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Stability of AML1 (Core) Site Enhancer Mutations in T Lymphomas Induced by Attenuated SL3-3 Murine Leukemia Virus Mutants

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Murine retrovirus SL3-3 is highly T lymphomagenic. Its pathogenic properties are determined by the transcriptional enhancer of the U3 repeat region which shows preferential activity in T cells. Within the U3 repeats, the major determinant of T-cell specificity has been mapped to binding sites for the AML1 transcription factor family (also known as the core binding factor [CBF], polyomavirus enhancer binding protein 2 [PEBP2], and SL3-3 enhancer factor 1 [SEF-1]). SL3-3 viruses with AML1 site mutations have lost a major determinant of T-cell-specific enhancer function but have been found to retain a lymphomagenic potential, although disease induction is slower than for the SL3-3 wild type. To compare the specificities and mechanisms of disease induction of wild-type and mutant viruses, we have examined lymphomas induced by mutant viruses harboring transversions of three consecutive base pairs critical to AML1 site function (B. Hallberg, J. Schmidt, A. Luz, F. S. Pedersen, and T. Grundström. J. Virol. 65:4177–4181, 1991). Our results show that the mutated AML1 sites are genetically stable during lymphomagenesis and that ecotropic provirus numbers in DNA of tumors induced by wild-type and mutant viruses fall within the same range. Moreover, proviruses were found to be integrated at the c-*myc* locus in similar proportions of wild-type and mutant SL3-3-induced tumors, and the mutated AML1 sites of proviruses at c-*myc* are unaltered. In some cases, however, including one c-*myc*-integrated provirus, a single-base pair change was detected in a second, weaker AML1 binding site. By DNA rearrangement analysis of the T-cell receptor β-locus, tumors induced by the AML1 site mutants are found to be of the T-cell type. Thus, although the AML1 site mutants have weakened T-cell-specific enhancers they are T-lymphomagenic, and wild-type- and mutant-virus-induced tumor DNAs are similar with respect to the number of overall ecotropic and c-*myc*-integrated clonal proviruses. The SL3-3 wild-type and AML1 site mutant viruses may therefore induce disease by similar mechanisms.

Murine leukemia viruses (MuLVs) are nonacute transforming retroviruses, which can induce hematopoietic tumors after a period of weeks to months following inoculation of newborn mice of susceptible strains. The disease induction pattern varies among isolates; among the well-studied isolates, Moloney MuLV (Mo-MuLV) and SL3-3 MuLV are potent inducers of thymic lymphomas, while the Friend MuLV induces erythroleukemias (3, 6, 29). The induction of hematopoietic tumors by MuLVs is a multistep process that is not fully understood. However, several studies have provided evidence that insertional mutagenesis plays an important role. By this step, integration of proviral DNA near a cellular proto-oncogene(s) of an appropriate target cell leads to aberrant expression of the gene(s) (6, 9, 14, 29, 35, 40, 41).

For a number of virus isolates, a major determinant of leukemogenicity has been mapped to transcriptional enhancer sequences in the viral long terminal repeats (LTRs) (5, 7, 15). These enhancers have been found to influence cell type specificity, oncogenicity, disease specificity, and latency period for development of neoplasm (5, 9, 11, 16, 33). The exact mechanisms by which virus enhancer structures determine disease induction are not known. First, the enhancer effect on provirus transcription may determine the level of expression of a viral gene product in the target cell or the virus load in target tissues (17, 27). However, in the case of thymic lymphomagenesis it was found that infection of target tissue is not sufficient for subsequent disease development (11, 12). Second, in insertional mutagenesis the proviral enhancer structure may determine its ability to deregulate the expression of a host gene (14).

The MuLV transcriptional enhancer sequences are composed of densely packed binding sites for transcription factors, and point differences among isolates in the sequences of these sites may have a major influence on the pathogenic properties of the virus. Proteins of the core binding factor (CBF)/polyomavirus enhancer binding protein 2 (PEBP2)/SL3-3 enhancer factor 1 (SEF-1) family bind to sequences in the transcriptional enhancers of T-lymphomagenic SL3-3 and Mo-MuLV (32, 33, 38, 39). Transcription factors of this family are composed of one of a group of α-subunits with sequence-specific DNA-binding properties and a non-DNA-binding β-subunit. The α-subunits are expressed in a tissue-restricted manner, whereas the β-subunits are found ubiquitously (21, 22, 42). One of the α-subunits is the mouse homolog of the product of the human AML1 gene (1, 2). Various names for these proteins and their recognition sequences are currently in use. In this report we have chosen to refer to the transcription factor complexes of this family as AML1 proteins and to their binding sites as

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AML1 sites (previously referred to as core, inverse core, and SEF-1 sites 1 and 2). AML1 has been found to play a role in early hematopoiesis (23) and to bind the enhancers of several genes specifically expressed in T cells, including the T-cell receptor (TCR) chain genes, TCR-γ, -δ, and -β (12, 13, 25, 26).

