JOURNAL OF VIROLOGY, Jan. 1995, p. 446–455 Vol. 69, No. 1
0022-538X/95/$04.00+0

Long Terminal Repeat Enhancer Core Sequences in Proviruses Adjacent to c-*myc* in T-Cell Lymphomas Induced by a Murine Retrovirus

HARRY L. MORRISON, BOBBY SONI, AND JACK LENZ*

Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York 10461

Received 28 June 1994/Accepted 17 October 1994

The transcriptional enhancer in the long terminal repeat (LTR) of the T-lymphomagenic retrovirus SL3-3 differs from that of the nonleukemogenic virus Akv at several sites, including a single base pair difference in an element termed the enhancer core. Mutation of this T-A base pair to the C-G sequence found in Akv significantly attenuated the leukemogenicity of SL3-3. Thus, this difference is important for viral leukemogenicity. Since Akv is an endogenous virus, this suggests that the C-G in its core is an adaptation to being minimally pathogenic. Most tumors that occurred in mice inoculated with the mutant virus, called SAA, contained proviruses with reversion or potential suppressor mutations in the enhancer core. We also found that the 72-bp tandem repeats constituting the viral enhancer could vary in number. Most tumors contained mixtures of proviruses with various numbers of 72-bp units, usually between one and four. Variation in repeat number was most likely due to recombination events involving template misalignment during viral replication. Thus, two processes during viral replication, misincorporation and recombination, combined to alter LTR enhancer structure and generate more pathogenic variants from the mutant virus. In SAA-induced tumors, enhancers of proviruses adjacent to c-*myc* had the largest number of core reversion or suppressor mutations of all of the viral enhancers in those tumors. This observation was consistent with the hypothesis that one function of the LTR enhancers in leukemogenesis is to activate proto-oncogenes such as c-*myc*.

Murine leukemia viruses (MuLVs) are retroviruses that induce hematopoietic tumors after a period of weeks to months following inoculation of susceptible newborn mice (2, 22, 86). The leukemogenic potential of MuLVs varies among virus isolates. Some induce thymic lymphomas, while others cause erythroleukemia or myeloid leukemia. Others are nonleukemogenic. Recombination studies using molecular clones of MuLV genomes have shown that the most important genetic determinant of viral leukemogenicity is a transcriptional enhancer within the long terminal repeat (LTR) (9, 18, 35). The nature of the LTR enhancer determines both the tissue specificity of the disease and the potency of the virus. Transcription assays using various types of hematopoietic cells have shown that the cell type preference of LTR enhancer activity correlates with the tissue target for leukemogenicity (7, 71, 80). In general, enhancers of viruses that cause tumors in a particular type of cell are 2 to 20 times more active in that type of cell than enhancers of other viruses. Presumably, this difference in transcriptional activity accounts for the difference in viral leukemogenicity by affecting specific events in the process of leukemogenesis.

Several steps have been defined in leukemogenesis by MuLVs (2, 22, 86). During thymic lymphomagenesis by such viruses as SL3-3 (SL3) and Moloney leukemia virus (Mo-MuLV), virus infection is established in the thymus. The nature of the LTR enhancer affects this process (19, 62). However, infection of the target tissue is not sufficient for leukemogenesis to ensue (21). Some nonleukemogenic, mink cell focus-forming (MCF) viruses are equally proficient as leukemogenic MCF viruses in establishing virus infection (49, 50). Holland et al. (27) found that the LTR enhancer sequences determine whether these viruses are pathogenic. Therefore, the LTR enhancers affect one or more additional events in leukemogenesis.

Viral thymic lymphomagenesis involves a preleukemic phase that is characterized by hyperplasia in multiple hematopoietic lineages (22). During this period, ecotropic viruses often recombine with endogenous viral sequences to generate MCF viruses (26, 78, 88). Studies of a nonleukemogenic variant of Mo-MuLV with a mutant enhancer region showed it to be highly infectious but incapable of inducing preleukemic hyperplasia or propagating MCF viruses (5, 6, 15–17). These findings suggest that preleukemic hyperplasia is necessary for disease and that MCF *env* gene expression may play a role in this process. Expression of MCF *env* gene products was shown to render interleukin-2- or erythropoietin-dependent cells factor independent (37, 38, 64, 84). This suggests that the MCF SU/TM protein can stimulate hyperplasia of T cells and other hematopoietic cells in an autocrine manner via growth factor receptors. The nature of the LTR enhancer may affect the levels of *env* gene expression.

Hyperplastic cells in the T-lymphocyte lineage may be precursors of clonal or oligoclonal tumors. Tumor cells contain proviruses integrated adjacent to cellular proto-oncogenes. The most common proviral integration sites in T-cell lymphomas are c-*myc* and *pim-1* (89). Proviral integrations near c-*myc* cluster at certain sites up to 270 kb from the gene and appear to activate its expression (12, 32, 87). Nearly all proviruses within 2 kb 5′ of the first exon of c-*myc* are in the opposite transcriptional orientation (12, 39, 48, 58, 69, 76). On the basis of the relative orientations of the proviruses and c-*myc* in tumors induced by various retroviruses, it was hypothesized that a viral enhancer, presumably the known enhancer in the LTR, is responsible for c-*myc* activation (12, 55), although this is difficult to test directly. Likewise, LTR enhancers have been

* Corresponding author. Mailing address: Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-3715. Fax: (718) 430-8778. Electronic mail address: lenz@aecom.yu.edu.

hypothesized to activate other proto-oncogenes (31, 47, 89). In the case of *pim-1*, most proviruses are found in the 3′ untranslated region and in the same transcriptional orientation as the gene (67, 68). This results in a fusion transcript where LTR sequences replace most of the 3′ untranslated sequences normally found in *pim-1* mRNA, thereby increasing its stability. It is uncertain whether proviruses integrated into the 3′ untranslated sequences also act by an enhancer mechanism to stimulate the *pim-1* promoter.

The SL3 enhancer consists of two tandem 72-bp repeats that contain binding sites for multiple nuclear factors including CBF (4, 42, 81), S-CBF (4), Myb, Ets-1, (41), NF-1/CTF (45), SEF2 (13), and the glucocorticoid receptor (8). The binding site for CBF and S-CBF is an element that was termed the enhancer core because a similar, although not identical, sequence is present in the enhancers of all MuLVs as well as in other enhancers (24, 30, 35, 74). Mutations in the cores were reported to decrease the leukemogenicity of Mo-MuLV and SL3 (25, 97) or to alter the tissue target for transformation by Mo-MuLV (73). The SL3 and Akv core elements differ by a single base pair (35). This difference affects enhancer activity specifically in T cells (4). It also alters the binding of at least one factor present in the nuclei of mammalian hematopoietic cells (4). Therefore, we wished to test whether the single base pair difference was critical for the leukemogenicity of SL3. In this study, we constructed a mutant virus containing only this single base pair difference relative to SL3. It induced lymphomas with a surprisingly high incidence. Therefore, we undertook an analysis to determine whether reverted viruses were present in mice that developed tumors. Most tumors contained mixed populations of viruses with rearrangements in the enhancers that appear to have occurred during viral replication. The presence of multiple viruses in the tumor DNA offered a means to test whether the nature of the viral enhancer affected the capacity to activate c-*myc*.

