Pathogenic Determinants in the U3 Region of Recombinant Murine Leukemia Viruses Isolated from CWD and HRS/J Mice

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Recombinant murine leukemia viruses (MuLVs) from high-leukemia-incidence mouse strains typically acquire pathogenic U3 region sequences from the genome of the endogenous xenotropic virus, *Bxv-1*. However, a recombinant virus isolated from a leukemic HRS/J mouse and another from a CWD mouse contained U3 regions that lacked genetic markers of *Bxv-1*. The U3 regions of both recombinants were derived from the endogenous ecotropic virus *Emv-1* and had retained a single enhancer element. However, compared with that of *Emv-1*, the U3 region of each of the recombinant viruses contained five nucleotide substitutions, one of which was shared. To determine the biological significance of these substitutions, chimeric ecotropic viruses that contained the U3 region from one of the two recombinant viruses or from *Emv-1* were injected into NIH Swiss mice. All three of the chimeric ecotropic viruses were leukemogenic following a long latency. Despite the presence of an enhancer core motif that is known to contribute to the leukemogenicity of the AKR MuLV SL3-3, the HRS/J virus U3 region induced lymphomas only slightly more rapidly than the allelic *Emv-1* sequences. The chimeric virus with the U3 region of the CWD recombinant caused lymphomas more frequently and more rapidly than either of the other two viruses. The results support the hypothesis that one or more of the five nucleotide substitutions in the U3 regions of the recombinants contribute to viral pathogenicity. Comparison of DNA sequences suggests that the pathogenicity of the CWD virus U3 region was related to a sequence motif that is shared with *Bxv-1* and is recognized by the basic helix-loop-helix class of transcription factors.

The development of spontaneous lymphomas in the high-leukemia-incidence mouse strains CWD, HRS/J, AKR, and C58 has been linked to the expression of endogenous ecotropic murine leukemia viruses (MuLVs) and the subsequent generation of pathogenic recombinant viruses (1, 12, 14, 15, 20, 50, 52, 55, 58). The recombinant viruses are thought to represent the most pathogenic virus species in vivo, as they are invariably associated with tumor tissues and often accelerate the onset of disease when inoculated into neonatal mice of susceptible strains (6, 7, 15, 50, 52, 54, 58). Typically, the genomes of the recombinant viruses contain ecotropic virus sequences in the *gag* and *pol* genes, while portions of the *env* gene are inherited from endogenous polytropic viruses (12, 15, 52, 53, 58). The U3 region sequences of most HRS, AKR, and C58 recombinant viruses and about 25% of CWD recombinants are inherited from the endogenous xenotropic provirus, *Bxv-1* (12, 15, 33, 34, 50, 55, 58).

Previous studies by Holland and coworkers demonstrated that chimeric recombinant MuLVs that contained the U3 region of *Bxv-1* are more pathogenic than viruses that contained the allelic sequences from the AKR endogenous ecotropic virus, AKV623 (20, 21). This finding suggests that the reproducible incorporation of the *Bxv-1* U3 region sequences into the genomes of the recombinant viruses is related to a selection for a pathogenic determinant that is absent in the U3 region sequences of the endogenous ecotropic viruses (20). While the identity of the pathogenic determinant(s) is not known, it is likely located near or within the viral enhancer. This hypothesis is supported by the observation that the

pathogenicity of exogenous ecotropic MuLVs such as Friend, Moloney, Gross passage A, and SL3-3 is influenced by sequences within the enhancer element (5, 10, 29, 45, 49). These sequences are bound by transcription factors, such as NF-1, Ets-1, and the core-binding proteins. Complex interactions between these proteins regulate enhancer function which, in turn, presumably influences viral oncogenicity and target cell specificity (2-4, 8, 10, 16, 23, 30, 31, 35-39, 42, 45-48, 57, 59).

We have studied two leukemogenic recombinant MuLVs that lack genetic markers of the *Bxv-1* U3 region. The two viruses, CWM-T15 and PTV-1, were each recovered from spontaneous thymic T-cell lymphomas that developed in a CWD and an HRS/J mouse, respectively (15, 50). The U3 region sequence of CWM-T15 differed by only five nucleotides from that of the endogenous ecotropic parent virus, *Emv-1*, and three of these five differences were clustered immediately 3' of the enhancer core (53). The same substitutions have been detected in recombinant viruses in about one-third of spontaneous CWD lymphomas (34). Previous studies have shown that the U3 region of PTV-1 genome also contained *Emv-1*-related sequences (15). These observations raised the possibility that a small number of specific substitutions in the ecotropic virus-derived U3 region contributed to the pathogenicity of these two recombinant viruses. To address this possibility, we first determined the nucleotide sequence of the U3 region of the PTV-1 genome and then generated and compared the pathogenicities of chimeric viruses that contained the U3 region sequences of PTV-1, CWM-T15, or *Emv-1*. These results provide insights about the nucleotide substitutions within the U3 region that promote viral leukemogenicity and the process by which pathogenic recombinant viruses are generated in vivo.