Three major studies have addressed the influence of point mutations in the AML1 sites on MuLV pathogenicity. Speck and coworkers (33, 34) found that a double-base pair mutation in the AML1 site of the Mo-MuLV enhancer changed the disease specificity of the virus from lymphomas to erythroleukemia. For the T-lymphomagenic SL3-3 virus, the two AML1 sites in the repeat sequences differ in their nucleotide sequences and in their affinities for AML1 proteins. Hallberg et al. (11) found that mutations of three consecutive base pairs in the stronger AML1 site, site I, led to a lower incidence and a prolonged latency of disease induction. While a 3-bp mutation in the weaker AML1 site, site II, of SL3-3 had only a minor effect on pathogenicity, a virus with mutations in both AML1 sites was only weakly leukemogenic. In this study it was shown that the introduced mutations were present in bulk PCR-amplified proviral DNA from tumors (11).

In a recent report (19) the leukemogenicities of SL3-3 mutant viruses harboring point mutations in AML1 site I were found to be reduced. Analysis of proviral enhancer sequences of integrations near c-*myc* in the DNAs of five tumors revealed either reversion of the original point mutation back to the wild type or an additional point mutation in AML1 site I, changing the sequence to that of the T-lymphomagenic Soule MuLV (19). While the reversion of enhancer sequences in proviruses integrated at the c-*myc* locus indicated a role for enhancer strength in c-*myc* deregulation, it also precluded the direct analysis of the pathogenic properties of a virus with an AML1 site-weakened enhancer.

To elucidate the role of AML1 site impairment in the oncogenic potency and specificity of SL3-3 virus, we have analyzed tumor material available from the study of Hallberg et al. (11). Using this material we have asked whether the tumors induced by AML1 site mutant viruses are of the T-cell type and whether there is an obligatory selection for more potent variants during the infection of the animals. We report that tumors induced by an AML1 site mutant are of T-cell origin and that proviruses with the input enhancer which is mutationally weakened in T-lymphoid cells can participate directly in insertional mutagenesis of a host gene.

MATERIALS AND METHODS

Tumors. Tumors originated from experiments described previously (11). Newborn NMRI mice were injected with SL3-3 virus or SL3-3 virus harboring a 3-bp mutation (GTT→TGG) in AML1 binding site I of the direct repeats. Control mice were mock injected with complete medium. Tumors were diagnosed on the basis of gross appearance of lymphoid organs as previously described (11, 28) and histologically according to the cytologic and anatomic criteria described by Pattengale (24).

Southern blotting and hybridization. These procedures have been described previously (30).

DNA probes. The ecotropic-virus-specific probe employed was a gp70 *Sma*I fragment from the Akv MuLV (positions 6240 to 6570). The immunoglobulin (Ig) heavy-chain probe was a *Bam*HI-*Eco*RI fragment from the J region of the murine Ig heavy-chain gene (denoted pJ11 [18]). The remaining probes (c-*myc*, TCR-J1, and J2) were prepared by PCR with the primers described below. The PCR amplification products were electrophoresed in a 1.5% (wt/vol) low-melting-point agarose (SeaKem; FMC Products) gel; fragments were excised from the gel and purified by phenol-chloroform extractions or with the Wizard PCR Preps DNA purification system (Promega).

PCRs, Dynabead-streptavidin purification, and sequencing of PCR fragments. These procedures have been described previously (30, 31).

Preparation of oligonucleotides. Oligonucleotides (DNA Technology ApS, Aarhus, Denmark) were synthesized with an Applied Biosystems Instruments 381B DNA synthesizer and purified with oligonucleotide purification cartridges.

Primer sequences. The proviral primers for amplification of the 5′ LTR and 3′ LTR cellular flanking sequences have been described previously (30, 31). In addition to these primers, an SL3-3-specific primer in the 5′ untranslated region was employed (5′-GATGCCGGCACACACACACACACACTCTCCC-3′; located between positions 867 and 896 in SL3-3 provirus [1 is the first base in the 5′ LTR]). c-*myc* primers a and b (Fig. 3A), which were used to generate the probe for Southern hybridizations, have been described previously (30). The sequence of c-*myc* primer c (Fig. 3A) was 5′-TGTCTGCCCGCTGCAATGGGCAAAGTTT-3′. The sequences of the TCR-β J1 region primers used to generate the probes for Southern hybridizations were as follows: upstream primer, 5′-GGGTCCCATCAGCTCTTTGGGAG-3′ (located at positions 1261 to 1283); downstream primer, 5′-GGGTTCCAGATGGGAAGGGACGACTCTGT-3′ (located at positions 1970 to 1942). Positions in parentheses correspond to the sequences in the GenBank/EMBL databases, accession no. X01018 and M23825. The sequences of the TCR-β J2 region primers used to generate the probe for Southern hybridizations were as follows: upstream primer, 5′-GCTTCTTGGCAACTGCAGCGGGGAGT-3′ (located at positions 1295 to 1320); downstream primer, 5′-CTGGGTCTCCAACACTGCTTCAAGTG-3′ (located at positions 2058 to 2033). Positions in parentheses correspond to the sequence in the GenBank/EMBL databases, accession no. K020802.

Analysis of number of direct repeats. PCR fragments of tumors wt-5, wt-13, GTT-4, and GTT-6 containing the LTR direct repeats were obtained by using proviral-specific primers against c-*myc*-specific primers. The PCR products were digested with *Dra*I and *Pst*I or *Cel*II and separated by electrophoresis on 1.5% agarose gels.

