously shown to disrupt binding of the cognate factor to DNA, except for the single base pair mutation in one of the CBF binding sites, core I, which induces only a twofold reduction in CBF binding (23, 37, 39, 40, 43). Infectious virus was generated by transfection of the full-length genome into NIH 3T3 fibroblasts. The spread of infectious virus particles in the cultures was monitored by the presence of reverse transcriptase activity in culture supernatants. All SL3 enhancer mutant viruses exhibited reverse transcriptase activity comparable to that of wild-type SL3 and yielded similar titers in XC plaque assays.

To confirm the integrity of the LTR mutations in the viral stocks, the enhancers of proviruses in cellular DNA from infected NIH 3T3 fibroblasts were amplified by PCR analysis and

analyzed by DNA sequencing. Primers that hybridized to sequences at the 5' end of U3 and to the 5' untranslated sequence of viral DNA (Fig. 1) were used to amplify proviral 5' LTR sequences. These primers distinguished SL3 and the LTR mutants from endogenous MuLVs. The PCR products were resolved on 2% agarose gels. A 749-bp fragment was the predominant PCR product, indicating that viruses with two enhancer repeats predominated in the viral stocks, although PCR products corresponding to LTRs with one or three 72-bp units were also detected. This finding is consistent with previous evidence that these viruses exist as quasispecies with variable numbers of enhancer repeats (27). PCR-amplified fragments were directly sequenced to confirm the presence of the mutations. Both the Myb and Ets site mutations were maintained.

Tumor induction by viruses with mutated enhancer sequences. Virus stocks generated for each enhancer mutation were tested for tumorigenicity by injection of newborn mice of two different mouse strains, NIH/Swiss (Fig. 2) and AKR/J (Table 1). Parallel controls were performed with wild-type SL3 and a previously described mutant virus, SAA (27), that has a 1-bp mutation in the core I element (Fig. 1). Since AKR/J mice have a high spontaneous incidence of T-cell lymphomas that first appear about 7 months after birth, these mice were maintained only until they reached the age of 7 months.

The Ets site mutant induced disease in both NIH/Swiss (Fig. 2) and AKR/J (Table 1) mice, with only a modest increase in the latent period to disease onset. The increase in the mean latent period in each strain was significant in a Student *t* test ($P = 0.0018$ for NIH/Swiss and $P = 0.023$ for AKR/J). The Ets mutant induced disease with a mean latent period that was significantly shorter than that of the core I mutant, SAA ($P = 0.029$ for NIH/Swiss and $P = 0.045$ for AKR/J). We conclude that mutation of the Ets site of SL3 had a modest but statistically significant effect on the tumorigenicity of SL3.

In contrast, mutation of the Myb site almost eliminated the ability of SL3 to induce tumors. Only 3 of 11 NIH/Swiss mice developed tumors following inoculation with the Myb mutant (Fig. 2). Curiously, all three developed the tumors at about 7 months of age. Only one of eight AKR/J mice developed a tumor before 7 months of age (Table 1). Thus, mutation of the Myb site eliminated much of the lymphomagenicity of SL3. The Myb mutation reduced tumorigenicity to a greater extent than the core I mutation did in both mouse strains (Fig. 2; Table 1). This result indicates that the Myb site in the LTR enhancer is crucial for the pathogenicity of this virus.

LTR structures of tumor-derived proviruses. It was important to test the stability of the viral Ets and Myb mutations during the process of tumorigenesis. Previous data showed that the majority of mice that developed lymphomas following inoculation with the core I mutant virus, SAA, contained proviruses with reversions of the original mutation (27). To analyze the LTR structures of proviruses in tumors, genomic DNA was purified from leukemic spleens and thymuses and used as a template for PCR amplification of proviral 5' LTRs as previously described (27). Products were analyzed from 10 of the

TABLE 1. Viral tumorigenicity in AKR/J mice

Virus	No. of mice inoculated	No. of mice with tumor by 8 mo	Avg latency (days)
SL3	12	12	71
Myb mutant	8	1	186
Ets mutant	6	6	91
SAA	8	8	109