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FIG. 1. Comparison of nucleotide sequences of the U3 regions of endogenous ecotropic MuLV and the recombinant MuLVs PTV-1 and CWM-T15. The sequences are compared with those of AKV623. *Emv-1* and *Emv-3* are the two endogenous ecotropic viruses found in HRS/J and CWD mice. The dots indicate homology with the AKV623 sequences, and substitutions are shown by the insertion of the nucleotide symbol in the appropriate position. The sequence between the two asterisks is duplicated in AKV623; each of the other viruses contains a single enhancer element. The brackets enclose the enhancer core region sequences that are recognized by the oligonucleotide probes described in Materials and Methods. The underlined sequences denote sites recognized by transcription factors, oligonucleotide primers, and restriction enzymes. ECOLTR5 is the 5' PCR oligonucleotide primer, while ECOLTR3 indicates sequences recognized by the 3' primer. Transcription factors: CTF/NF-1, CCAAT-binding transcription factor or nuclear factor 1 (35, 39, 46); core, enhancer core-binding proteins (37, 46, 57); Lvb/ets, Lvb and/or Ets-1 protein (16, 38, 46); GRE, glucocorticoid response element (3, 46). The box encloses a second E-box sequence that is unique to recombinant MuLVs.

MATERIALS AND METHODS

Mice. NIH Swiss mice were obtained from the small animal section of the National Institutes of Health and maintained at the University of Virginia vivarium.

Construction of chimeric proviruses. Standard recombinant DNA techniques were used to replace the U3 sequences of an infectious clone of the AKR ecotropic MuLV AKV623 with those of the endogenous ecotropic virus, *Emv-1*, the CWD recombinant virus, CWM-T15, or the HRS/J recombinant, PTV-1 (Fig. 1). The hybrid proviruses are referred to as AKL-E1, AKL-T15, or AKL-P1, respectively. Plasmid pAKV623 was digested with *Bss*HII and *Pst*I to obtain a proviral fragment that lacked the U3 region sequences 3' of the *Pst*I site in the long terminal repeat. This fragment was purified by electrophoresis through a low-melting-point agarose gel and was added to a ligation reaction that contained pBR322 plasmid DNA that had been cleaved with *Pst*I and treated with calf intestinal alkaline phosphatase and the *Pst*I-*Bss*HII U3 region fragment of either *Emv-1* or PTV-1 (32). The *Emv-1* U3 sequences were obtained by *Pst*I and *Bss*HII digestion of the pN22 plasmid that contained a permuted clone of *Emv-1* (gift of Rex Risser). The same restriction enzymes were used to remove the U3 region sequences from a subclone of the PTV-1 provirus (54). Competent *Escherichia coli* cells were transformed with aliquots of the ligation mixtures, and colonies that were resistant to tetracycline were screened for

plasmids that contained AKV623 proviral fragments with PTV-1 or *Emv-1* U3 sequences (32). The AKL-T15 provirus was constructed in a similar manner except that the *Pst*I-*Pvu*I fragment that contained the U3, R, U5, and 5' leader regions of the CWM-T15 provirus (53) was ligated to the *Pvu*I-*Pst*I fragment of the AKV623 provirus and DNA of plasmid pUC13 that had been digested with *Pst*I and dephosphorylated. Competent *E. coli* cells were transformed with the ligation products, and ampicillin-resistant colonies were screened for plasmids that contained the hybrid provirus (32). The DNA sequences of the R, U5, and 5' leader regions of the three plasmids were identical except that the 5' leader region of AKL-T15 contained a substitution of a cytidine for a thymidine 52 residues from the 3' end of U5.

Double-stranded DNA sequencing. The sequences of the U3 region of the AKL-E1, AKL-P1, and AKL-T15 plasmids were verified by the double-stranded DNA dideoxynucleotide termination method, using a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) and [α - 35 S]dATP (56).

DEAE-dextran transfection. The plasmids that contained the hybrid proviruses were digested with *Pst*I and then incubated at a high DNA concentration (100 μ g/ml) in the presence of T4 DNA ligase (New England Biolabs, Beverly, Mass.) at room temperature for 4 h. Under these conditions, the reaction products contained high-molecular-weight DNA concatamers that included proviruses with a reconstituted 3'

LTR. Five micrograms of the ligated DNAs was mixed with 10 μ g of carrier salmon sperm DNA, 50 ml of DEAE-dextran (25 mg/ml), 5 ml of serum-free minimal essential medium (Gibco/BRL, Bethesda, Md.), and 50 mM Tris-Cl (pH 7.5). The mixture was preincubated for 5 min at room temperature and then added to NIH 3T3 cells that were 80% confluent (100-mm-diameter cell culture dishes; Costar, Cambridge, Mass.) and had been washed twice with phosphate-buffered saline (PBS). After incubation of the cells for 1 h at 37°C, 100 mM chloroquine was added, and the cells were incubated for an additional 2 h. The cells were washed twice with PBS and fed with minimal essential medium with 10% fetal calf serum (Gibco/BRL) and 2 mg of Polybrene per ml. The cells were monitored and fed fresh medium when necessary and were subcultured every 3 to 5 days. After five or more passages of the cells, culture supernatant was collected, centrifuged briefly to remove cellular debris, and stored at -70°C. Aliquots were later thawed and tested for viral particles that contained reverse transcriptase activity (43).