Sequence comparison. Nucleotide sequences were compared with sequences in the GenBank and EMBL databases by using the Wisconsin Package EGCG [version 8.1.0 (a), May 1996] FASTA program with the parameter "word size: 6."

Nucleotide sequence accession numbers. Provirus flanking nucleotide sequences have been assigned the EMBL data bank accession numbers given in Table 1.

RESULTS

Tumors induced by AML1 site mutant virus. Previously (11), a mutant SL3-3 enhancer with a 3-bp mutation in AML1 site I was constructed, changing the sequence from 5′-TATCTGTGGTTA-3′ to 5′-TATCTGTGTGGA-3′ (Fig. 1). The GTT mutation was chosen because in vitro protein-DNA complex formation with AML1 proteins was abolished when this mutation was present (38). Analysis of plasmid constructs with the mutated enhancer showed that transcription was lowered twofold in T lymphocytes but not in other cell lines. Complete SL3-3 viruses with the GTT mutation, termed GTT-mutant viruses, were found to replicate in cell culture and to infect thymus and spleen cells after injection into newborn mice of the NMRI strain. Within an observation period of 300 days 28 of 46 animals developed tumors (11). Eight lymphomas were chosen for DNA analysis. Table 1 gives the latency periods of disease induction and tumor localizations for the eight tumors.

Tumor DNA from thymus or lymph nodes was analyzed by Southern blotting with an ecotropic provirus-specific MuLV *env* probe. After cleavage with *Hin*dIII, which cleaves once in the SL3-3 provirus, provirus-host junction DNA fragments would be detected. By this approach the tumors were analyzed for clonality or oligoclonality with respect to individual integrated ecotropic proviruses. In the DNA of all tumors with the exception of GTT-4, clonal or oligoclonal integrations of hybridizing proviruses could be detected (Table 1). In most cases the hybridizing bands were of equal intensity, possibly reflecting true clonality. In some cases one or two extra bands of reduced intensity were observed. In DNA of the GTT-4 tumor, repeated analyses failed to detect hybridizing bands, indicating a lack of clonally integrated ecotropic proviruses (see below).

Tumor characterization. The reduced T-lymphoid specificity of the GTT-mutant transcriptional enhancer led us to perform a phenotypic analysis of tumor cell type. The majority of the mice injected with GTT-mutant viruses developed a lymphoblastic malignant lymphoma, i.e., a large-cell high-grade malignant lymphoma, with considerable enlargement of the thymus. However, there were rare cases without this macroscopically massive involvement of the thymus. In order to further distinguish

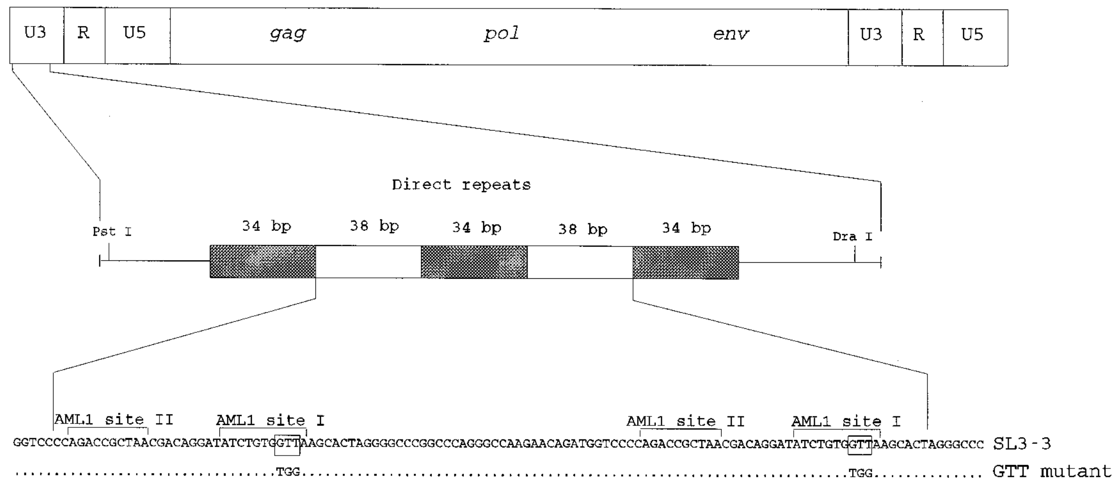

FIG. 1. Mutations in the AML1 binding site I in SL3-3 MuLV. The upper panel shows a schematic representation of the SL3-3 proviral organization. The enlarged lower panels indicate the structure (middle) and the sequence (bottom) of the direct repeats of the U3 region, depicting the introduced 3-bp GTT mutation in both AML1 binding sites I.

between cells of the B and T lineages, Southern blot hybridizations with probes for the TCR-β chain and the Ig heavy chain were done. To determine rearrangements in the TCR-β chain, two DNA probes covering joining regions 1 and 2 (TCR-J1 and TCR-J2, respectively) were used. Probe localizations and examples of hybridization patterns are shown in Fig. 2. The presence of germ line and rearranged bands is summarized in Table 1. In DNA of all eight tumors rearranged bands