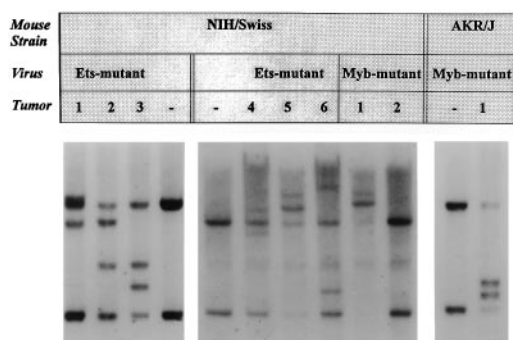


FIG. 3. Southern blot analysis of TCR β rearrangements in tumors induced by the Ets and Myb mutants. Six different tumors induced by the Ets mutant in NIH/Swiss mice are numbered 1 through 6. Two different tumors induced by the Myb mutant in NIH/Swiss mice and one tumor induced in an AKR/J mouse are also shown. Negative control DNAs from livers of uninfected mice of the corresponding strains are indicated by -. DNA samples were digested with *Hpa*I, and Southern blots were hybridized to 86T5, a cDNA clone for the murine TCR β locus (16).

Ets mutant-induced tumors and three of the Myb mutant-induced tumors. Multiple products were detected in all of the tumors. As previously described (27), these consisted of LTRs that contained variable numbers of 72-bp enhancer repeat units. No amplified products were detected in parallel control experiments performed with tissues from uninfected mice. The PCR products from the tumors were excised from the gels and directly sequenced.

Sequence analysis demonstrated that all integrated proviruses from mice inoculated with the Ets and Myb mutant viruses maintained the original point mutations. No reversions were apparent. The sequences of the remainder of the enhancer regions were also intact, indicating that no potential suppressor mutations were present. Thus, viruses that retained the Ets or Myb mutations were the predominant forms in the tumors.

Characterization of the types of tumors induced by the Myb and Ets mutant viruses. SL3 appears to induce strictly T-cell lymphomas. Mo-MuLV also induces T-cell lymphomas. However, mutation of the Ets site in Mo-MuLV resulted in erythroleukemias in about 25% of inoculated mice (37). Therefore, it was important to test whether mutation of the Ets and Myb sites altered the tissue target for transformation by SL3. T-cell lymphomas induced by SL3 generally involve an enlargement of the thymus, and most show evidence of rearrangement of a TCR β locus. In about 70% of SL3-induced lymphomas, the thymus, spleen, and lymph nodes are grossly enlarged, although in about 30% of the tumors, the enlargement of the thymus is less than fivefold relative to age-matched controls. All mice inoculated with the Ets-1 mutant virus showed gross pathologies typical of thymic lymphomas that included enlarged thymus, spleen, and peripheral and mesenteric lymph nodes. We performed an analysis of TCR β gene rearrangements on DNA from six of the Ets mutant-induced tumors. TCR β gene rearrangements were seen in all six (Fig. 3). Thus, it appears that the tumors induced by the Ets mutant are T-cell lymphomas.

Tumors developed in four mice that were inoculated with the Myb mutant. Gross pathologies of the four tumors were similar in that the spleens were greatly enlarged but little to no thymic enlargement was evident. DNA was obtained from three of the tumors and screened for TCR β rearrangements. Figure 3 shows that one of the two tumors in NIH/Swiss mice had a TCR β rearrangement. The tumor induced by the Myb

mutant in an AKR mouse also showed a TCR β rearrangement. The NIH/Swiss tumor without a detectable TCR β rearrangement was also tested for a rearrangement of the IgH D-J region. A rearrangement was detected (data not shown). Thus, all three of the examined tumors induced by the Myb mutant were lymphoid, and at least two were T-cell lymphomas.

Correlation between viral lymphomagenicity and transcriptional activity on the *c-myc* promoter. In about 20% of the T-cell lymphomas induced by MuLVs such as SL3 and Moloney, the *c-myc* gene is a common proviral integration site. Evidence exists that the SL3 LTR enhancer must have an appropriate structure to be able to activate *c-myc* during the process of lymphomagenesis (27). In addition, comparison of the SL3 and Akv LTR enhancers for the ability to activate transcription from the two major *c-myc* promoters in plasmids that contained the *c-myc* promoters linked to the CAT reporter gene showed that the Akv enhancer was almost unable to activate them in T cells (8). In contrast, the SL3 enhancer activated the *c-myc* promoters as much as 50-fold in some T-cell lines (8). The near lack of activity of the Akv enhancer on *c-myc* in T cells was consistent with the hypothesis that this virus is not lymphomagenic in T cells, at least in part because it cannot sufficiently activate *c-myc* and, possibly, other proto-oncogenes (8, 27). Therefore, it was of interest to investigate the effects of c-Myb and Ets-1 mutations on the ability of the SL3 LTR enhancer to activate the *c-myc* promoters.