MATERIALS AND METHODS

Mutation of the core elements. The core elements in two tandem 72-bp repeats in the LTR of SL3 were mutated by using a plasmid subclone of the SL3 LTR. A 772-bp *Pst*I-to-*Bgl*II fragment that contained virtually all of the 5′ LTR and part of the 5′ untranslated region of SL3 was inserted into the *Pst*I and *Bam*HI sites of pGEM3Z(−) (Promega). A 91-bp *Eco*RV-to-*Apa*I (both of which cleave within the enhancer repeats) fragment that contained both core elements was excised from the resulting plasmid and replaced by a 91-bp synthetic oligonucleotide that had the SL3 T-to-Akv C transition in each core to generate a plasmid termed pG-SAA. The structure of the resulting enhancer was confirmed by sequencing. A 447-bp fragment containing the enhancer was then excised by digestion with *Pst*I, removal of the 3′ overhang with T4 DNA polymerase, and digestion with *Bss*HII. The last enzyme cleaves SL3 at the U3-R boundary in the LTR. This fragment was then used to replace the corresponding sequences in a plasmid clone of the SL3 LTR linked to the chloramphenicol acetyltransferase (CAT) gene, pSU3-CAT (7). pSU3-CAT was digested with *Nde*I (which cleaves just 5′ to the LTR), and the 5′ terminus was filled in using the Klenow fragment of *Escherichia coli* DNA polymerase I and then digested with *Bss*HII to generate a 3.5-kbp fragment. The two fragments were ligated together to generate pSAA-CAT with the SAA LTR linked to the CAT gene.

To generate an infectious viral subclone of the SAA LTR linked to the remainder of the genome of SL3, pG-SAA was digested with *Bss*HII and *Eco*RI. The latter enzyme cleaves in the plasmid vector sequences. A 3.8-kbp fragment containing the U3 portion of the LTR and vector sequences was isolated. The remainder of the SL3 genome was isolated as a 7.7-kbp *Bss*HII-to-*Eco*RI fragment from a plasmid subclone of SL3 (36). The two fragments were linked together to generate pSAA. This plasmid contained a single LTR. To generate infectious virus, the viral sequences were excised from this plasmid by digestion with *Pst*I, ligated to form concatemers with two identical LTRs, as previously described, and used for transfection of NIH 3T3 fibroblasts (35, 36).

CAT assays. CAT assays were performed using three murine T-cell lines (L691-6, WEHI 7.1, and SL3B), the human T-cell line Jurkat, the murine erythroleukemia cell line MEL DS-19, and the murine fibroblast line NIH 3T3. Most cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 U of streptomycin per ml. Jurkat cells were grown in RPMI 1640 with the same supplements. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and the same antibiotics. Transfections were performed as previously described by the DEAE-dextran method (71). Cells (5×10^6) were pelleted and resuspended in 1 ml of serum-free medium containing 250 μg of DEAE-dextran per ml and 5 μg of supercoiled plasmid DNA. They were incubated for 1 h at 37°C with 100% humidity and 7.5% CO_2, pelleted, resuspended in 10 ml of medium plus serum, and harvested 2 days later.

Leukemogenicity assays. SL3 and SAA virus production in transfected cultures was monitored regularly by reverse transcriptase assays. Once viral production was high, at about 2 weeks posttransfection, frozen viral stocks were prepared, and infectious titers were determined by endpoint dilutions of the stocks. Newborn mice (less than 48 h old) were injected intraperitoneally with about 0.1 ml of culture supernatant containing 5×10^3 to 1×10^4 infectious units of virus. Mice were monitored several times per week. Moribund individuals were sacrificed and examined for gross pathological changes including enlargement of the thymus, spleen, liver, and peripheral and mesenteric lymph nodes. Lymphomatous tissues were stored frozen for future analysis.

PCR amplification of proviral LTRs. PCR was performed with cellular genomic DNA as a template. The PCR primer pair employed was at positions −475 to −442 and 274 to 247 relative to the viral transcriptional initiation site. The sequences were 5′-TTCATAAGGCTTAGCCAGCTAACTGCAG-3′ and 5′-GATGCCGGCACACACACACACACTCTCCC-3′. To amplify proviruses adjacent to c-*myc* or *pim-1*, an LTR primer was used in conjunction with an oncogene-specific primer. For c-*myc*, five primers, from positions 122 to 95, 296 to 269, 627 to 600, 1342 to 1316, and 1688 to 1659 relative to an *Xba*I site located 1.5 kbp 5′ of exon I (12), were tried in individual reactions. The sequences were 5′-TACTACGCTGTGCATTCTGTACAATCCC-3′, 5′-AGTAAAGTGGCGG CGGGTGCGCTCTACC-3′, 5′-AGTAATAAAAGGGGAAGGCTTGGGTTT G-3′, 5′-GTGGGGAGCCGGGGAAAGAGGAGGAGGA-3′, and 5′-TCCCT CTGTCTCTCGCTGGAATTACTACAG-3′. For *pim-1*, two primers located in exon 6, 5′-ATTAAATGGTGCCTGTCCCTGAGACCGT-3′ and 5′-GTCAGA CCCTCCAGGGAGGGAGAGCTTG-3′, were tried. PCR amplifications were performed in 100 μl with 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.25 mM each primer, 0.025 U of Ampli/*Taq* polymerase (Perkin-Elmer Cetus) per ml, and 5 to 10 ng of genomic DNA per ml. PCR mixes were heated to 94°C and then subjected to a 30-cycle program (1 min at 94°C, 1 min at 64°C, and 3 min at 72°C) and a 10-min extension step using a thermal cycler (Perkin-Elmer Cetus). Most reactions generated multiple products. These were resolved by electrophoresis through nondenaturing 5% polyacrylamide gels in 1× Tris-borate-EDTA (TBE) buffer at 10 V/cm for 100 Vh · cm. Gels were stained in 0.5 μg of ethidium bromide per ml in 1× TBE. Individual bands were excised, the gel was crushed, and DNA was eluted in 100 μl of 10 mM Tris-HCl (pH 8)–1 mM EDTA at 60°C for 16 h.

DNA sequencing. Templates were prepared for DNA sequencing using asymmetric PCRs. A 5-μl volume of an eluted PCR fragment was subjected to asymmetric PCR under the conditions described above, except that one primer was used at 0.5 mM and the other was used at 0.01 mM. DNA sequencing was performed by the chain termination method using Sequenase (U.S. Biochemicals) with a plus-strand primer from positions −150 to −171 (5′-ACAAGGAA GTACAGAGAGGC-3′) or a negative-strand primer from positions −364 to −382 (5′-TTGAAACTGTTGTTGTTTAGC-3′). Reaction products were resolved on 6% polyacrylamide wedge gels in 1× TBE at 100 W for 3 h.