TABLE 1. Tumor data

Tumor no.	Mouse age (days)[a]	Tumor characterization: No. of TCR fragments with[b]: J1 probe		J2 probe		No. of heavy-chain fragments[c]		No. of ecotropic proviral integrations[d]	Accession no. of provirus flanking sequences[e]
		G	R	G	R	G	R		
GTT-1	154	0	0	0	2	1	0	3 (1)	Y11798
GTT-2	170	0	2	0	1	1	0	1	Y11799
GTT-3	174	0	0	0	2	1	0	3 (1)	Y11800 Y11801 Y11802 Y11803 Y11804
GTT-4	177	0	2	0	2	1	0	0	Y11805[f] Y11806[g]
GTT-5	197	1	0	1	1	1	0	1 (1)	Y11807
GTT-6	204	0	1	0	1	1	0	4	Y11808[f] Y11809
GTT-7	255	0 (1)	0	0	1 (1)	1	0	1 (2)	Y11810
GTT-8	300	0	1	0	1	1	0	2	Y11811 Y11812 Y11813

[a] The age at death of each mouse.
[b] The number of germ line (G) or rearranged (R) fragments observed in Southern blot hybridization with probes covering either the TCR-β chain J1 or J2 region. Numbers in parentheses indicate additional weak hybridizing fragments.
[c] The number of germ line or rearranged Ig heavy-chain fragments observed in Southern blotting analysis.
[d] The number of ecotropic proviral integrations determined by Southern blot hybridizations. Numbers in parentheses indicate additional weak hybridizing fragments.
[e] From each tumor one to five provirus flanking sequences were amplified by a two-step PCR method (30, 31) and were assigned the indicated EMBL accession number.
[f] c-*myc* sequence.
[g] 18S rRNA sequence.

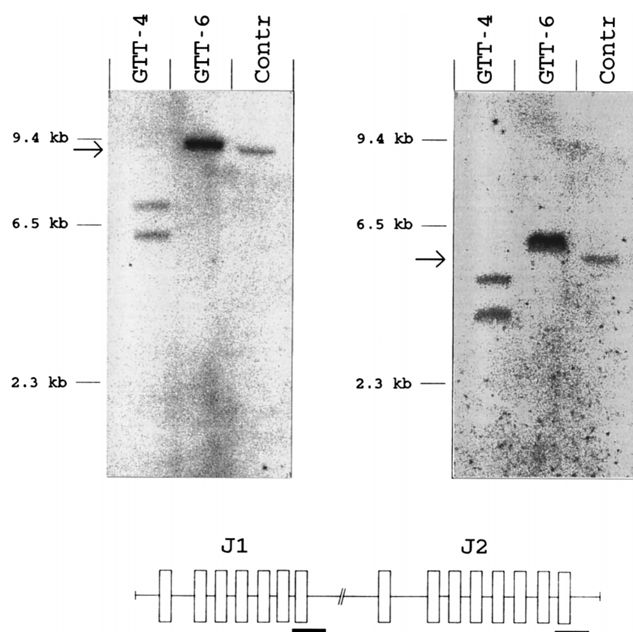

FIG. 2. TCR-β chain rearrangement. DNAs from tumors GTT-4 and GTT-6 and control DNA (DNA from the liver of mock-injected control mice [Contr]) were digested with *Hin*dIII and hybridized with a TCR-J1 and a TCR-J2 probe. Arrows indicate the sizes of the germ line fragments. The locations of the probes (black bars) are shown below (J1 and J2, joining regions 1 and 2, respectively, of the TCR).

were observed with the J1 probe and/or the J2 probe. In some cases, germ line-sized bands were present as well. It should be noted that the structure of the TCR-β locus and the rearrangement pathways predict that the J1 but not the J2 region may be lost by rearrangement. In agreement with this notion we observed J2 hybridizing bands in all cases. By analysis with an Ig heavy-chain probe, no rearrangements were detected. Thus, by TCR-β and Ig heavy-chain gene rearrangement analyses, all eight tumors were found to be of the T-cell type. Judging from the complexity and the equal intensities of the bands, all lymphomas might be clonal with respect to a rearrangement of one or two TCR-β alleles, although other interpretations cannot be excluded.

Proviral enhancer sequences and integration sites. DNA samples of the eight GTT-mutant tumors chosen (Table 1) were analyzed with respect to the structure of the U3 enhancer. A fragment encompassing the tandem repeat sequence was amplified by PCR with the upstream U3 primer d (Fig. 3B) and an SL3-3-specific primer in the 5′ untranslated region. Agarose gel electrophoretic analysis of the PCR products identified one or more provirus-specific bands (data not shown). By this kind of analysis, the appearance of more than one band has been found to reflect the presence of more than one type of U3 structure in a given tumor (8, 19). Our data are compatible with a fluctuation in the number of U3 tandem repeats in the proviruses of a tumor, an interpretation which is supported by analysis of c-*myc*-integrated proviruses (see below). Sequence analysis of the PCR products in all cases confirmed the presence of the introduced mutations. In DNAs of tumors GTT-2, GTT-4, and GTT-5 a single nucleotide alteration in all copies of AML1 site II was observed, changing the sequence from AGACCGCTAA to AGACCGTTAA. In DNA of tumor GTT-1 all copies of the AML1 site II sequence showed a single nucleotide change to AGACCGCTTA. These alterations may have an influence on the binding of AML1 proteins to the site, since transversion of the base pair in either position from AGACCGCTAA to AGACCGATAA and AGACCGCTCA, respectively, have been shown to reduce binding (39). Hence, although the base pair positions as such are not neutral in terms of protein binding, information on the exact effect of the observed nucleotide alterations on AML1 site functions is not available. Besides these point differences no clear nucleotide changes were observed in the repeat regions of DNAs of the eight GTT tumors. Sequence analysis of SL3-3 wild-type tumor DNAs of this and other series has not identified similar alterations in AML1 site II. Although this type of PCR sequence analysis confirmed that the introduced mutations could be recovered from tumor DNA, it did not exclude the presence of a mixture of types of repeat sequences, among which some may be of functional importance. In fact, in previous work we have recovered a variant U3 structure with increased enhancer strength in T-lymphoid cells from another tumor induced by the GTT-mutant of SL3-3 MuLV (8).

