Germ Line Insertions of Moloney Murine Leukemia Virus in the TLL Mouse Causes Site-specific Differences in Lymphoma/ Leukemia Frequency and Tumor Immunophenotype

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Abstract. *Background: Moloney murine leukemia virus (Mo-MLV) has proven valuable for studies of the pathogenesis of malignant lymphoma. Inoculation of newborn mice induces T cell lymphoma with 100% incidence. The TLL (T cell lymphoma/leukemia)-strain was previously established and was shown to spontaneously develop T cell lymphoma at high frequency. Materials and Methods: Differential screening of cDNA libraries was performed to discover an involvement of Mo-MLV and genomic sequencing was used to identify the chromosomal position of Mo-MLV proviral integration sites. Immunophenotypes of the tumors were established by flow cytometry. Disease frequency curves were created according to the Kaplan-Meier method. Results: Two independent Mo-MLV germ line integrations were characterized on chromosomes 2 and 14, giving rise to two substrains of mice denoted TLL-2 and TLL-14. The chromosomal position of the integrated provirus affected the frequency of disease, as well as the immunophenotype of the tumors. Conclusion: The data suggest that factors influencing the transcriptional activity of the chromosomal regions, leading to differences in proviral expression, could underlie the observed difference in tumor frequency.*

Mouse models for human disease are irreplaceable tools for studying the pathogenesis of many complex disorders, since the mouse shares anatomical, immunological and genomic similarities with humans. In the case of hematological malignancies, a large number of model systems are available (1). Several of the commonly used inbred laboratory mouse strains spontaneously develop lymphoma and/or leukemia and the variation among strains is to some extent dependent on the expression of endogenous leukemia viruses and virus controlling genes.

Moloney murine leukemia virus (Mo-MLV) is considered the prototype of a simple retrovirus, and inoculation of newborn mice induces T cell lymphoma with 100% incidence and a mean latency of 3-4 months (2). Mo-MLV-induced tumors generally have the phenotypes of immature T cells and the U3 region of the long terminal repeat (LTR) of the virus largely controls the pathogenicity and cell type of the resulting tumor (3).

Previous studies have also shown that Mo-MLV infection of pre-implantation mouse embryos can lead to integration of the virus into the germ line, with subsequent Mendelian transmission of the proviral DNA to following generations (4, 5). Upon infection of newborn mice with murine leukemia viruses, or activation of endogenous viruses during mouse development, a multistep process is started, including generation of mink-cell focus-inducing recombinants, early virus-induced changes in hematopoesis and proviral insertional activation of cellular proto-oncogenes (2). From studies of the different mouse strains carrying Mo-MLV germ line integrations, it is known that the chromosomal position of the inserted provirus is important for its activation during development (5). The proviral insertion site is essentially random with respect to cellular DNA sequences, showing a mild preference for actively transcribed regions (6). However, more recent studies have suggested that integration specificity is mainly determined by local structural features of the DNA, such as methylated CpG dinucleotide repeats (7, 8).

The establishment of a mouse strain (TLL) that spontaneously develops T cell lymphoma/leukemia at a high

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Key Words: Moloney murine leukemia virus, T cell lymphoma, germ line, proviral integration, oncogenesis.

frequency was previously reported by our group (9). In this study, this mouse model is further characterized and two independent germ line integrations of Mo-MLV are described, providing a plausible explanation for the observed phenotype. The detailed comparison of two substrains containing independent germ line integrations demonstrates that the chromosomal position of the integrated viral genome affects the frequency of disease as well as the immunophenotype of the tumors. Together, these models provide additional tools for investigating the oncogenesis of retrovirally-induced tumors and could also be used in therapeutic studies.

Materials and Methods

Animals. All animals were housed under specific pathogen-free (SPF) conditions at the animal facility at Umeå University, Sweden. For determining disease frequencies, TLL and B6 mice were crossed, the offspring were screened by Southern blot or PCR for the presence of MMLV in the germ line, and were then followed for up to 12 months. At the first sign of disease such as hunched back, rugged fur and/or swollen abdomen, the mice were sacrificed and further analyzed.