Southern blots. Southern blotting was performed to detect LTR fragments containing the enhancer repeats in genomic DNA from tumor tissue. DNAs were digested with *Eco*RI and *Kpn*I. About 30 μg of DNA was resolved on 2% agarose gels in 1× TAN (71) buffer and then transferred to a nylon membrane. A 72-bp *Bst*NI fragment from the SL3 enhancer was used as a probe. The final wash was performed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 61°C. Densitometry was performed on an autoradiograph of the blot with a laser densitometer.

To detect rearranged c-*myc* fragments, genomic DNAs were digested with either *Eco*RI or *Kpn*I. To detect rearranged *pim-1*, N-*myc*, or L-*myc* fragments, DNAs were digested with *Eco*RI. About 10 μg of DNA was resolved on 0.7% agarose gels in 1× TAN buffer and transferred to nylon membranes. For c-*myc*, the probe employed was a 1.4-kbp *Xho*I fragment from a plasmid clone of murine c-*myc* cDNA, p*myc*54 (75). For *pim-1*, the probe used was a 2.4-kbp *Sac*I fragment of *pim-1* genomic DNA (14). For L-*myc*, a cDNA was used (33).

RESULTS

Leukemogenicity of the SL3 core mutant. The LTR enhancers of SL3 and Akv consist of tandem repeats located 173 bp upstream of the transcription initiation site (Fig. 1A). The enhancer of SL3 contains two 72-bp repeats, with one core motif present in each. The Akv enhancer consists of two 99-bp repeats, each containing a core. The SL3 core (TGTGGTT AA) differs from the Akv core (TGTGGTCAA) by a single T-C transition. To test the importance of this difference for viral leukemogenicity, the Akv C was substituted for the T in

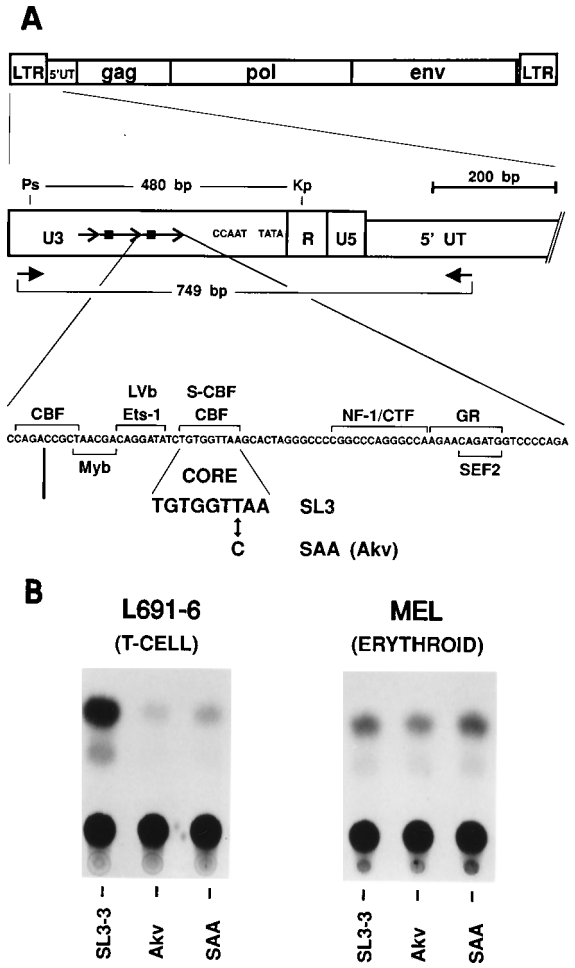

FIG. 1. Structure of the SL3 enhancer and activity in transient expression assays. (A) The three maps show the structure of the SL3 genome, the structure of the viral LTR, and the sequence of the viral enhancer. The sequence consists of one 72-bp repeat and five bases of the next unit upstream. Binding sites for several cellular factors are shown. The position of the core element is indicated, including the position of the 1-bp difference in the cores of Akv and SAA. In addition, the map of the full LTR shows the positions of the *Pst*I and *Kpn*I sites used for Southern blotting and the 749-bp product of PCR amplification. (B) CAT assays using the LTRs of the three viruses are shown for two cell lines.

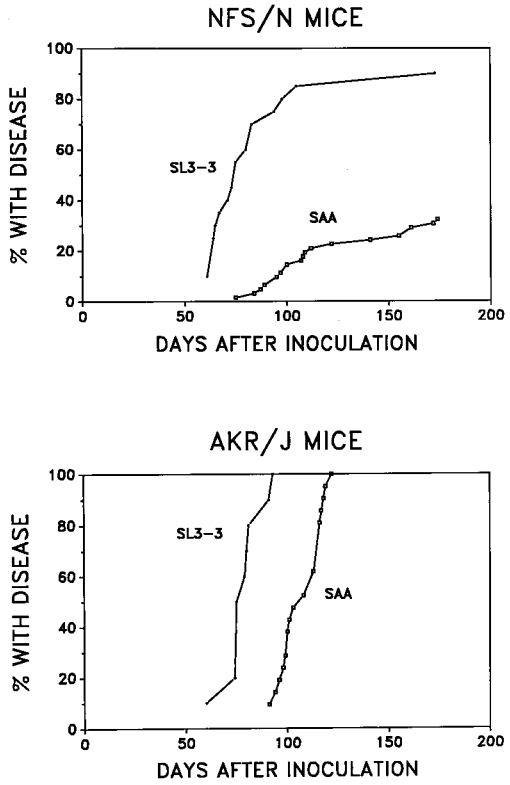

FIG. 2. Leukemogenicity assays in NFS/N and AKR/J mice.

both copies of the core in a plasmid clone of the SL3 LTR. Previous experiments demonstrating that the T-to-C change diminished transcriptional activity specifically in T lymphocytes were performed with LTRs that lacked one repeat unit (4). We first tested the activity of the enhancer with both cores mutated in transient expression assays in hematopoietic cells. Examples are shown in Fig. 1B. In all four T-cell lines tested, L691-6, Wehi 7.1, SL3B, and Jurkat, the mutations eliminated most of the difference in activity between the SL3 and Akv LTRs. In non-T cells, they had little or no effect. Therefore, the core mutations affected the activity of enhancers with two repeat units and did so specifically in T lymphocytes.