Leukemogenicity assays. The titer of infectious thawed virus was determined by syncytium formation in rat XC cells (44). Virus titers were 3.5×10^4 PFU/ml for AKL-T15, 4.0×10^4 PFU/ml for AKL-P1, and 3.0×10^4 PFU/ml for AKL-E1. From 0.05 to 0.1 ml of thawed medium that contained one of the three viruses was injected into the peritoneum of neonatal NIH Swiss mice that were less than 48 h old. The mice were weaned at about 3 weeks of age and then monitored for signs of illness or enlarged spleens. Animals that became severely ill were sacrificed by metaflane inhalation; animals that died suddenly in the cages were refrigerated until necropsy, usually within 24 h of death. The spleen, thymus, and any tumor tissue were removed, and aliquots were frozen for isolation of DNA and immunohistochemistry or placed in formalin for fixation for histopathology. Three mice from each injection group were sacrificed at 3 months of age to verify viral infection. These mice were considered preleukemic and were not included in the leukemogenicity assays.

DNA isolation, Southern blotting, and hybridization. DNA was extracted from fresh or frozen tumor tissue as previously described (9, 50). For Southern blot studies, 5 μ g of DNA was digested with the appropriate enzymes, and the products were separated by electrophoresis into 0.7 or 1.0% agarose gels. The fragments were then blotted to nylon membranes (Sure-blot; Oncor) (41). The membranes were incubated in prehybridization buffer (4 $\times$ SSCP [480 mM NaCl, 60 mM sodium citrate, 60 mM Na₂HPO₄, 20 mM NaH₂PO₄], 1 $\times$ BFP [200 mg of bovine serum albumin per ml, 200 mg of Ficoll per ml, 200 mg of polyvinylpyrrolidone per ml], 1% sodium dodecyl sulfate [SDS], 1.25 mg of salmon sperm DNA per ml) at 65°C for at least 2 h. The prehybridization solution was removed and replaced with 2×10^6 to 8×10^6 cpm of ³²P-labeled probe per ml in 4 $\times$ SSCP-1 $\times$ BFP-1% SDS-10% dextran sulfate, and the mixture was incubated for 18 to 24 h at 65°C. The membranes were washed to a final stringency of 0.1 $\times$ SSCP at 65°C, blotted dry with filter paper, and exposed to X-Omat RP-5 film at -70°C with intensifying screens.

DNA probes. Gene rearrangements were detected by hybridization of Southern blots to probes for the immunoglobulin heavy-chain joining region (J_H) and light-chain joining region (J_K) (Roger Perlmutter, University of Washington) and sequences from the constant region of the beta-chain gene of the T-cell receptor (TCR β), which hybridizes to both the Cb1 and Cb2 regions of TCR β (Tak Mak, University of Toronto). The probes were excised from the plasmid vectors by the appropriate restriction enzyme digest and labeled with [α -³²P]dATP to 2×10^6 to 8×10^6 cpm/ml by the standard random prime

labeling procedure (Boehringer Mannheim, Indianapolis, Ind.). Unincorporated label was removed by chromatography on a Sephadex G-50 column (13).

Histology and expression of immunophenotypic markers. Tissues were preserved in formalin and embedded in paraffin. Two-micrometer sections of tissue were stained with hematoxylin and eosin. The tumors were classified according to the terminology of Pattingale and Taylor (40), with modifications to coordinate terminology with the Working Formulation for the classification of human lymphomas. To detect expression of immunophenotypic markers, frozen tissue was embedded in OCT compound (Tissue-Tek) and cut into 5- μ m sections. After fixation in acetone, separate sections were stained with monoclonal antibody raised against Thy 1.2 (Becton Dickinson, Mountain View, Calif.), an antigen present on T lymphocytes or B220 (gift of I. Weissman, Stanford University), an antigen present on pro-B, pre-B, and mature B cells, or with rabbit antiserum that recognizes mouse immunoglobulin M (IgM) (gift of P. Isakson, Pharmasci Inc.) which is produced by pre-B cells and B cells. Endogenous biotin was blocked with an avidin-biotin blocking kit (Vector, Burlingame, Calif.). After incubation with the primary antibody, the sections were washed and exposed to a solution with rabbit anti-goat IgG or mouse-absorbed rabbit anti-rat IgG antibodies that were conjugated to biotin. After additional rinses with buffer, binding of the secondary antibodies was detected by the addition of avidin-biotin horseradish peroxidase complex, hydrogen peroxide, and 3'-3' diaminobenzidine substrate stain. Following the substrate stain, the tissue sections were counterstained with Harris's hematoxylin. The tumors were scored positive for expression of the antigen if more than one-half of the cells were stained by the antibody. Sections of normal spleen tissue that were stained with the same reagents were used as controls.