Screening of mice. DNA was isolated from mouse spleen, thymus and tail biopsies, according to standard methods to be used for screening with Southern blot. The probe used was a MMLV U3LTR probe (10), kindly provided by Prof. A. Berns, Netherlands Cancer Institute, NL. For PCR screening, crude preparations of tail biopsies were subjected to PCR with primers specific for the two integrations (primer sequences available upon request).

Differential screening of cDNA libraries. The initial RNA was isolated from 10^3 $CD4^+$ tumor cells and MACS-sorted (Miltenyi Biotech, Bergisch Gladbach, Germany) $CD4^+$ cells from thymus tissue of a healthy B6 mouse. RNA was prepared using the Ultraspec RNA isolation system (Biotecx, Houston, TX, USA). The synthesis and amplification of cDNA was performed according to Brady (11, 12) with the exemption of the cell numbers used. The cDNA libraries were generated using the pre-digested Lambda Zap II cloning kit, according to manufacturer's instructions (Stratagene, La Jolla, CA, USA) and screened using radiolabelled DNA probes from tumor tissue (S) and control tissue (K). The clones representing differentially-expressed cDNA, found by comparing the S and the K probes, were selected and the identified clones were divided into 48 groups based on RFLP. Representative clones from these groups were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI automated sequencer.

Immunophenotyping. For immunophenotyping, the following antibodies were used: anti-CD8-FITC, anti-CD4-PE, anti-TCRαβ-CyC, anti I-Ab,d-biotin and anti-H-2kb-biotin (all obtained from BD Biosciences Pharmingen, San Diego, CA, USA). In all events in which biotinylated antibodies were used for primary stainings, secondary stainings were performed using avidin-APC conjugate (BD biosciences Pharmingen). The stained cells were analyzed using the FACSCalibur (Becton Dickinson) and CellQuestPro software (Becton Dickinson).

Isolation of the proviral insertion sites. The splinkerette-PCR method was previously described (13, 14) and modified according to Mikkers *et al.* (14). The obtained fragments were subcloned in the TOPO-TA vector system (Invitrogen, Carlsbad, CA, USA) and sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) on an ABI automated sequencer.

Viremia. RNA from mouse serum was prepared using the QIAamp MinElute Viral Spin Kit (Qiagen, Germany). The collected RNA was used in an RT-PCR reaction using Random Hexamer (Applied Biosystems) and the obtained cDNA was subjected to a PCR reaction with primers specific for the Mo-MuLV envelope gene, (primer sequences available upon request).

Expression analysis. For Northern blot and RT-PCR analysis, RNA was prepared with the guanidine isothiocyanate-cesiumchloride method. For Northern blot, 5-10 μg of RNA was hybridized with amplified fragments of 220-500 bp size, chosen randomly from exons within the genes or putative genes flanking the proviral integration site. For RT-PCR, the collected RNA was used in an RT reaction using Random Hexamer (Applied Biosystems) and the obtained cDNA was subjected to a subsequent PCR reaction with primer pairs from the same genes as above, but located over exon-intron borders to avoid amplification of contaminating DNA (primer sequences available upon request). For TaqMan, RNA was prepared from tumor tissue or cells from thymus and spleen from Mo-MuLV-positive and wild-type animals using RNeasy® Mini kit (Qiagen), and the RT reaction was performed as described above. Expression analyses of *Sef-pending* and *Hesx1* were carried out with Assay-on-demand (Applied Biosystems), using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems).

Statistics. Disease frequency curves were constructed using the Kaplan-Meier method and differences in outcome between the TLL substrains were tested with the log rank test. The Mann-Whitney rank sum test for independent groups was used to compare age of onset between the different substrains, and Pearson's Chi-square test was used for the comparison of immunophenotypes. The level of statistical significance was defined as $p<0.05$ (two-sided). All data was analyzed using SPSS 11.0.1 software for Windows (SPSS, Chicago, IL, USA).