The enhancer regions of the LTRs of Ets and Myb mutants were inserted into a plasmid containing the first exon and 5'-flanking sequences of the *c-myc* gene linked to the CAT reporter gene (Fig. 4A). This plasmid contained the two major promoters of *c-myc*, P1 and P2. Enhancer sequences were placed in the opposite transcriptional orientation in relation to *c-myc*, as this is the manner in which they are situated in most of the tumors where a provirus is integrated adjacent to *c-myc*. Activities of the mutated enhancers were compared to those of the wild-type SL3 and Akv enhancers by transfection into cultured T cells (Fig. 4B).

As previously shown (8), the SL3 LTR enhancer strongly stimulated transcription from the *c-myc* promoters compared to the activity of the *c-myc* promoters alone (Fig. 4B). In contrast, enhancer sequences of nonleukemogenic Akv gave little to no activation. Mutation of the c-Myb binding site greatly reduced the ability of the SL3 enhancer to activate the *c-myc* gene promoters. Interesting, the levels of transcription were reduced to almost the same levels seen with the Akv enhancer. Thus, mutation of the Myb site strongly reduced both the lymphomagenicity of SL3 and the capacity of the viral LTR enhancer to activate the *c-myc* promoters.

Mutation of the Ets-1 binding site had less of an effect on *c-myc* promoter activity (Fig. 4B). In the mouse T-cell lines SL3H and L691, the Ets site mutation reduced transcription twofold or less. In the human T-cell line Jurkat, it resulted in about a fivefold decrease in activity. The Ets mutation had less of an effect on transcription than the single base pair mutation in the enhancer of the core I mutant virus, SAA. Mutation of the core II site had no effect on activity in any of the cell lines. Thus, mutation of the Ets site resulted in only a modest decrease in lymphomagenicity of SL3 and had less of an effect on transcription from the *c-myc* promoters than mutations in the Myb or core I sites. These findings show that the mutations that most inhibited the ability of the viral enhancer to activate the *c-myc* promoters also caused the greatest reduction in viral lymphomagenicity.

SL3 is integrated adjacent to *c-myc* in about 15% of the lymphomas induced by this virus (27). We tested whether the Ets and Myb site mutants of SL3 were integrated adjacent to

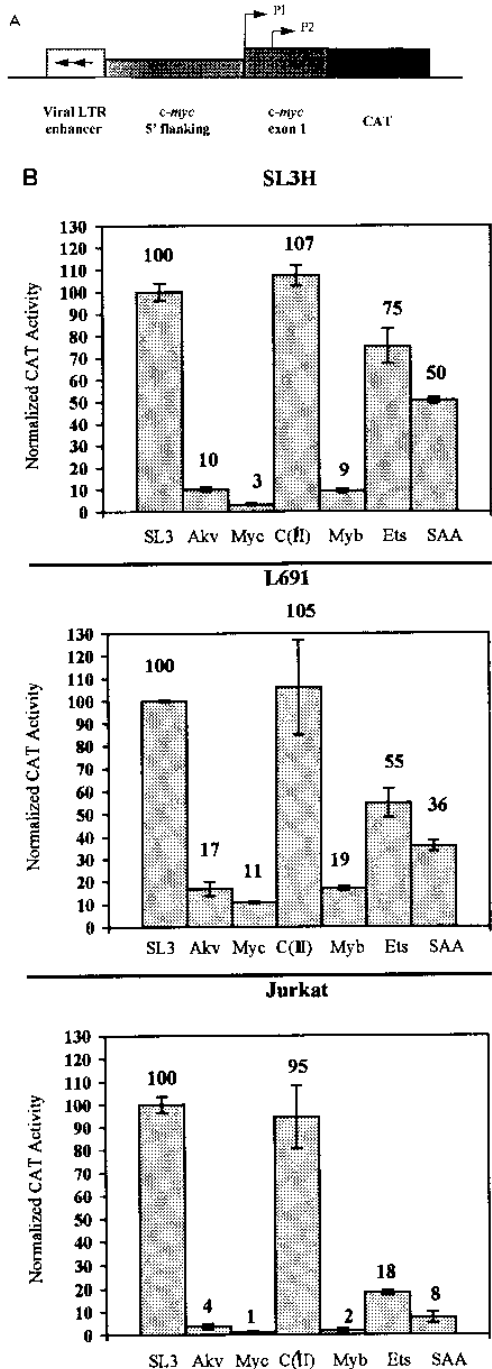


FIG. 4. (A) Organization of the sequences within the LTR-Myc-CAT plasmids. The arrows in the viral enhancer indicate the 5'-to-3' orientation of the enhancer on the viral coding strand. P1 and P2 indicate the initiation sites and direction of transcription from the two major promoters of the mouse *c-myc* gene. (B) Relative transcriptional activities of the SL3 and mutated viral enhancers. Activities were tested in three different T-cell lines, SL3H, L691, and Jurkat. Each experiment was performed four times, and the means were calculated. Activities are shown normalized to that of the SL3 enhancer in each cell line. The number above each bar indicates the activity. Error bars indicate 1 standard deviation. The identities of the enhancers are shown underneath the horizontal axis of each graph. Myc indicates the plasmid with no viral enhancer. C(II), core II.