To test for effects of the core mutation on leukemogenicity, the mutated LTR was then linked to the remainder of the genome of SL3 in a plasmid clone. Infectious virus was generated by transfection of this DNA into NIH 3T3 fibroblasts. Since the plasmid clone had only a single LTR, both LTRs in the resulting virus were the same. The resulting virus, termed SAA, was identical to SL3 except for the two single base pair transitions, one in each core. In NFS/N mice, SAA generated a lower incidence of disease and a longer average latent period than SL3 (Fig. 2). In AKR/J mice, both viruses induced disease in 100% of inoculated mice. However, the SAA-inoculated mice developed disease more slowly than the SL3-inoculated mice. For the assays in both strains, application of Student's *t* test confirmed that the difference in mean latency of disease between SL3- and SAA-inoculated mice was highly significant ($P < 0.001$). However, since the mutation had large effects on enhancer activity in T cells (Fig. 1B), it was surprising that SAA was as potent as was observed. Therefore, we considered the possibility that revertant viruses that were the proximal cause of disease may have been generated.

Variability in the number of enhancer direct repeats in tumor proviruses. To determine whether the SAA virus underwent reversion during the preleukemic phase, DNAs were extracted from the tumors and used as templates for PCR amplification of the proviral LTRs. To amplify SL3 or SAA LTRs specifically, a PCR primer pair (25) was employed that amplified a 749-bp viral fragment that included virtually all of the 5′ LTR and a portion of the 5′ leader downstream of the LTR (Fig. 1A). The primer in the leader region had six base pair differences at the 3′ end from the sequence in the endogenous Akv virus (3). Thus, the primers did not amplify endogenous viral sequences. The resulting products were a mixture of LTRs from all of the SL3 or SAA proviruses present in a particular tumor.

Surprising results were observed when the amplified LTRs from the SAA-induced tumors were analyzed (Fig. 3A). All of the PCRs generated a product with the expected 749-bp length. However, there were usually additional products that were larger or smaller. PCR amplification using tumor DNAs from SL3-3-inoculated mice also yielded similar, multiple-size products. To investigate the possibility that the products of

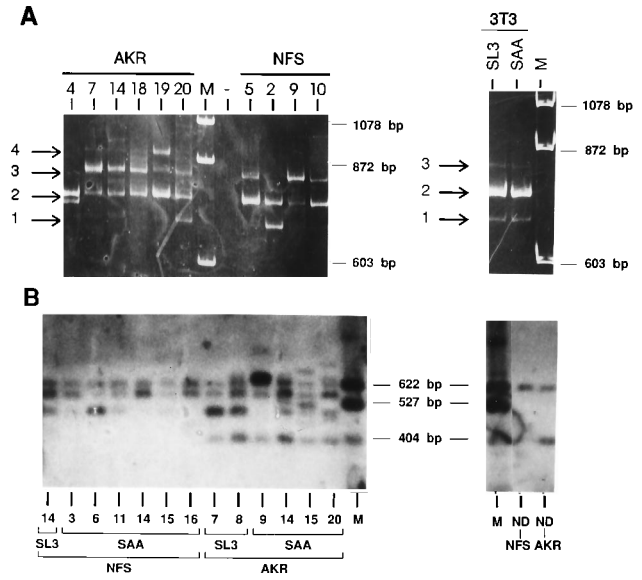

FIG. 3. PCR and Southern blot analyses of proviral LTRs in lymphomas. (A) PCR amplification of proviral 5′ LTRs. The positions of the primers used are diagrammed in Fig. 1A. LTRs were amplified from lymphomas in AKR or NFS mice. Individual mice are indicated by the numbers. LTRs were also amplified from SL3- and SAA-infected NIH 3T3 fibroblasts. Arrows show positions of fragments that contain one, two, three, or four tandem 72-bp repeats. The lanes marked M are size markers included in the electrophoresis. No template DNA was included in the lane marked with a minus. Negative control DNAs from either uninfected NIH 3T3 cells or from the liver of a 3-month-old uninfected AKR mouse included in PCRs did not yield any products with these PCR primers, since they are SL3 specific (25). (B) Southern blot analysis of tumor proviral LTRs hybridized with a 72-bp SL3 enhancer probe. Lymphoma DNAs were digested with *Pst*I and *Kpn*I, which cleave the viral LTRs as shown in Fig. 1A. The left panel shows tumor DNAs from SL3- or SAA-inoculated NFS or AKR mice. Numbers refer to individual mice. The right panel shows DNAs from tissues of uninfected mice (ND). M indicates size markers from pBR322 DNA digested with *Msp*I and hybridized to a pBR322 probe.

unexpected mobilities involved rearranged LTRs, one with an apparent length of about 820 bp was subcloned into a plasmid vector. Analysis of this product by restriction enzyme digestion revealed that it was an amplified LTR with a third, tandem copy of a 72-bp direct repeat. Subsequent DNA sequencing (described below) showed that most of unexpected size products were in fact insertions or deletions of tandem, 72-bp units. One hypothesis that could explain the presence of mixtures of proviruses with various numbers of enhancer repeats is that the rearrangements may have occurred during PCR amplification. Jumping artifacts can occur during PCR if 3′ ends prematurely form within a repeat unit that, upon the next cycle, hybridize to alternative complementary sequences in another repeat (65).

Therefore, it was important to determine whether the rearranged LTRs were present within the proviruses in the tumor DNAs. For this purpose, Southern blotting was performed on a panel of tumor DNAs and DNAs from normal tissue of both mouse strains (Fig. 3B). The restriction enzymes used, *Pst*I and *Kpn*I, cleave the viral LTRs on opposite sides of the enhancer repeats (Fig. 1A). A gel-purified, radiolabeled, 72-bp fragment consisting of a single SL3 repeat unit was used as a probe. This procedure detected a fragment of approximately 600 bp in the NFS/N germ line and fragments of about 400 and 600 bp in the AKR/J germ line (Fig. 3B).

The expected size of an SAA LTR fragment with two 72-bp enhancer repeats was 480 bp. This fragment was detected in all six of the SAA-induced NFS/N tumors examined (Fig. 3B). In addition, all six contained a band of about 550 bp, corresponding to that expected for an LTR fragment with three tandem copies of the 72-bp unit. Fragments corresponding to the LTRs containing two or three repeat units were also detected in AKR/J tumors induced by SAA. One tumor (AKR SAA-9) generated a fragment of about 625 bp that corresponded to an LTR containing four 72-bp tandem repeats. Another tumor (AKR SAA-15) generated several fragments with unusual sizes, including two of about 520 and 450 bp. Subsequent sequencing analysis (described below) showed that these corresponded to proviruses containing deletions within a 72-bp unit. In addition, fragments with the sizes expected for LTRs containing two or three repeats could be detected in tumors induced by wild-type SL3. In summary, the pattern of LTR fragments detected in Southern blots correlated with that seen with the PCR products in all of the tumors. We conclude that proviruses with various numbers of enhancer repeat units commonly occur in both SAA- and SL3-induced tumors. Similarly, Brightman et al. (5) reported the frequent appearance of a third repeat unit in mice infected with an LTR variant of Mo-MuLV.