Classification of tumor immunophenotype. In the absence of rearrangements, the J_H probe hybridizes to a 6.4-kb *Eco*RI fragment, the J_K probe identifies a 22-kb *Hpa*I fragment, and the TCR β probe hybridizes to 11.6- and 6.1-kb *Hpa*I fragments. The tumors were classified as pre-B cell if the DNA contained a J_H rearrangement in the absence of J_K or TCR β rearrangement and the cells reacted with the B220 antibody. Tumors were defined as B cell if both J_K and J_H rearrangements were detected, TCR β rearrangements were absent, and the cells reacted with the IgM antibody. T-cell tumors were defined by detectable TCR β rearrangements in the DNA in the absence of J_K rearrangements and if the cells stained with Thy 1.2 antibody of tissue sections. J_H rearrangements were seen in some of the T-cell lymphomas as previously reported (19, 51).

PCR amplification of the viral sequences. The U3 region of the acquired proviral DNAs was amplified from the DNA of the control and tumor tissue by PCR. Five hundred nanograms of genomic DNA or 1 ng of plasmid DNA was mixed with 1 $\times$ PCR reaction buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μ M each of the deoxynucleoside triphosphates, 0.2 μ M each of the oligonucleotide primers (ECOLTR5 and ECOLTR3), and 2 U of *Taq*I polymerase. The primers correspond to sequences that are unique to the 5' and 3' portions of the U3 regions of the ecotropic proviruses (ECOLTR5, 5'-AAACAAGAACAAGGAAGT; ECOLTR3, 5'-TTCCCGGGTCTCTTGAAACTGTTGTTG) (Fig. 1). The PCRs were performed in a Perkin-Elmer Cetus Thermo-Cycler for 25 to 30 cycles of denaturing for 30 s at 94°C, annealing for 30 s at 50°C, and extension for 30 to 45 s at 72°C, with a final extension of 5 min. These conditions allowed for amplification of all injected viral sequences, including AKL-P1, which has a one-base-pair mismatch in the region of the 5' primer (Fig. 1). Each set of reactions was heated to 80°C for 2 min prior to the

addition of the nucleotides to avoid nonspecific annealing of the primers. The products of the PCR were visualized after electrophoresis through 1.8% agarose gels. Approximately 200 ng of each PCR product was transferred to a nylon membrane for Southern blot analysis as described above.

Oligonucleotide labeling and hybridization. Oligonucleotides that corresponded to the noncoding strand of the core region sequences of CWM-T15 (T15), PTV-1 (P1), or *Emv-1* (E1) (Fig. 1) were synthesized at the University of Virginia Protein and Nucleic Acid Sequencing Center. Under appropriate conditions, these oligonucleotide probes hybridize specifically to the corresponding core region sequences although on occasion there is a slight degree of cross-hybridization between the *Emv-1* and PTV-1 sequences that differ by a single nucleotide (34) (data not shown). The 5' ends of the oligonucleotides were labeled by incubation with [γ - 32 P]dATP in 1 $\times$ kinase buffer (70 mM Tris-Cl [pH 7.6], 10 mM MgCl₂, 5 mM dithiothreitol) with 25 U of polynucleotide kinase (New England Biolabs) for 45 min at 37°C. Unincorporated label was removed by chromatography on a Sephadex G50-50 column. Approximately 5×10^6 to 10×10^6 cpm of the labeled probe per ml was incubated with the PCR Southern blots for 18 to 24 h in hybridization solution (1 M NaCl, 20 mM Tris-Cl, 6 mM EDTA, 10 $\times$ BFP, 1% SDS, 0.5% Nonidet P-40, 100 mg of salmon sperm DNA per ml, 50 mg of yeast RNA per ml). Unbound probe was removed by washing the membrane in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature for 15 min, 2 $\times$ SSC–0.1% SDS at 50°C for 15 min, 1 $\times$ SSC–0.1% SDS at 50°C for 15 min, and finally 0.1 $\times$ SSC–0.1% SDS at 50°C for 5 min. The membranes were exposed to X-Omat RP-5 film at –70°C with intensifying screens.