c-myc by Southern blotting as previously described (27). None of the tumors examined in this study (Fig. 2; Table 1) contained a provirus within the 20-kbp *EcoRI* fragment that contains the *c-myc* gene (28a). However, the number of tumors generated in this study was too low to determine whether the mutation of the Myb or Ets site in fact disrupted the ability of the virus to utilize *c-myc* in the process of lymphomagenesis.

DISCUSSION

Mutation of the Myb site drastically reduced the lymphomagenicity of SL3, while mutation of the Ets site had only a modest, though statistically significant, effect. Previous studies showed that the core I site was also very important for lymphomagenicity of SL3 whereas the core II site had only modest effects (15, 27). Thus, the Myb and core I sites appear to be the most important sites in the SL3 LTR enhancer for viral lymphomagenicity. The Ets and core II sites appear less important quantitatively, although both do affect viral lymphomagenicity.

Interestingly, no correlation between the relative importance of a particular site for lymphomagenicity of SL3 and its conservation among MuLVs was evident. The Myb and core II sites that are just upstream of the Ets and core I sites appear to be unique to SL3 and the very similar Gross passage A virus (20, 23). Presumably, this was due to the addition of these novel sites into the genome of a precursor virus that lacked them. The acquisition of one of the unique sites, the Myb site, was more crucial for viral lymphomagenicity than the other, since mutation of core II had only modest effects on tumorigenic potential (Fig. 2 and reference 15).

Likewise, the highly conserved sites in the viral enhancers differed in their importance for viral lymphomagenicity. The Ets (LVb) site and the adjacent core element, core I in SL3, are present in all MuLVs (14), although some sequence variation has occurred within them, particularly in the core element (14). The core I site was previously shown to be important for lymphomagenicity by SL3 (15, 27). However, mutation of the Ets site had only a minor effect. These results argue that it is not possible to predict the relative importance of particular transcription factor binding sites for viral lymphomagenicity based on evolutionary conservation.

Endogenous ecotropic MuLVs like Akv are at best weakly leukemogenic (19). Akv is the likely evolutionary precursor of SL3 (20). The Myb site is not present in the LTR enhancer of Akv (Fig. 5). Thus, the acquisition of the Myb site was crucial in the derivation of highly lymphomagenic SL3 from Akv. It was previously demonstrated that the single base pair change between the core I element of SL3 and the core element of Akv was important for acquisition of pathogenicity (27). In addition, the appearance of the core II element in SL3 also slightly increased pathogenicity (15). Thus, three changes in transcription factor binding sites, a mutation in one CBF binding site (core I), the addition of a c-Myb binding site, and the gain of a second CBF binding site (core II), contributed to the development of highly lymphomagenic SL3. Conversely, the absence of these sites is correlated with the minimal pathogenicity of the endogenous ecotropic MuLVs.

Both SL3 and Mo-MuLV induce T-cell lymphomas. The LTR enhancers of both viruses contain homologous Ets (LVb) and adjacent core (core I in SL3) sites (Fig. 5). Thus, the cellular transcription factors that appear to be responsible for the ability of SL3 and Mo-MuLV to cause T-cell lymphomas are, in part, shared. However, the Myb site is not present in the LTR enhancer of Mo-MuLV. Thus, the highly T-cell lymphomagenic properties of SL3 and Mo-MuLV appear to be