To allow the analysis of single rather than multiple proviruses, we wanted to identify sequences flanking individual proviruses of tumor DNAs. Sequences of proviral-host junctions in the eight GTT tumors were identified by PCR amplification with a provirus-specific and a partly degenerate primer (30, 31). Among the 16 junction sequences identified (Table 1), two corresponded to the c-*myc* promoter region and one to a sequence in 18S ribosomal RNA, while the remaining junctions showed no obvious homology to sequences in the GenBank/EMBL databases.

Enhancer structures of proviruses integrated near c-*myc*. DNA of the eight GTT tumors was analyzed by Southern hybridizations of *Hin*dIII-digested DNA with a probe of the c-*myc* promoter. Clonal rearrangements were found in tumors GTT-4 and GTT-6, whereas the other six tumor DNAs showed only the germ line configuration of the c-*myc* promoter region (data not shown). Fragment sizes of tumors GTT-4 and GTT-6 were in accordance with the integration sites found by PCR, indicating that these junction sequences represent clonal proviral integrations in the c-*myc* promoter (Fig. 3A). Moreover, fragment size analysis of Southern hybridizations with an ecotropic provirus-specific probe confirmed a clonal integration of a provirus in this position in the case of tumor GTT-6. The lack of clonal ecotropic proviruses in DNA of tumor GTT-4 suggested that the provirus in the c-*myc* promoter may have acquired nonecotropic *env* sequences. The structure of this provirus was analyzed by a Southern hybridization of DNA digested by *Hin*dIII and *Eco*RI with the c-*myc* promoter probe (data not shown). This analysis mapped an *Eco*RI cleavage site to the proviral *env* region in a position characteristic of SL3-3 nonecotropic *env* recombinants (36, 37). We infer that the clonal provirus in c-*myc* of GTT-4 DNA harbors U3 sequences in both copies of the LTR that have been derived from the injected SL3-3 mutant (see below) and polytropic sequences in the *env* region.

Twenty tumors from mice infected with wild-type SL3-3 were examined previously (30), and 4 of these showed clonal provirus integration near c-*myc* (Fig. 3A). Two of these (tumors wt-5 and wt-13) were used for comparison to the GTT-mutant-induced tumors. The provirus integrations in DNA from tumors wt-13 and GTT-6 were found to be in the same transcriptional orientation as the c-*myc* gene, while the integrations in tumors wt-5 and GTT-4 were in the opposite orientation (Fig. 3A). Tumors wt-5, wt-13, GTT-6, and GTT-4 developed after latency periods of 82, 114, 177, and 204 days, respectively.