Results

The previously described TLL mouse strain (9) arouse during an attempt to target the IgVH locus. Unexpectedly, all the generated chimeras and a majority of their offspring developed a lymphoma/leukemia phenotype that was shown to segregate from the targeted mutation. To identify the genetic element causing the development of lymphoma, a differential screen between a cDNA library from $CD4^+$ T cells from normal thymus tissue and a cDNA library generated from $CD4^+$ lymphoma cells was performed. Clones representing cDNA overexpressed in the tumor were isolated and the sequence analysis revealed that 42% of these clones were derived from Mo-MLV.

To investigate the basis for the observed involvement of Mo-MLV in the TLL mice, a Southern blot analysis of DNA

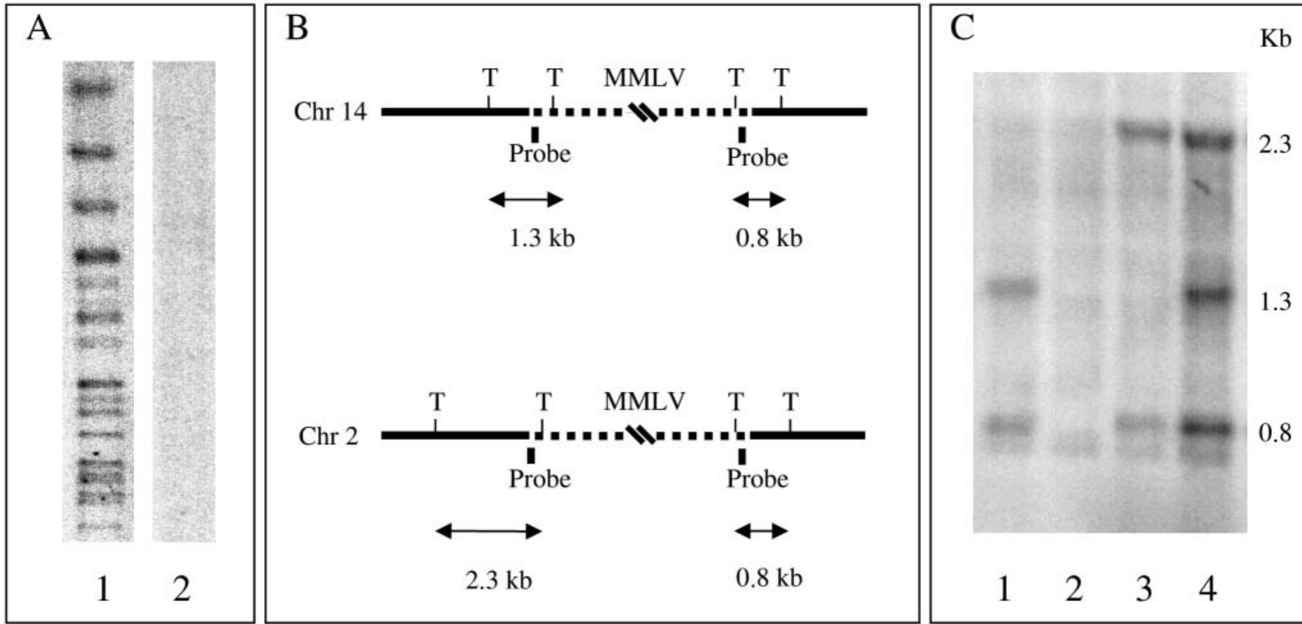

Figure 1. *(A) Southern blot on Taq I digested ES cell DNA hybridized with a Mo-MLV U3LTR probe. Lane 1=Mo-MLV targeted ES cell DNA showing multiple integrations of the virus; lane 2=ES cell clone from same original ES cell clone as ES cell in lane 1, but showing no retroviral integrations. (B) Schematic map of Mo-MLV integrations on chromosomes 2 and 14. T=Taq I restriction sites. The total length of the Mo-MLV proviral genome is 8.3 Kb. (C) Southern blot of Taq I-digested tail DNA hybridized with Mo-MLV U3LTR probe. Lane 1= TLL-2; lane 2= wild-type B6; lane 3= TLL-14; lane 4=TLL-2/14.*