The relative amounts of proviruses with different numbers of enhancer repeats were quantified by densitometry. Since DNA fragments with larger numbers of repeats should hybridize proportionately more probe, the densitometric determinations were adjusted to the number of repeat units that each contained. This analysis showed that the number of enhancer repeats in tumor DNAs was quite variable. In the 10 SAA-induced tumors examined, 6 had one form that was at least three times more abundant than any other. Specifically, in one tumor (AKR 9), a structure with four repeats predominated. In three tumors (NFS 3, 14, and 16), the three-repeat form was present at the highest levels. In one tumor (AKR 15), a 2.5-repeat form was most abundant. In one (NFS 6), the two repeat form predominated. In the other four tumors (NFS 11, NFS 15, AKR 14, and AKR 20), the two- and three-repeat LTR forms were present at about equal levels (less than a 1.4-fold difference). Thus, Southern blotting showed that the two- and three-repeat structures were the most common, and they frequently occurred in the same tumors. Since these two forms were also the predominant PCR products, this showed that the PCR products were generated at approximately the same relative levels at which they were present in a tumor. In particular, the PCR did not grossly overproduce the two-repeat structure relative to the three-repeat structure.

Most SAA-induced tumors contain revertants. To determine whether reverted proviruses were present in the SAA-induced tumors, sequencing was performed directly on PCR products without prior subcloning. The products were separated by size on polyacrylamide gels. Sequences were obtained for the enhancer repeats and the immediately adjacent sequences of 74 PCR fragments from SAA-induced tumors in 38 mice. A total of 36 of the tumors contained proviruses with two enhancer repeats (Table 1). Most also contained proviruses with three repeats. Several contained proviruses with one or four repeats. In addition, seven of the mice had tumors with proviruses containing small deletions within one or more of the repeat units. Tumors induced by wild-type SL3 also had variable numbers of enhancer repeats (Table 1). Analysis of the proviruses was also performed on DNAs extracted from the spleens of five 10-month-old SAA-inoculated mice that had failed to develop lymphomas. These tissues contained only proviruses with one or two detectable enhancer repeats. We conclude that viruses with additional copies of the enhancer are frequently selected during the process of leukemogenesis.

Of the 38 SAA-inoculated mice that developed lymphomas, 26 had one or more proviruses in which at least one of the

TABLE 1. Numbers of animals with viral enhancers with various numbers of repeats

No. of repeats or enhancer type[a]	No. of animals in which virus was detected		
	SAA	SL3	SAA nondiseased
1	9	2	5
2	36	10	5
3	29	8	0
4	3	0	0
Nonintegral	7	0	0
Total no. of animals	38	10	5

[a] Nonintegral, mice with proviral enhancers that had internal deletions within one or more 72-bp repeats.

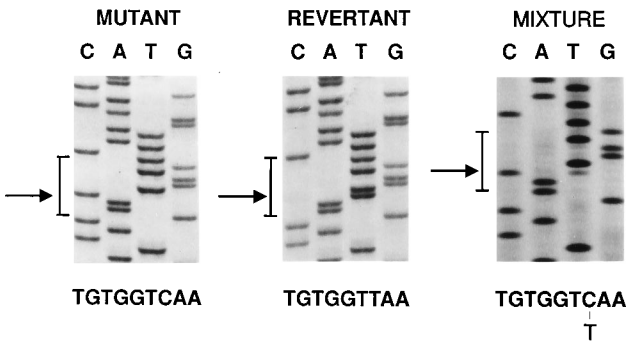

FIG. 4. Sequencing analysis of the core region of lymphomas in SAA-inoculated mice. Examples of the core regions from three separate tumors are shown.

enhancer core mutations had reverted to the SL3 sequence (Table 2). Only 8 of the mice had tumors in which revertants were not detected (Table 2). Examples of the LTR sequence analysis are shown in Fig. 4. Of the 74 sequenced PCR fragments from tumors in SAA-inoculated mice, 29 exhibited a mixture of two different core sequences (Fig. 4C). In summary, core sequence reversions were detected in about 70% of the tumors, and most tumor DNAs contained multiple, proviral LTR structures.

Eleven tumors had proviruses that retained the original mutation but also had a new mutation at a different base pair within the core (Table 2). In three different mice, the third base pair in the core had the same transition (TGCGGTCAA). This same core sequence was previously observed in the enhancer of the T-cell lymphomagenic virus Soule MuLV (12). On the basis of these two facts, it is likely that these represent second-site suppressor mutations within the SAA core element. A different second-site mutation was detected in six other SAA-inoculated mice, in which the base pair just 3′ to the original change underwent the same transversion (TGTG GTCTA). This mutation, termed T*, was found in six independent tumors. Thus, we feel that it is also a suppressor mutation, although proof of this will require testing viruses that have the T* core structure for leukemogenicity. Six tumor DNAs contained both a provirus with a reverted core and another provirus with a suppressor mutation in the core. Two other core mutations were detected, each in one mouse (Table 2). In summary, about 75% of the tumors in SAA-inoculated mice had proviruses with reversions or potential suppressor mutations within the core element. We conclude that the nature of the core element was highly important for viral leukemogenicity. Thus, one or more steps in the leukemogenic process were sensitive to the precise sequences of the cores and the transcription factors that bind differentially to them. Viruses that could perform these processes more effectively were selected during leukemogenesis.

Further support for these conclusions was obtained by sequencing the enhancers from 20 proviruses in 10 SL3-induced tumors. All maintained the original SL3 core structure. This indicated that this core was highly potent and was not strengthened by additional mutations. We also determined

TABLE 2. Infected tissues with at least one provirus containing the indicated core sequences

Element	Sequence[a]	No. of animals (% of total no.)[b]		
		SAA tumor	SL3 tumor	SAA nondiseased[c]
SL3	TGTGGTTAA	26 (68)	10 (100)	0 (0)
Soule	TGCGGTCAA	3 (8)	0 (0)	0 (0)
T*	TGTGGTCTA	6 (16)	0 (0)	0 (0)
Other	TGTGGTCGA	1 (3)	0 (0)	0 (0)
Other	TGTAGTCAA	1 (3)	0 (0)	0 (0)
Akv-SAA (only)[d]	TGTGGTCAA	8 (21)	0 (0)	5 (100)

[a] Core sequences are shown. Underlined nucleotides differ from the sequence of the Akv-SAA core.

[b] Numbers of animals of each category in which the core sequence was detected. Thymuses or spleens from 38 diseased SAA-inoculated mice, 10 diseased SL3-inoculated mice, and 5 nondiseased SAA-inoculated mice were used.

[c] DNA was isolated from the spleens of these animals.

[d] Mice in which all of the sequenced PCR products had the Akv-SAA core structure.