In order to investigate if any changes had occurred in the

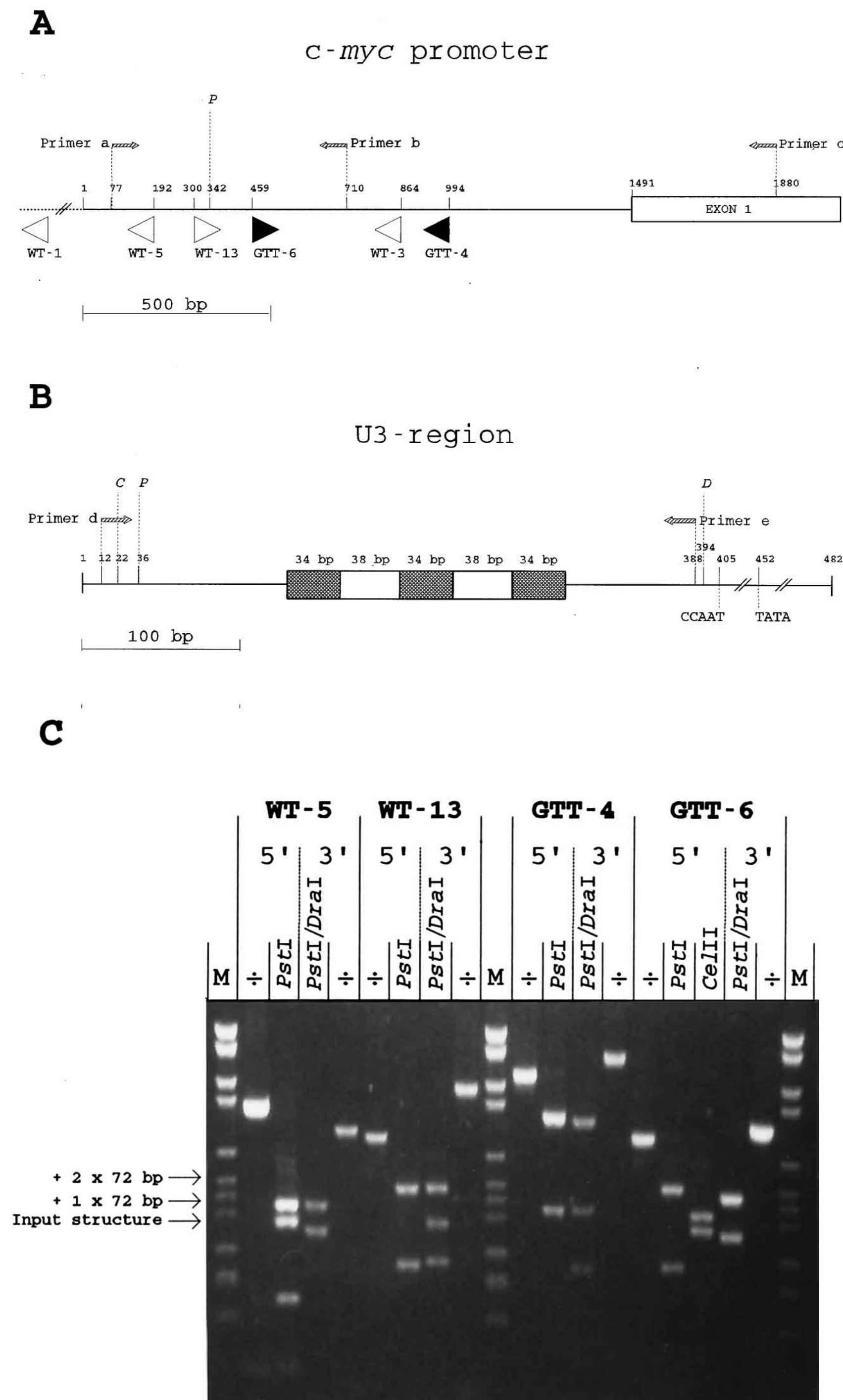

FIG. 3. Repeat structure of proviruses integrated in c-*myc*. (A) Positions and orientations of proviruses integrated in the c-*myc* promoter. Positions and orientations are indicated by triangles; a solid triangle symbolizes GTT-mutant integration, while an open triangle symbolizes wild-type SL3-3 integration. The locations of primers a, b, and c, which were used together with SL3-3 U3-specific primers (shown in panel B) to amplify the direct repeats of the integrated proviruses (panel C), are shown. The position of the single *Pst*I (*P*) restriction site is indicated. The position numbering corresponds to a c-*myc* promoter sequence from GenBank and EMBL (nucleotide sequence accession no., M12345). (B) The U3 region of SL3-3 showing the positions (1 denotes the first base in U3) of the *Pst*I (*P*), *Cel*II (*C*), and *Dra*1 (*D*) restriction sites and the locations of the primers used for amplification of the direct repeat of the proviruses integrated in the c-*myc* promoter (panel C). Primer e was used for the 5′ LTR amplification, and primer d was used for 3′ LTR amplification. (C) Repeat number analysis. The PCR amplifications of the 5′ LTR and 3′ LTR c-*myc* junction fragments (primers shown in panels A and B) were cleaved with *Pst*I and *Dra*I (3′ LTR) or *Pst*I (5′ LTR; for GTT-6 DNA, *Cel*II was used instead of *Pst*I [see text]). The arrows at the left indicate the expected size of the input direct-repeat-containing fragment from the *Pst*I and *Dra*I cleavage and the sizes of fragments containing one and two extra repeats. ÷, the uncleaved PCR fragment; M, molecular size markers (from top to bottom, 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234/220, and 154 bp).

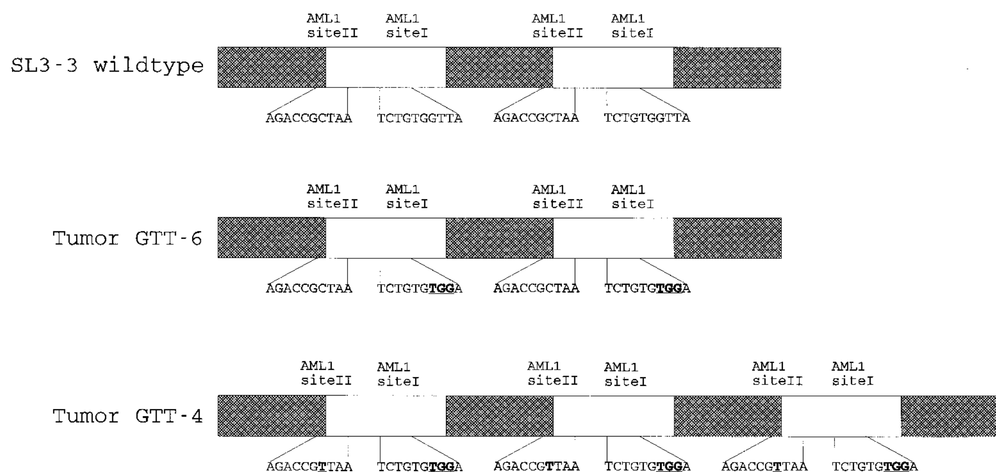

FIG. 4. Structure of U3 direct repeats in GTT-mutant proviruses integrated in the c-*myc* promoter. The sequences of the AML1 binding sites I and II are shown. The triple-nucleotide mutation in AML1 site I recovered in the GTT-mutant viruses as well as the point mutation detected in AML1 site II in the GTT-4 tumor are underlined and in boldface. The remaining sequences of the repeat region were found to be identical to the SL3-3 wild type (sequences not shown).