derived from the E14 ES cell line used in the initial IgVH locus targeting experiment was performed using a Mo-MLV U3LTR probe (Figure 1A). Multiple integrations of Mo-MLV were observed in this cell line, but related ES cell clones derived from the same source clone were negative for Mo-MLV integrations, indicating that the bands on the blot represented newly aquired Moloney proviruses. Multiple integrations were also seen in the early generations of backcrossing the TLL mice to B6. Initially, backcrossed offspring were selected for further breeding based on a sustained high tumor frequency. At a later stage, as it became clear that germ line integrated Mo-MLV underlied the tumor formation, offspring were selected based on screening for specific viral integrations. Over time, individual Mo-MLV integrations segregated and were consecutively lost during backcrossing. Two substrains, representing two separate integrations, were kept for further characterization after more than ten generations of backcrossing (Figure 1B,C).

The chromosomal localization of the two germ line retroviral insertions was determined using the Splinkerette PCR method. The obtained genomic sequences (available upon request) flanking the provirus were compared to NCBI and Celera annotated databases using the BLAST (basic local alignment search tool) program and the integrations were positioned on chromosomes 2 and 14. Consequently, the two substrains were denoted TLL-2 and TLL-14. No deletions of chromosomal DNA were found at the proviral insertion sites and sequencing the proviral genome of the TLL-14 mice showed on average 97% homology to the Mo-MLV sequence reported in GenBank (accession no. AF462057), meaning that no major deletions or mutations were found in the proviral genome.

To investigate the influence of the chromosomal position of the integrated provirus on lymphoma/leukemia development, the two TLL substrains were compared with regard to the frequency of the lymphoma/leukemia and immunophenotype of the lymphoma. Also included in these analyses were mice containing both of the retroviral integrations, here denoted TLL-2/14.

As illustrated in Figure 2A, the TLL-14 substrain developed tumors with a higher frequency than TLL-2 ($p=0.001$). Moreover, in the mice carrying both integrations, the frequency was further increased. In addition to the observed difference in tumor frequency, the mean age of lymphoma onset was found to significantly differ between TLL-2 and TLL-14 ($p=0.002$), between TLL-2 and TLL-2/14 ($p<0.001$) and between TLL-14 and TLL-2/14 ($p=0.046$), (Figure 2B).

The lymphomas were subsequently immunophenotyped using antibodies for the T cell markers CD4 and CD8 and flow cytometric analysis. As shown in Table I, the TLL-14 substrain and the combined strain (TLL-2/14) displayed a