Statistical analysis. The results of the leukemogenicity assays were analyzed by the Medlog software package from Information Analysis Corporation, Incline Village, Nev. This program compares Kaplan-Meier estimates of survival curves of all injected animals and tests the two survival curves for equality by the Mantel-Haenszel log rank test. The program censors animals that did not develop lymphoma or developed nonlymphoid tumors. The analysis, however, assumes that until they were removed from the study, the censored animals had the same chance of developing disease as the noncensored mice. The differences in disease latency of mice injected with the AKL-P1 or AKL-E1 viruses were also compared by the Wilcoxon rank-sum test (11).

RESULTS

Comparison of the U3 region sequences of the HRS/J PTV-1 and CWD CWM-T15 recombinant viruses. PTV-1 is a recombinant polytropic virus that was recovered from a spontaneous HRS/J thymic lymphoma and has been shown to induce T-cell lymphomas when inoculated into newborn mice of susceptible strains (15, 53, 55). Unlike the genomes of most HRS/J and AKR recombinant viruses, the genome of PTV-1 lacked markers of the U3 region sequences of *Bxv-1* and appeared to retain sequences of one of the two endogenous ecotropic viruses, *Emv-1* or *Emv-3* (Fig. 1) (15, 55). We determined the nucleotide sequence of the U3 region of PTV-1 as found in proviral fragments that were originally cloned into bacteriophage lambda vectors (54). As shown in Fig. 1, the U3 region of PTV-1 was highly homologous to those of the endogenous ecotropic proviruses but lacked the *Emv-3*-specific substitutions at position 182 and 188. Therefore, the U3 sequences of PTV-1 were probably derived from *Emv-1* but had acquired five substitutions, including a C-to-T transition within the viral

enhancer region. This latter base pair substitution, which may be the result of spontaneous mutation, created a core sequence that is not found in endogenous MuLVs but is present in the enhancer regions of the highly leukemogenic SL3-3 and Gross passage A MuLVs (10, 29, 34). The SL3-3 core sequence has been reported to bind a specific transcription factor and contribute to viral pathogenicity and T-cell tropism (2, 17, 30, 36).

CWM-T15 is a recombinant polytropic virus that was recovered from a spontaneous T-cell lymphoma in a CWD mouse. This recombinant virus accelerates the onset of B- and T-cell lymphomas in CWD mice when injected as a phenotypic mixture with an endogenous ecotropic virus (50, 53). As previously reported, the U3 region of CWM-T15 is also derived from *Emv-1* and contains four substitutions that are distinct from the substitutions found in U3 region of PTV-1 (Fig. 1) (53). Three of the substitutions in the CWM-T15 U3 region are clustered just 3' of the enhancer core region but 5' of the site recognized by the NF-1 transcription factor (Fig. 1). This sequence motif was also found in another CWD recombinant virus isolated from a B-cell lymphoma and can be detected in about one-third of spontaneous CWD tumors (34).

Construction and pathogenicity of chimeric MuLVs that contained the different U3 region sequences. The U3 region sequences of the AKR endogenous ecotropic virus, AKV623, were replaced by those of *Emv-1*, CWM-T15, or PTV-1 by standard recombinant DNA techniques (Fig. 2). AKV623, which is closely related to *Emv-1*, *Emv-3*, and other endogenous ecotropic viruses, is considered to be weakly pathogenic or nonpathogenic (6, 7, 29). To obtain infectious viruses, the DNAs of the modified proviruses were introduced into NIH 3T3 cells by the DEAE-dextran transfection procedure. The viruses produced by the transfected cells were designated AKL-E1 (*Emv-1* U3 sequences), AKL-T15 (CWM-T15 U3 region sequences), and AKL-P1 (PTV-1 U3 region sequences). The genetic structure of the viruses was confirmed by T₁ oligonucleotide fingerprint of the viral RNAs (data not shown) and by analysis of the U3 sequences that were amplified by PCR (see below).

To determine if the slight differences in the U3 regions of the chimeric viruses influence viral pathogenicity, different litters of neonatal NIH Swiss mice were inoculated with one of the chimeric viruses. NIH Swiss mice were chosen because they have a low incidence of spontaneous lymphomas and lack the endogenous *Bxv-1* xenotropic virus as well as endogenous ecotropic proviruses. This reduced the probability that recombination in vivo would alter the U3 region sequences of the injected viruses. All three of the chimeric viruses were infectious in this strain, as proviral sequences could be detected in the splenic DNA of normal preleukemic mice that were sacrificed at 3 months of age (see below).