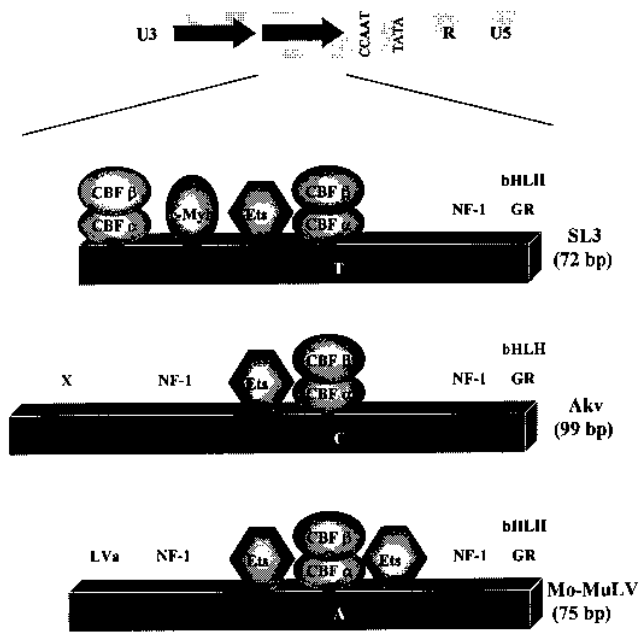


FIG. 5. Comparison of the SL3, Akv, and Mo-MuLV enhancers. The organization of one enhancer repeat unit is shown for each virus below the general organization of the viral LTRs. Geometric figures are used to represent c-Myb, the Ets family of factors, and the subunits of CBF. Two Ets binding sites in Mo-MuLV are shown. The one to the left of the CBF binding sites is also known as the LVb site, and the one to the right is also called the LVc site (36, 38). Sites for NF-1, the glucocorticoid receptor (GR), and bHLH factors are shown. X in Akv indicates a binding activity of unknown identity (26). LVa in Mo-MuLV also indicates a binding activity of unknown identity (36). The positions of the homologous core elements in the three viruses are indicated by the single nucleotides within the boxes. These represent the positions where the three core elements, TGTGGTAA in SL3, TGTGGTCA in Akv, and TGTGGTAA in Mo-MuLV (20, 36), differ.

due to the evolution of transcription factor binding sites that are, in part, different.

Mo-MuLV contains a second Ets binding site, called the LVc site, that is situated downstream of the core element and is not found in either SL3 or Akv (Fig. 5). However, mutation of the LVc site had no effect on the lymphomagenicity of Mo-MuLV (37). Besides the Ets (LVb) and core elements, two other sites were found to be important for lymphomagenicity of Mo-MuLV, the NF-1 site and the glucocorticoid response element (GRE) (37). The mutation in the GRE also presumably disrupted an overlapping site that likely binds basic helix-loop-helix (bHLH) factors including SEF2 and ALF1 (9, 28). The NF-1 and the GRE/bHLH sites appear to be conserved in MuLVs including SL3 and Akv. Thus, it is uncertain what distinguishes highly T-cell lymphomagenic Mo-MuLV from weakly pathogenic Akv. It would also be interesting to know whether the NF-1 and GRE/bHLH sites are important for T-cell lymphomagenicity by SL3.

Cotransfection studies with transcription factors showed that CBF could not activate transcription through the conserved core element of SL3 and Mo-MuLV unless a heterologous transcription factor was bound to a neighboring site. In the case of Mo-MuLV, the activity of CBF required the binding of a member of the Ets family of factors to the Ets (LVb) site (38, 42). In the case of SL3, CBF required either a factor at the Ets site or the binding of c-Myb to the Myb site (43). It is interesting that the cooperative stimulation of transcription by CBF and c-Myb did not require an intact Ets site (43). Thus, the appearance of the Myb site in SL3 may have reduced the

necessity for binding of an Ets family member. This is consistent with modest effect of the Ets site mutation on the lymphomagenicity of SL3 seen here. An alternative speculation to explain the limited importance of the Ets site in SL3 is that DNA binding by an Ets family member might be less affected by the mutation of the Ets site when both CBF and c-Myb are bound at the flanking sites that are present in this virus.

It is interesting to note the correlation between the reduction in transcriptional activity and the reduction in viral lymphomagenicity. Mutations that had the greatest effect on the ability of the viral enhancer to activate the *c-myc* promoters also had the greatest effect on viral lymphomagenicity. This is consistent with the idea that the transcriptional activity of the LTR enhancers determines viral lymphomagenicity. It is curious that although the relationship between effects on transcriptional activity and viral lymphomagenicity is proportional, it does not appear to be linear. It is also interesting that the Myb site mutation reduced the activity of the SL3 enhancer to the same level as that of the Akv enhancer on the *c-myc* promoters. The inability of the LTR enhancer of the virus with Myb site mutation to activate *c-myc* promoters could certainly contribute to its greatly decreased lymphomagenicity. It remains to be tested whether these mutations show similar effects on the promoters of other proto-oncogenes.

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