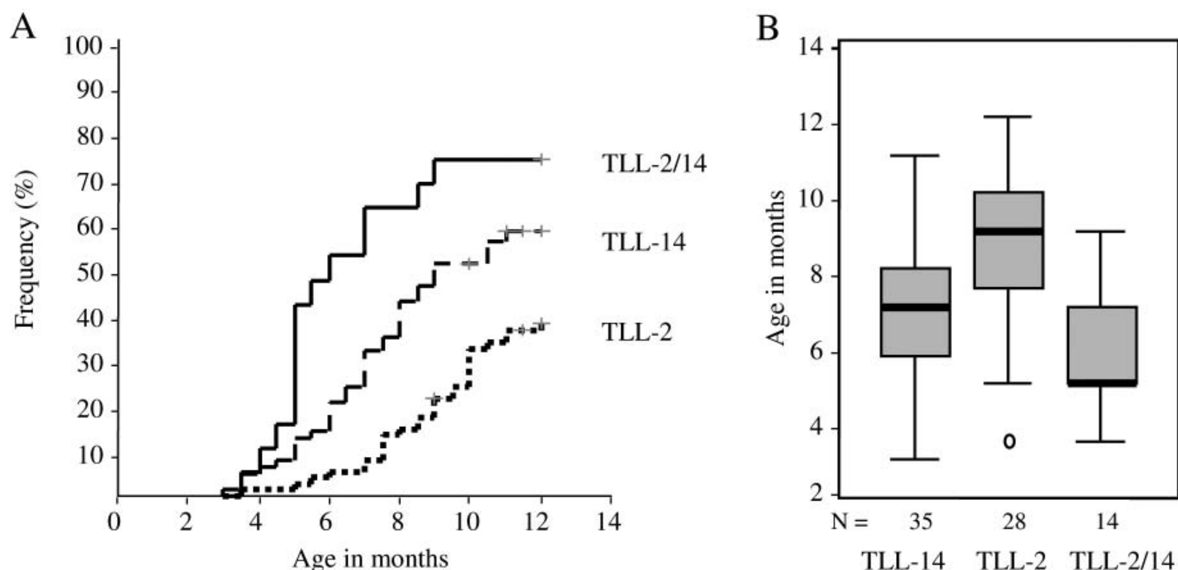

Figure 2. *(A) Spontaneous lymphoma frequency in the different substrains of TLL mice. TLL-2 (28 out of 75 mice), TLL-14 (35 out of 58 mice) and TLL-2/14 mice (14 out of 19 mice). (B) Box plot showing mean age of lymphoma onset in the TLL-2, TLL-14 and TLL-2/14 mice. o represents outlier.*

Table I. *Immunophenotype of tumors from TLL mice.*

Immunophenotype	TLL-14	TLL-2	TLL-2/14
($CD4^+/CD8^+$ or $CD4^-/CD8^-$)			
immature	65.7%	33.3%	57.1%
mature ($CD4^+$ or $CD8^+$)	25.7%	51.9%	35.7%
not classified	8.6%	14.8%	7.1%
number of tumors	35	27	14

majority of lymphomas with a mature immunophenotype, characterized by the cell surface expression of either CD4 or CD8. In contrast, more than half of the lymphomas arising in the TLL-2 substrain displayed an immature CD4/CD8 double-positive or CD4/CD8 double-negative phenotype. A fraction of the lymphomas could not be classified as being mature or immature based on FACS analysis of the CD4/CD8 expression. When comparing the TLL-2 and TLL-14 substrains, the difference in immunophenotype was statistically significant (p=0.04).

To determine the influence of genotype on lymphoma development, TLL-14 mice were mated and offspring homozygous for the Mo-MLV integration were generated. Twenty-nine homozygous TLL-14 mice were followed until death due to lymphoma or 12 months of age. The lymphoma frequency in TLL-14 homozygous mice is displayed in Figure 3. The age of onset of lymphoma in the

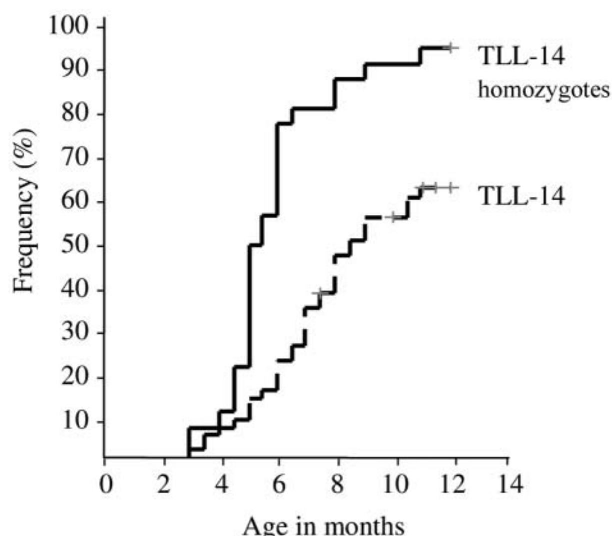

Figure 3. *Spontaneous lymphoma frequency in homozygous TLL-14 mice carrying two Mo-MLV proviral copies in the germ line, compared to TLL-14 mice carrying one proviral copy. TLL-14 homozygotes (27 out of 29 mice), TLL-14 (35 out of 58 mice).*