Each of the viruses was found to induce malignant lymphomas after a long latency (Table 1). The incidence of lymphomas in the AKL-E1-injected mice was 55.5%, a surprisingly high frequency since endogenous ecotropic viruses are thought to be weakly pathogenic (6, 7, 29). AKL-P1 induced lymphomas in about the same proportion of animals, while 79.2% of the AKL-T15-injected animals developed lymphoma. The Kaplan-Meier disease plots for the three groups of animals were analyzed for statistical differences by the Mantel-Haenszel log rank test (Fig. 3a and 3b). This method of evaluation allows comparison of the projected incidence of lymphoma and takes into account those animals that died of diseases other than lymphoma. AKL-T15 induced lymphomas more frequently and more rapidly than AKL-E1 or AKL-P1, with significance values of $P < 0.0001$ (Fig. 3a) and $P = 0.0101$,

a

b

FIG. 2. Strategy for the construction of chimeric proviruses. (a) Plasmid vectors that contained the U3 sequences of the three different viruses are shown in the top row, and plasmids with the modified reconstituted proviruses are shown in the bottom row. The U3 region sequences were obtained by digestion of plasmids pN23 and P1LTR with *Pst*I and *Bss*HI and plasmid T15LTR with *Pst*I and *Pvu*I. Plasmid pAKV was digested with *Pst*I and *Bss*HI (or *Pvu*I) to obtain a large proviral fragment with the structural genes of AKV623. The appropriate U3 region and AKV623 proviral fragments were then ligated together and inserted into the pBR322 or pUC13 vector that had been digested with *Pst*I and treated with calf alkaline phosphatase. LTR, long terminal repeat. (b) Schematic of the genomes of the chimeric viruses.

respectively. Despite an increase in the number of early deaths and shorter disease latency, the Kaplan-Meier plot of the AKL-P1-injected mice did not differ significantly from that of the mice injected with AKL-E1 ($P = 0.5220$ (Fig. 3b). However, a second analysis by the Wilcoxon rank-sum test revealed that the 3.3-month difference in the average disease latency in these two groups was statistically significant ($\alpha < 0.05$) (11).

The majority of the lymphomas were of B-cell origin (84.4%), and most were classified as large-cell lymphomas (Table 1). There were no clear differences in the immunologic or histologic phenotype of the tumors in the three groups of animals. The high proportion of B-cell tumors may be related to the long latency (40), which presumably reflects the decreased rate of replication of the chimeric ecotropic viruses compared with other more pathogenic MuLVs, such as SL3-3 or Moloney MuLV, that commonly induce thymic lymphomas by 6 months of age. No T-cell lymphomas were seen in the AKL-E1-injected mice; however, four of the animals injected with AKL-T15 and three mice injected with AKL-P1 developed T-cell tumors. Four of these T-cell tumors were classified as lymphoblastic lymphomas. The difference in the projected incidence of T-cell lymphomas in the AKL-T15- and AKL-E1-injected mice approached statistical significance ($P = 0.0522$) (data not shown). Since the average latency for T-cell tumors was less than for B-cell tumors (11.1 and 16.9 months, respectively), the higher incidence of T-cell lymphomas contributed to the shorter disease latency of the mice injected with AKL-T15 and AKL-P1. However, the difference in the incidence and latency of the B-cell lymphomas in the mice injected with AKL-T15 and AKL-E1 was also statistically significant (data not shown).

Analysis of the enhancer core region of proviruses associated with tumor tissues. Because of the long disease latency, the possibility existed that the U3 region sequences of the injected viruses was altered in vivo by mutation or recombination with endogenous viruses. PCR was used to amplify the U3 regions of the proviral DNAs from tumors or tissues from preleukemic mice. The primers for the reaction (ECOLTR5 and ECOLTR3) corresponded to sequences that are unique to the 5' and 3' ends of the U3 region of ecotropic MuLVs (Fig. 1). Southern blots of the 158-bp PCR products (for those

viruses that contained a single enhancer region) were sequentially hybridized to oligonucleotide probes that corresponded to the different enhancer core region sequences of the injected viruses (Fig. 1). In the majority of cases, the amplified DNAs hybridized to the probe that corresponded to the enhancer core region sequences of the injected virus (Fig. 4). In addition, DNA sequence analysis of the amplified U3 region of proviruses from an AKL-E1-injected animal revealed no changes in the enhancer sequences (data not shown). The PCR products from some of the tumor DNAs hybridized to various degrees with probes that did not match those of the injected virus. For example, the signal from the DNA product of one of the AKL-P1-induced tumors was greater with the *Emv-1* (E1) probe than with the PTV-1 (P1) core region probe (Fig. 4, AKL-P1, lane 5). Also, the PCR fragments of two AKL-T15-injected mice reacted with probes that recognize the *Emv-1* (E1) or PTV-1 (P1) enhancer sequence probes (Fig. 4, AKL-T15, lanes 1 and 19). Most likely, a portion of the proviruses in these tumors had enhancer core region sequences that had been altered in vivo as a result of spontaneous mutation or recombination. Alternatively, the discordant hybridization pattern may have reflected DNA contamination or another type of PCR artifact. In any case, the number of tumors with anomalous hybridization patterns was not sufficient to alter the interpretation of the leukemogenicity assays.