1	No.	2	No.	3	No.	4	No.
T	4	TT	8	TTT	3	TTTC	2
So	1	TT*	1	SoSoSo	1		
T*	1						
C	7	TC	7	TTC	10		
		CT	3	TCT	0		
		CT*	1				
		CC	12	CTT	1		
				CSoSo	1		
				CT*T*	1		
				TCC	1		
				CTC	0		
				CCT	1		
				CCC	1		

FIG. 5. Summary of enhancer core structures in SAA-induced tumors. Structures of the core elements of the viral enhancers are shown. Left-to-right order of core sequences corresponds to the promoter-distal-to-promoter-proximal order of the enhancer repeats. C, the Akv-SAA core structure; T, the SL3 core structure; So, indicates the core structure found in Soule MuLV (TGCG GTCAA); T*, the core structure with an A-to-T substitution in the base immediately 3′ to the SAA mutation (TGTGGTCTA). Thus, CSoSo signifies an enhancer with three 72-bp repeats with the SAA core sequence in the promoter-distal repeat and the Soule MuLV core sequence in the two others. The numbers at the top of the figure refer to the numbers of 72-bp repeats in the viral enhancer. LTRs with internal deletions within the enhancer repeat were excluded from this analysis. The numbers in the no. columns refer to the numbers of tumors that contained viruses with an enhancer of the given structures.

the core structures of proviruses in NIH 3T3 fibroblast cultures infected with the SAA virus. While LTRs containing one, two, or three 72-bp repeats could be seen (Fig. 3), only the original mutated core structure (TGTGGTCAA) was detected. Thus, the reversions and suppressor mutations were not selected during passage on fibroblasts.

LTR structures from 10 proviruses in spleens of 10-month-old, SAA-inoculated mice that failed to develop lymphomas were also analyzed. All maintained the mutated core structure of SAA (Table 2). In addition, we examined the sequences of the enhancer repeats in the portion outside the core elements in the SAA- and SL3-induced tumors. No mutations were detected in the enhancer repeats of any of the 20 sequenced proviruses in SL3-induced tumors. In the SAA tumors, six single-base differences within the 72-bp repeats but outside the cores were detected. Thus, most of the enhancers had no changes other than those in the core elements. We conclude from these studies that enhancer core mutations do not accumulate in the absence of selective pressure that favors the viruses with the altered sequences.

Enhancer structures in SAA-induced tumors. Insight regarding the mechanisms underlying the formation of the variant LTRs detected in lymphomas in SAA-inoculated mice was obtained by analyzing their structures. Figure 5 presents a summary of the LTRs of 66 proviruses in the 38 tumors. The structures are represented by a one-letter abbreviation for the sequence of the core in each of the tandem repeats. Only LTRs in which all bases were unambiguous were included. Thus, if a mixture of core sequences was detected in two or more repeat units, that LTR was excluded. Several points are noteworthy. In LTRs with one or two repeats, many maintained the mutated core structure. However, in LTRs with three or four repeats, virtually all had reversions or potential suppressor mutations. Most had the change in two or three of the repeats, and these always involved adjacent units. The structures TCT and CTC were never detected (Fig. 5).

These results are consistent with the interpretation that the duplication events to generate additional enhancer repeats occurred subsequent to the single base changes in the core elements. A model of these events is shown in Fig. 6. Template misalignment to generate a triplicated structure always results in the central repeat being identical to one of the flanking units (Fig. 6A). Thus, the pattern of enhancer structures that was observed may reflect, at least in part, the constraints on the types of structures that can form during reverse transcription. Figure 6B details one of the possible mechanisms by which the triplicated enhancer structures could form. Variant LTRs generated by the indicated combination of misincorporation and polymerase jumping might result in a virus with an increased capacity to induce lymphomas. Alternatively, more effective enhancer structures may form by single base mutations after the increase in the number of enhancer repeats. If so, this would indicate that the relative frequency of variant structures (Fig. 5) reflects a relative growth advantage in mice conferred by particular enhancers. Indeed, it is interesting to note that the structure TTC was detected 10 times while CTT was detected only once (Fig. 5). Perhaps the former is a stronger enhancer. It is also conceivable that the absence of the structures TCT and CTC might indicate that these are relatively poor enhancers. However, we hypothesize that the absence of these two structures is more likely a consequence of formation of the triplicated enhancers by template misalignment. In summary, the structures of the enhancers seen in the tumors are presumably a consequence of both the mechanisms by which they were generated and the selective pressures that favored certain variants. By identifying the processes that favor certain LTR structures, insight may be gained into the mechanisms by which the viral enhancers participate in leukemogenesis.

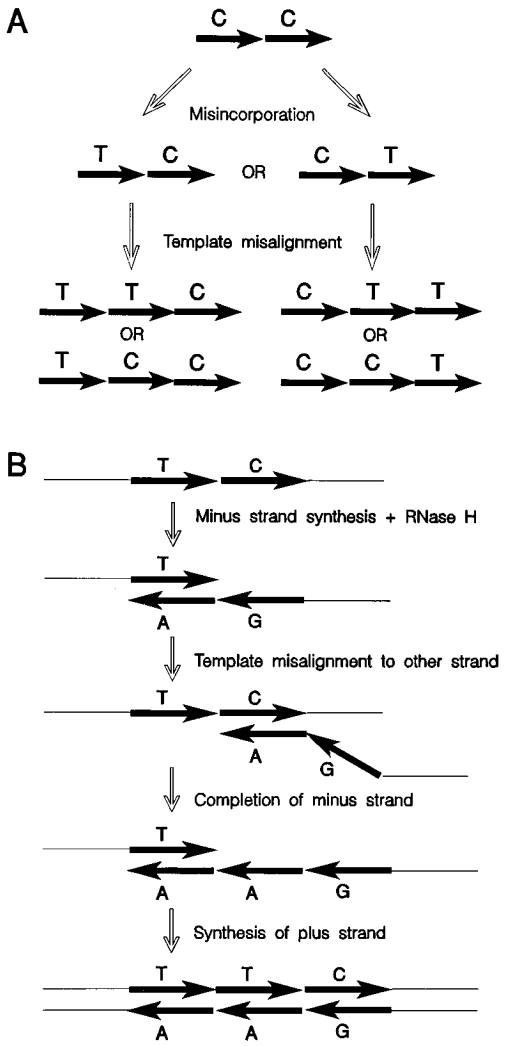

FIG. 6. Generation of an extra enhancer repeat unit by template misalignment errors during reverse transcription. (A) Sequential misincorporation and template misalignment events to generate structures with three tandem repeats, two of which have reverted cores. Arrows represent the individual 72-bp repeats. In the first step, one of the cores of SAA incurs a single base pair mutation. In the second step, template misalignment results in generation of an extra repeat unit. Details of the polymerase jump are shown in panel B. Each two-repeat construct can give rise to two distinct, three-repeat products. They differ on the basis of whether the misalignment occurs before or after the variable nucleotide in the core is copied. Note that the two intermediates give rise to different final products. (B) One mechanism of template misalignment to generate an additional repeat unit by the forced copy choice mechanism (10). An equally plausible model can be drawn for the strand displacement-assimilation model (72). The first line shows an RNA template containing two enhancer repeats with different cores. Minus-strand DNA synthesis proceeds into the second repeat with concomitant RNase H digestion of the template. At this point, polymerization stalls, perhaps because of a nick in the RNA template. The nascent minus strand then hybridizes to the second viral RNA molecule by complementary bases at the 3′ end. However, the 3′ end hybridizes to the misaligned repeat in the second RNA. Continued polymerization of the minus strand then ensues and is followed by synthesis of the plus strand.