homozygous mice was 5.7 months, compared to 7 months for the heterozygous mice. The disease frequency and age of onset for TLL-14 homozygous mice resembled that for TLL-2/14 mice, suggesting that the number of proviral copies in the germ line is important for disease outcome, irrespective of where in the genome the retroviral integrations are located.

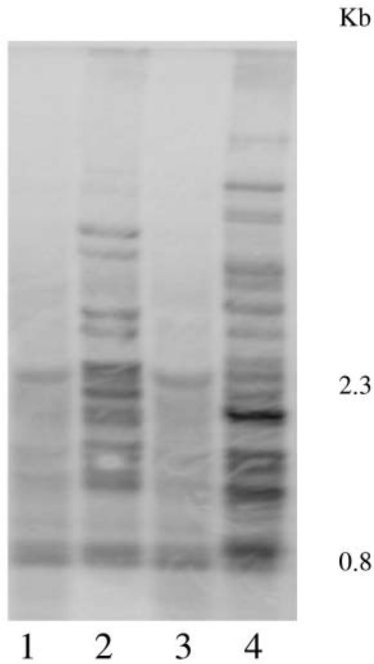

Figure 4. *Southern blot showing two examples of secondary integrations in lymphoma-derived DNA compared to tail-DNA. Prior to agarose gel separation, DNA was digested with TaqI and hybridization was carried out with the MoMLV U3LTR probe. Lane 1=tail-DNA from TLL-2 mouse; lane 2=lymphoma-derived DNA from same mouse as lane 1; lane 3=tail-DNA from TLL-2 mouse; lane 4=lymphoma-derived DNA from same mouse as lane 3.*

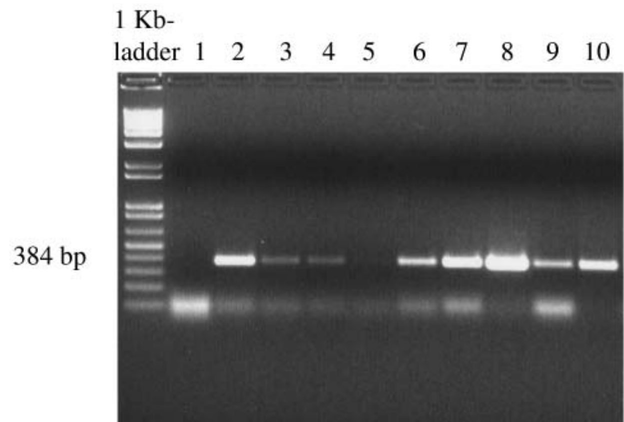

Figure 5. *RT-PCR performed on serum samples from TLL mice at different ages. The primers used were specific for Mo-MuLV-derived sequences (env-gene). Lane 1=C57/BL6 control mouse. Lanes 2-10=TLL mice aged from 2.5 to 8 months.*

The Mo-MLV infection process is characterized by the generation of mink cell focus-inducing viruses and secondary integrations of proviral DNA in the target cells, subsequently leading to lymphoma development (2). In order to investigate the presence of secondary proviral integrations in the tumors of TLL mice, a Southern blot of ten tumor-derived DNA samples from each substrain and the combined strain was performed using the Mo-MLV U3LTR probe. This revealed a number of secondary retroviral integrations in the lymphomas compared with tail-DNA (Figure 4). The average number of secondary integrations was ten, ranging from five to 20. No difference in the average number of secondary integrations between the substrains was seen (data not shown). Viral RNA sequences were detected by PCR in serum samples from TLL mice at different stages of development, meaning that these mice were viremic during a period preceding lymphoma development (Figure 5).