GTT-mutant provirus integrated near c-*myc*, fragments containing the LTR direct repeats and the flanking c-*myc* DNA were PCR amplified (Fig. 3C). The fragments were obtained with primer e or d (Fig. 3B) for amplification of 5′ LTR- and 3′ LTR-flanking DNA, respectively, together with relevant c-*myc* primers a, b, or c (Fig. 3A). To estimate the number of direct repeats in U3 regions of the four proviruses, the PCR fragments were cleaved by *Dra*I and *Pst*I (Fig. 3B) and analyzed by agarose gel electrophoresis (Fig. 3C). Since the GTT-6-derived 5′ LTR fragment was not cleavable by *Pst*I, apparently as a result of a single nucleotide alteration, cleavage at the adjacent *Cel*II site (Fig. 3B) was performed instead (Fig. 3C). In all cases the determined sizes of cleaved and uncleaved fragments could be explained on the basis of the determined positions of integration into c-*myc* (Fig. 3A) and a fluctuation in the numbers of 72-bp repeat units present in U3. In tumors wt-13, wt-5, GTT-4, and GTT-6 we identified fragments with sizes corresponding to 4, 3, 3, and 2 copies of the direct repeats, respectively, from both the 5′ and 3′ LTRs.

The nucleotide sequence of the U3 repeat region was determined in both the 5′ and 3′ LTR regions in independent PCRs. The sequence analysis confirmed the repeat number assignments and showed conservation of the GTT mutations at AML1 site I for both GTT-4 and GTT-6 (Fig. 4). Moreover, GTT-6 showed no alterations in the remaining part of the repeat sequence, while the GTT-4 provirus at the c-*myc* locus carried the AML1 site II single-nucleotide alteration (Fig. 4) that was also seen in U3 sequences determined for GTT-2, GTT-4, and GTT-5 after overall proviral repeat PCR amplification. No alterations of the repeat element sequence were detected for the c-*myc*-integrated proviruses of the wt-5 and wt-13 tumor DNAs.

DISCUSSION

Introduction of mutations into the AML1 sites of the SL3-3 enhancer reduces its transcriptional activity in T-lymphoid cells and causes a reduction in leukemogenicity of the virus. In the present work we have asked whether the reduced incidence and prolonged latency period observed for AML1 site mutants reflect (i) a requirement for the emergence of revertants or other potent variants, (ii) a shift in specificity, possibly towards more slowly developing tumor forms, or (iii) a reduced disease potential without change in specificity. To address these questions we have analyzed tumor material available from a previous study by Hallberg et al. (11). As reported, the SL3-3 wild-type virus caused disease in all animals with an average latency period of 3 to 4 months. The introduction of three nucleotide changes in critical positions of AML1 site I was found to reduce disease incidence to about 60% within an observation period of 300 days (11). Here, we have analyzed tumors induced by one such AML1 site I mutant, the GTT-mutant virus. We determined if reversion or other nucleotide alterations within the mutated sites were observed with any significant frequency. In all eight tumors included in the study the nucleotide sequences of the mutated AML1 sites were found to be retained in PCR-amplified U3 regions. Such PCR products are presumably derived from a population of clonal and nonclonal proviruses of which only some may have played a role in tumor development. It was therefore of interest to study individual proviruses, in particular those believed to be involved in the process. Two of the eight tumors carried clonal proviruses in the c-*myc* promoter region. Analysis of these two proviruses confirmed a lack of reversion of the AML1 site I mutant in tumor DNA. This indicates that viruses with impaired AML1 sites may contribute directly to insertional mutagenesis, thus arguing against an obligatory reversion step.

Morrison et al. (19) found a comparable reduction in SL3-3 leukemogenicity as a result of only a single critical T-to-C nucleotide change in AML1 site I. In proviral DNA of tumors induced by this mutant virus, frequent C-to-T reversion was observed, and the authors hypothesize that most of the tumors induced by the mutant virus with the T-to-C change were caused by revertants or mutants with potential suppressor functions. These results are at variance with the conservation of the 3-bp mutations in proviruses of tumor DNA observed in our work. Presumably, a simultaneous reversion of all three mutated base pairs in the GTT-mutant virus is unlikely. A single nucleotide change may not be sufficient to re-create an AML1 site with partial function, thus making complete reversion of the site through sequential mutation and selection steps improbable. Alternatively, other nucleotide alterations of the mutated site may contribute to enhancer strength and pathogenicity. Such single-nucleotide transition mutants, which are likely to be mutants with suppressor mutations, were observed (19). One recurring AML1 site transition created a sequence

previously found in the T-cell lymphomagenic Soule MuLV. In the work reported here point mutations in impaired AML1 site I were not detected in any of the tumor DNAs, again supporting the interpretation that the function of this site was abolished in a manner that did not allow functional restoration by a single base pair mutation.