DISCUSSION

The results of these studies are consistent with the hypothesis that the U3 regions of the HRS/J recombinant virus PTV-1 and the CWD recombinant CWM-T15 contained pathogenic determinants that are not found in the endogenous ecotropic virus parent, *Emv-1*. These determinants had only a modest effect on the viral phenotypes, which were manifest as an acceleration in the onset of disease. In the case of the CWM-T15 U3 region, an increase in the overall incidence of lymphomas was also seen. These results contrast with earlier studies that showed that a chimeric AKV623 virus that contained the U3 sequences of SL3-3 rapidly induces T-cell tumors in NIH Swiss mice (29). The increased pathogenicity of the SL3-3 U3 region is likely related to duplications, rearrange-

TABLE 1. Immunophenotypes of virus-induced malignant lymphomas from injected NIH Swiss mice^a

Tumor type	AKL-T15 (n = 24)		AKL-E1 (n = 18)		AKL-P1 (n = 29)	
	Incidence (%)	Latency (mo; mean $\pm$ SD)	Incidence (%)	Latency (mo; mean $\pm$ SD)	Incidence (%)	Latency (mo; mean $\pm$ SD)
All lymphomas	19 (79.2)		10 (55.5)		16 (55.1)	
B cell	13	16.0 $\pm$ 2.3	8	18.6 $\pm$ 1.9	13	16.0 $\pm$ 2.9
Pre-B cell	2	15.6 $\pm$ 2.9	2	18.2 $\pm$ 2.1	0	
T cell	4	10.4 $\pm$ 3.1	0		3	12.2 $\pm$ 4.8
Other	6		10		13	

^a The immunophenotypes were determined by gene rearrangement and immunohistochemical staining assays as described in Materials and Methods. The other category includes mice without detectable disease or with nonlymphoid tumors. In some instances, the number of mice in the disease groups adds up to more than the total mice since three animals had more than one disease (lymphoma and adenocarcinoma).

FIG. 3. (a) Comparison of the incidence of lymphoma in mice injected with AKL-E1 or AKL-T15 (Kaplan-Meier projection). (b) Comparison of the incidence of the lymphoma in mice injected with AKL-E1 or AKL-P1 (Kaplan-Meier projection).

ments, and substitutions of the enhancer sequences (17, 29, 30, 31, 37). Presumably, the high degree of homology in the nucleotide sequences of the U3 regions of the chimeric viruses tested here was responsible for the relatively small differences in the phenotypes. The modest contribution of the CWM-T15

U3 region to the pathogenicity of the chimeric virus is similar to that seen with the U3 region sequences of the endogenous xenotropic virus *Bxv-1*. These latter sequences are frequently incorporated into the genomes of spontaneous recombinant MuLVs and have been shown to be necessary but not sufficient

FIG. 4. Hybridization of oligonucleotide probes to Southern blots of proviral U3 sequences amplified by PCR. The boxes enclose Southern blots of PCR products from DNAs of animals that were injected with the indicated viruses. The blots were incubated in sequential reactions to oligonucleotide probes that were specific to the enhancer core regions of AKL-T15 (T15), AKL-P1 (P1), or AKL-E1 (E1). The specificity of the probes was monitored by the hybridization to controls shown at the right. For AKL-T15-injected mice, lanes 3 to 7 and 9 to 19 are DNAs from mice with lymphoma, lanes 1 and 2 are DNAs from 3-month-old preleukemic mice, and lane 8 is DNA from a mouse that had no detectable disease. For AKL-E1-injected mice, lanes 6 to 9 are DNAs from mice with lymphoma, lanes 1 and 2 are DNAs from 3-month-old preleukemic mice, lanes 4, 11, and 12 are DNAs from mice with no detectable disease, and lanes 3, 5, and 10 are DNAs from mice with nonlymphoid tumors. For AKL-P1-injected mice, lanes 1, 3 to 5, and 7 are DNAs from mice with lymphoma, lane 6 is DNA from a mouse with no detectable disease, and lanes 2 and 8 are DNAs from mice with nonlymphoid tumors. Controls are PCR-amplified plasmid DNAs of PTV-1 (P), AKL-T15 (T), and AKV623 (A) (the enhancer core sequences of AKV623 are identical to those of *Emv-1*).

to confer full leukemogenicity to hybrid AKR recombinant viruses (20, 22).

The detection of pathogenic determinants in the U3 regions suggests that PTV-1 and CWM-T15 had a selective replicative advantage *in vivo* or were more oncogenic than similar recombinants that had retained the U3 region sequences of the endogenous ecotropic virus parent. This hypothesis is consistent with the observation that an independently isolated CWD recombinant as well as viruses from about one-third of spontaneous CWD lymphomas contain the same three nucleotide substitutions that are found near the enhancer core of CWM-T15 (34, 53). Recent studies from our laboratory indicate that these substitutions may not result from spontaneous mutations but are inherited from the U3 region sequences of an endogenous polytropic virus that is found in CWD mice but not other high-leukemia-incidence strains (33, 34). Consequently, recombinant viruses from CWD mice appear to be distinct from those found in other strains in that they may acquire pathogenic U3 region sequences from at least two noncancerous proviruses, *Bxv-1* or the putative polytropic donor of the CWM-T15-specific U3 region sequences (33, 34).