Whether the increased lymphoma frequency in TLL-14 mice reflected the action of the Moloney provirus itself on genes in the vicinity of the proviral insertion site on chromosome 14 was examined. Northern blot and RT-PCR analyses were carried out in order to look for enhanced expression of seven genes located close to the proviral integration site, comparing mice carrying the Mo-MuLV integration on chromosome 14 to unaffected C57/BL6 mice. No enhanced expression was detected with the probes/primer pairs used (data not shown). Two of the genes, *Hesx1* and *Sef-pending* (*Il17rd*), were selected based on their relative proximity to the provirus and on the previous known characteristics of the genes. The expression of these genes was investigated with TaqMan real-time PCR, comparing the expression in the thymus of mice carrying the proviral integration with unaffected C57/BL6 mice. Neither of the two genes showed any expression difference in the tissues analyzed (data not shown).

Discussion

In the present study, the existence of Mo-MLV in the germ-line of the TLL mice was demonstrated as the explanation of the lymphoma/leukaemia phenotype. The two substrains derived from the TLL mice differed significantly with respect to frequency of the disease and displayed differences in immunophenotypes of the tumors. No allelic variation was present in the TLL strain since all the crosses were on a pure C57BL/6 background. Hence, it is likely that host factors dependent on the chromosomal position of the integrated Mo-MLV provirus influenced the outcome of the TLL phenotype in the two substrains.

The difference between the TLL substrains could reflect the action of a gene – or several genes – activated or repressed by the Mo-MLV provirus itself. In both substrains, the provirus was integrated in chromosomal regions containing a number of actively transcribed genes as well as putative genes. In studies utilizing Northern blot and quantitative PCR

methods, we found no difference in gene expression of the genes located in the vicinity of the provirus, neither between the substrains nor between the TLL mice and wild-type B6 mice. Instead, factors influencing the transcriptional activity of the chromosomal regions may lead to differences in proviral expression and, subsequently, to the observed differences in tumor incidence. It could also reflect the time of expression of the Mo-MLV and its ability to infect target T cells at different stages of development. This was not investigated in the present study but is considered for future studies. The TLL-2 and TLL-14 mice produce infectious Moloney virus, as shown by the presence of virus-derived sequences in the bloodstream of mice at different ages and by the pattern of integration of newly acquired viral sequences in the developed tumors. The precise chromosomal position of the secondary integrations was not determined, as it was considered to be outside the scope of this study.

Retroviral etiology to hematological malignancies is common in mice, but, according to current knowledge, is very rare in humans (15). The human T cell leukemia/lymphoma viruses (HTLV type I and II) have been linked in the etiology of T-cell malignancies occurring in the Caribbean and southern Japan. HTLV-induced malignancies are difficult to treat with conventional chemotherapy (16) and, therefore, mouse models for this disease are valuable in the search for novel, more effective, therapies. The TLL model provides such a tool, as well as a valuable model for the studies of lymphomagenesis in general. During the last few decades, the focus of cancer therapy research has moved from conventional cytotoxic drugs towards the development of targeted therapies, inhibitors of angiogenesis and cancer vaccines (17-22). Good animal models are required to enhance knowledge in this field of research. Using the TLL model, an angiogenesis inhibitor was tested and was found to have an effect on tumor progression (23). Additional therapeutic studies are planned to elucidate the effects of combinations of novel therapies with conventional chemotherapy.

Acknowledgements

We thank Dr. Kristina Lejon for helpful technical assistance, Prof. Göran Roos and Dr. Fredrik Elgh for fruitful discussions and Dr. Kurt Lakovic for language corrections. This investigation was supported by grants from Lion's Cancer Research Foundation, Umeå University and the Cancer Foundation, Sweden.

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Received March 3, 2006
Accepted April 10, 2006