Structures of viral enhancers adjacent to c-*myc* and *pim-1*. One step in leukemogenesis that LTR enhancers might affect is the activation of proto-oncogenes (12, 55, 85). Proviral insertion near c-*myc* is associated with increased levels of

TABLE 3. Structures of LTR enhancers adjacent to c-*myc* or *pim-1*[a]

Oncogene and tumor[b]	Enhancer of provirus adjacent to c-*myc* or *pim-1*	Enhancers of all proviruses in tumor	
		No. of repeats	Enhancer structure
c-*myc*			
NFS-SAA-3	TTC	3	T T C
		2	T C
NSF-SAA-16	TTC	3	T T C
		2	C C
		1.5	C T*
NFS-SAA-11	TT	3	T T C
		2	T T
		3	T C C
		2	T C
NFS-SAA-13	SoSoC	3	So So C
		2	T T
		2	T C
NFS-SAA-17	SoSoSo	3	So So So
		2	T T
		2	T C
pim-1			
AKR-SAA-14	TTC	3	T/C T/C T/C
		2.5	ND[c] ND ND
		2	ND ND ND
		1.5	T* T*

[a] The nomenclature and abbreviations used are the same as those described in the legend to Fig. 5.
[b] NFS, a tumor in an NFS mouse; SAA, a tumor in an SAA virus-inoculated mouse. The numbers indicate the individual mice.
[c] ND, sequence data not determined.

c-*myc* mRNA in tumors relative to c-*myc* levels in normal thymus (69), although the increases are often quite small (58). By using somatic cell hybrids to separate tumor cell c-*myc* alleles, it was found that the allele with the provirus is expressed at higher levels (32). The hypothesis that LTR enhancers are important for proto-oncogene activation is difficult to test experimentally because mutations that disrupt enhancer structure affect viral replication. Thus, they also affect other steps in leukemogenesis, such as the establishment of viremia in the target tissue. Southern blotting, PCR, and sequencing analyses of the viral LTRs in tumors from SAA-inoculated mice showed that multiple proviruses were present in almost every one of the tumor DNAs. The LTR structures of the proviruses within a particular tumor DNA varied in both the number of repeats and the number of cores with reversion or potential suppressor mutations. This offered a means to test whether the nature of the LTR enhancer is indeed important for activating proto-oncogene expression. We reasoned that if it is, then the LTRs in a provirus adjacent to a proto-oncogene would be likely to have an enhancer with the largest number of reverted cores of all of the proviruses in that tumor. If it is not important, then the LTRs of proviruses adjacent to proto-oncogenes could be of any structure.

To investigate the structure of enhancers in proviruses adjacent to proto-oncogenes, the 38 tumor DNAs from SAA-inoculated mice were screened by Southern blotting for integrations adjacent to c-*myc*, a proto-oncogene known to be activated in MuLV-induced tumors. Seven tumors had rearrangements of c-*myc* alleles. To obtain the LTRs of adjacent proviruses, PCR was performed using one primer specific for the LTR and one specific for c-*myc*. Since the proviral integration sites spanned a region of several kilobases upstream of c-*myc*, several different c-*myc*-specific primers were employed. By this approach, LTRs adjacent to c-*myc* were amplified from five of the seven tumors. The enhancers of these were sequenced along with those of all of the other LTRs in those five tumors. These results are summarized in Table 3.

In each case, multiple LTRs were detected in the tumor DNA. These had enhancers consisting of variable numbers of repeats and different core structures. Of all of the LTRs in each tumor, the one with the most reverted or potential suppressor mutations in the core was adjacent to c-*myc*. In three of the tumors, the adjacent provirus had two enhancer repeats with reverted cores. In the other two, the adjacent proviruses had two or three enhancer repeats with the core structure of Soule MuLV. These two tumors also each had a provirus with two reverted cores. Additional experiments will be required to determine whether the Soule core structure is as potent as the reverted core structure. Nonetheless, it is interesting to note that although the Soule MuLV core structure was detected in only three of the 38 tumors, it was present in two of the five characterized LTRs adjacent to c-*myc*. It is also interesting to note that four of the five proviruses adjacent to c-*myc* had acquired a third enhancer repeat unit. These experiments provide genetic evidence consistent with the hypothesis that the nature of the viral LTR enhancer affects the ability to activate the expression of c-*myc* during lymphomagenesis.

Similarly, Miura et al. (43) found that a feline leukemia virus enhancer adjacent to c-*myc* had an enhancer triplication. Interestingly, the enhancer cores of feline leukemia viruses are identical to the SL3 core (77). Since feline leukemia virus proviruses in lymphomas frequently have enhancer duplications (44), it will be interesting to see if these also are correlated with the capacity to activate c-*myc* or other proto-oncogenes.

We also examined *pim-1* and N-*myc*, two other proto-oncogenes that are known to be activated by proviral insertion in MuLV-induced T-cell lymphomas (14, 90). In addition, we tested L-*myc* (33). Only 3 of the 38 tumors had rearranged *pim-1* alleles. Of these 3, 1 appeared to have the provirus within the 3′ untranslated sequences of the gene, the most common location of Mo-MuLV proviruses (67). This was the only LTR adjacent to *pim-1* that we were able to amplify. Sequencing of its LTR showed that it had the structure TTC, the same structure that was seen adjacent to c-*myc* in two of the five cases analyzed (Table 3). No rearrangements of N-*myc* or L-*myc* were detected in any of the tumors.

DISCUSSION

Introduction of the single base pair change into the core elements of the SL3 enhancer significantly reduced viral leukemogenicity. Moreover, most of the lymphomas that formed in SAA-inoculated mice contained viruses with reversions or potential suppressor mutations within the core. Therefore, we hypothesize that most of the tumors in SAA-inoculated mice were actually caused by mutated viruses with altered enhancers. This observation extends previous reports that mutations in the core elements of Mo-MuLV and SL3 affected viral leukemogenicity (25, 73, 97). In the studies reported here, the single base pair mutation in the core changed the sequence to that of Akv virus. Akv is nonleukemogenic when inoculated into newborn mice (46, 63). Therefore, the presence of the C in the core sequence of Akv and other endogenous, ecotropic retroviruses may represent an adaptation such that the virus replicated well enough to enter the germ line but did not confer too severe a leukemogenicity phenotype. In addition to

the single base in the core, the SL3 enhancer contains other differences relative to that of Akv. Sequences 5′ to the core element affect the ability of T lymphocytes to distinguish the SL3 and Akv cores (40). The fact that the SAA virus did induce tumors in a few mice while Akv is nonleukemogenic argues for the interpretation that the other differences between the SL3 and Akv enhancers are also important for viral leukemogenicity. One site 5′ to the core that affects viral pathogenicity was identified (25). Presumably, the full complement of transcription factors in T cells that bind to the SL3 enhancer is important for leukemogenicity.