Since we found no evidence of selection for mutational alterations within the sequence of the impaired AML1 site, the possibility of selection for alterations outside this sequence was examined. In fact, we have previously reported on three cases of tandem repeat sequence deletions in tumors induced by SL3-3 viruses with mutations at the AML1 site (8). These were from a GTT-mutant virus-induced tumor not included in the present study and from two tumors induced by another 3-bp transversion mutation at the same site (the ATC mutation) and by a mutant harboring the GTT mutation as well as a 3-bp transversion of the weaker AML1 site II. In all three cases the second-site deletions were found to confer increased T-lymphoid strength and specificity of the transcriptional enhancer (8).

In four of the eight GTT tumors single-nucleotide alterations in weaker AML1 site II were found in proviruses amplified from bulk DNA as well as in the c-*myc*-integrated provirus of one of these tumors. The alterations were of two types. We note that the only U3 sequence alterations observed were in the sequences of the AML1 sites not targeted by the introduced mutations and that such alterations are not frequently observed in tumors induced by the SL3-3 wild-type virus. The contribution of a functional AML1 site II to the pathogenicity of SL3-3 is only marginal, but this site apparently serves a back-up function, since mutants impaired in both AML1 sites exhibit strongly reduced pathogenicity (11). A detailed analysis of nucleotide sequence requirements for AML1 (SEF-1) protein binding was performed by Thornell et al. (38). They found that a nucleotide change of the site II sequence from AGAC CGCTAA to AGACCGATAA or AGACCGCTCA led to reduced binding. Thus, the nucleotide changes to AGACCGT TAA and AGACCGCTTA detected in this study occur in positions that play a role in binding. However, since the exact mutations have not been analyzed, an assignment of function to the AML1 site II changes observed is not possible. Recently, the importance of the c-Myb binding site adjacent to AML1 site II for SL3-3 lymphomagenicity was discovered (20). We note that the mutation detected in tumor GTT-1 DNA overlaps the c-Myb binding site and that the mutations found in tumors GTT-2, GTT-4, and GTT-5 are immediately adjacent to this site.

In one GTT tumor, a provirus of a recombinant *env* structure was integrated in the c-*myc* promoter region. Similar mink cell focus-forming virus type recombinants have previously been found in tumors induced by SL3-3 (36). In the case of the GTT-mutant tumor the recombinant *env* may contribute to disease induction; however, the U3 regions of this virus were of GTT-mutant origin.

Our results have confirmed previous reports on fluctuations in the number of U3 tandem repeats (8, 19). Moreover, no differences in repeat numbers between 5′ and 3′ LTRs of the same provirus were found, supporting the hypothesis that fluctuations occur at a preintegration step, most likely reverse transcription. In the four cases in which point mutations of AML1 site II are observed, these changes are found in all repeat elements, suggesting repeat sequence conversions as a result of additional repeat dynamics. No clear differences in repeat numbers between tumor-derived wild-type and GTT-mutant viruses are evident from our results, and no correlation between repeat numbers and latency period could be found.

The lack of enhancer reversion allowed analysis of the pathogenic spectra of AML1 site I-mutated viruses as such. To address the possibility of a change in tumor phenotype further, we analyzed tumors induced by the GTT-mutant virus for specific genetic rearrangements of the B or T lineages. Southern blotting analysis showed that all eight tumors had rearrangements in the TCR-β gene, while no evidence of rearrangements was found by using an Ig heavy-chain probe. Thus, all eight GTT-mutant-induced tumors were of the T-cell type. The effect of impairing SL3-3 AML1 site I is distinct from that observed for mutations of the equivalent sites of Mo-MuLV, for which Speck et al. (33) reported a change in disease specificity from lymphomas to erythroleukemia. Most likely, this discrepancy reflects a difference in the overall enhancer anatomies of the two viruses, although roles for the exact mutational alterations of the site cannot be excluded. The features of the SL3-3 virus that make it retain a strong T-cell specificity after knockout of what has been found to be its major T-cell-specific transcriptional *cis* element (4, 10) are not defined. Additional specificity may be conferred by AML1 site II, by other transcriptional elements of U3, or by other regions of the viral genome.

The finding that the tumorigenic targets of the GTT-mutant virus were of the T-cell type suggests the reduced strength of its transcriptional enhancer in the target cell. Conceivably, a reduction in enhancer strength may affect virus replication and virus load in the target tissue as well as the potency of insertional activation. It is possible that the prolonged latency period of the mutant results from reduced virus load in target tissues, although earlier measurements of virus titers in thymus and spleen do not point in this direction (11). Insertional mutagenesis seems to play a role in leukemogenesis by the SL3-3 GTT-mutant virus as it does in wild-type SL3-3-induced disease. In fact, the c-*myc* promoter region, which is a frequent target of the host genome in wild-type SL3-3 tumors, is associated with a similar frequency of provirus insertion in GTT-mutant tumors. Since the total numbers of clonal ecotropic proviruses in wild-type and mutant tumor DNAs were similar and since the c-*myc* integrations of the mutant proviruses were within the region affected by wild-type proviruses, our results have not revealed differences in insertional activation patterns between the two viruses. Possibly, weaker insertional activation by a provirus with a mutant enhancer may delay tumor manifestation because of slower growth and/or a requirement for additional mutations in the target cell genome.

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