The leukemogenicity of the control AKL-E1 virus that contained the U3 region of *Emv-1* was surprising in that the genome was derived entirely from two endogenous ecotropic viruses. The endogenous ecotropic viruses are considered to be weakly pathogenic, based on the results of leukemia acceleration or short-term leukemogenicity assays (6, 7, 24, 25, 27, 42, 58). However, the latency for disease in the AKL-E1-injected mice (mean of 18.5 months) suggests that the oncogenic potential of related endogenous ecotropic viruses was not appreciated in the earlier studies. The pathogenic effects were likely obscured by the background of spontaneous lymphomas in the high-leukemia-incidence strains or because the studies in the low-leukemia-incidence strains were terminated before the animals reached 18 months of age. In fact, we have observed that the AKV623 virus also induces lymphomas in NIH Swiss mice after a long latency (data not shown). Other less likely explanations for the oncogenicity of AKL-E1 is an interaction between two weak pathogenic determinants that are unique to each of the two parental viruses or that NIH Swiss mice are particularly susceptible to this chimeric endogenous MuLV.

Although there was no statistical difference in the Kaplan-Meier projections of the incidence of lymphoma between the mice injected with AKL-P1 and AKL-E1, the average disease latency in the AKL-P1-injected animals was significantly shorter. The minimal pathogenicity of the U3 region of PTV-1 was somewhat unexpected since one of the five substitutions had created an enhancer core motif that contributes to the oncogenicity and T-cell tropism of the SL3-3 MuLV (2, 17, 30, 36, 45). Taken together with previous studies, these observations strongly suggest that full pathogenicity of the SL3-3 U3 region requires sequences that are located outside the enhancer core. Similarly, the leukemogenicity of the PTV-1 recombinant likely depends on sequences located outside the U3 region since unlike AKL-P1, PTV-1 clearly accelerates the onset of T-cell lymphomas when injected into susceptible mouse strains (15, 52, 53). Regardless, the substitution in the enhancer core of PTV-1 remains a candidate for the pathogenic determinant that caused the modest decrease in tumor latency in the AKL-P1-injected animals.

The U3 region of the CWM-T15 recombinant virus was clearly pathogenic, as AKL-T15 induced both B- and T-cell lymphomas more frequently and more rapidly than the other chimeric viruses. This phenotype is similar to that of the CWM-T15 parent, which accelerates the onset of both types of

lymphomas when injected into CWD mice as a phenotypic mixture that contains endogenous ecotropic viruses (50, 53). The most likely candidates for the pathogenic determinants in the U3 region are the three nucleotide substitutions clustered near the core region of the enhancer that have the potential to influence enhancer function and viral pathogenicity (47, 53). Two of these substitutions are homologous to those seen in the allelic region of the *Bxv-1* enhancer and create a second consensus E-box sequence (CACCTGG). The other motif (CAGATGG) overlaps the glucocorticoid response element binding site that is found in the 3' end of the enhancers of each of the MuLVs studied here (8). The E-box sequence that is unique to the recombinant MuLVs is identical to the E4 box found in the enhancer of the cellular immunoglobulin heavy-chain gene and appears to influence the function of the enhancer (28). Related E-box sequences are also found in the enhancers of other lymphoid-specific genes, including those that encode immunoglobulin light chain and some of the T-cell receptor proteins (reviewed in reference 26). The E-box sequences contribute to enhancer function because they act as binding sites for the homodimers or heterodimers of the basic helix-loop-helix class of transcription factors (18, 26, 28). These observations raise the interesting possibility that basic helix-loop-helix proteins that are expressed in lymphoid cells bind to the recombinant virus-specific E-box motif to increase enhancer function. If so, AKL-T15 may have caused disease more rapidly because this virus replicated more efficiently in the lymphoid target cells or more readily transformed cells than did viruses whose enhancers lacked the second E box. Experiments to test the influence of the second E box on the function of the viral enhancers are in progress.

ACKNOWLEDGMENTS

S.C.L.-S. and A.C.M. are considered co-first authors.

This work was supported by Public Health Service grant CA 32995 from the National Cancer Institute (C.Y.T.) and Public Health Service Research Service Award AI 07957 from the National Institutes of Allergy and Infectious Diseases (A.C.M.). Some aspects of this work were supported by a research grant MV489 from the American Cancer Society.

We thank Cheryl Murphy for technical assistance and Lisa Morris for help in the preparation of the manuscript.

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