The single base pair difference between the SL3 and Akv cores alters the binding of a nuclear factor called S-CBF that preferentially recognizes the SL3 core (4). Thus, there is a correlation between decreased S-CBF binding and viral leukemogenicity, suggesting that binding of this factor to the viral enhancer is critical for leukemogenicity. A second factor also binds to the SL3 core. This factor has been alternately called CBF, SEF1, and S/A-CBF (4, 81–83, 93, 94), and we call it CBF here. We have not detected any difference in the capacity of this factor to bind to the cores of SL3 and Akv (3, 4), while others have reported that it binds better to the SL3 core (42, 81). CBF binds to the core elements in the enhancers of all MuLVs that have been tested, including T-lymphomagenic Mo-MuLV, T-lymphomagenic MCF-247 virus, erythroleukemogenic Friend MuLV, and a polycythemic strain of Friend spleen focus-forming virus in addition to SL3 and Akv (4, 74, 82, 93). All these core elements have slightly different sequences, except for those of Mo-MuLV and Friend MuLV, which are identical. Mutations that disrupted CBF binding to the Mo-MuLV core caused the virus to induce primarily erythroleukemias (73). Moreover, evidence exists that CBF is a mixture of multiple binding proteins (82, 83, 93, 94). Thus, individual components of the CBF mixture may actually be responsible for functionally distinguishing various MuLV enhancers. In summary, core binding factors are clearly critical in the process of viral leukemogenesis; however, the identity of the factor that actually distinguishes the SL3 and Akv enhancers remains uncertain.

The changes that occurred in the enhancers of SAA-infected mice appear to be the consequence of two processes acting in concert. Single nucleotide misincorporations during viral replication led to the reversions and potential suppressor mutations in the core. Template misalignment events during reverse transcription altered the number of tandem copies of the enhancer repeats. When a core mutation was followed by a misalignment event that generated an additional enhancer repeat, the altered core was present in two adjacent repeat units.

Retroviruses are well known to have relatively high frequencies of misincorporation during replication (11, 20, 23, 34, 56, 60, 61). This could account for the single base changes detected in the enhancers (Table 2). In addition to the normal error frequency of reverse transcriptase, evidence exists that a fraction of retroviruses in an infected population of cells may produce hypermutant viral progeny with misincorporation frequencies that may be 2 orders of magnitude higher than normal (53). When coupled with the high frequency of recombination during retroviral replication (28, 29), hypermutant genomes may be the origin of many of the single base changes that occur in a population of retrovirus-infected cells (53).

Two mechanisms may account for the variable number of tandem repeats. Since the viruses were generated by transfection of viral DNA, one might be homologous recombination involving transfected plasmid DNAs (59). Frequencies of intramolecular homologous recombination of about 10^{-2} and intermolecular events of 10^{-4} to 10^{-3} in transfection experiments have been reported (79). This process may have contributed to variability in repeat number at the beginning of the fibroblast infection. The second mechanism is by template misalignment errors (polymerase jumps) during reverse transcription (Fig. 6). Pathak and Temin (53) reported that one copy of 110-bp tandem repeats was deleted in 41% of viral progeny during a single round of replication. Internal deletions of unique viral sequences occur frequently during viral replication. These events are usually mediated by short homologous sequences flanking the deleted segment, although some appear to occur with no homology at the misalignment site (51, 52, 54, 57, 70, 91, 92, 95, 96, 98). On the other hand, duplications appear to occur far less frequently than deletions (54, 95). When a duplication event occurs by template misalignment from one tandem repeat unit to the adjacent one, the net result is three copies of the unit or a triplication. Our sequencing analysis (Fig. 5) provided evidence that the triplications present in the tumor proviruses occurred after the appearance of the single base changes in the core elements. Thus, they most likely occurred by misalignment errors during viral replication.

Deletions such as those detected in the enhancer repeats (Fig. 5) are believed to be formed when the 3′ end of a nascent DNA strand slips forward along the template strand (54, 95). On the other hand, the generation of tandem duplications would require nascent 3′ ends to slip backward along the template. It has been argued that duplications occur substantially less frequently than deletions because backward slippage is less likely to occur (54, 95). The block to such backward slippage might be due to the template strand being base paired to the nascent strand (54). In addition, since the RNase H activity of reverse transcriptase functions in parallel with the polymerase activity (66), then the degradation of the RNA template may preclude backward slippage during minus-strand DNA synthesis. These arguments to explain the observed preponderance of deletions over duplications suggest that most of these events occur by misalignment along a single template molecule. Since retroviral particles contain two copies of the genome, interstrand misalignments of nascent 3′ ends could also occur. If interstrand jumps also contribute significantly to changes in tandem repeat numbers, then they presumably would also have to favor deletions over duplications. Our experimental approach did not permit a measurement of the relative frequency of deletions and duplications in the enhancer region. We believe it is most likely that deletions occurred more frequently than duplications but that duplications that generated additional enhancer repeats conferred a selective advantage to viral genomes that contained them by increasing their potential to cause disease.

We hypothesize that the presence of proviruses with altered enhancers in tumors in SAA-inoculated mice was due to selection of the genomes that contained them. LTR enhancers likely play multiple roles in lymphomagenesis, and selection might occur at any step. An increased capacity of the virus to replicate in the target tissue is one likely step. Even a modest growth advantage can allow one virus to outgrow another if sufficient rounds of replication occur (1, 11). Evidence exists that LTR sequences also affect the ability of env recombinant viruses to propagate (5, 6). In addition, LTR enhancer sequences may directly affect cellular proliferation and transformation. They likely affect expression of viral gene products such as *env* that may play a role in stimulating target cell growth (37, 84). In addition, the ability of a particular LTR enhancer to activate a proto-oncogene may result in the

growth of tumor cells containing a provirus with a particular enhancer. The data reported here provide evidence consistent with the hypothesis that, at least for c-*myc*, proviral LTR enhancers do affect the capacity to activate an adjacent proto-oncogene.

ACKNOWLEDGMENTS

We thank Vinayaka Prasad for helpful discussions.

This work was supported by NIH grant CA44822. J.L. was supported by a Hirschl-Caulier Career Scientist award. H.L.M. was supported by NIH training grant GM7288. Synthesis of oligonucleotides was supported in part by NIH Cancer Center grant CA13330.

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