Origins of Enhancer Sequences of Recombinant Murine Leukemia Viruses from Spontaneous B- and T-Cell Lymphomas of CWD Mice

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Recombinant murine leukemia viruses from the highly leukemic mouse strains AKR, HRS, and C58 usually acquire pathogenic U3 region sequences from the endogenous xenotropic virus, *Bxv*-1. However, the majority of tumors from another highly leukemic strain, CWD, contained recombinant viruses that lacked *Bxv*-1-specific sequences. The nucleotide sequence of the U3 regions of two such CWD recombinants was nearly identical to that of the endogenous ecotropic virus parent *Emv*-1, but they shared three nucleotide substitutions immediately 3' of the enhancer core. These substitutions were found in recombinant proviruses from about one-third of spontaneous CWD lymphomas as determined by an oligonucleotide hybridization assay of proviral fragments that had been amplified by PCR. Analysis of amplified endogenous murine leukemia virus sequences suggested that the three nucleotide substitutions in the CWD viruses were inherited from an endogenous polytropic provirus that is absent in the other highly leukemic strains. On the basis of these and previous studies, we propose that CWD recombinants acquire pathogenic U3 region sequences through recombination with an endogenous polytropic virus or *Bxv*-1 and that the pathogenicity of these sequences may be related to a sequence motif that is known to bind members of the basic helix-loop-helix class of transcription factors.

The high incidence of spontaneous lymphomas in the inbred mouse strains AKR, HRS, C58, and CWD is related to the expression of endogenous ecotropic murine leukemia viruses (MuLVs) early in life. These viruses subsequently recombine with other endogenous viruses to generate pathogenic polytropic recombinant viruses. The genomes of these recombinants contain polytropic virus sequences in the envelope gene and nonectropic sequences in the U3 region (1, 7, 13, 17, 47, 48, 50, 51, 53, 54). The U3 region sequences contain the viral enhancer and promoter elements and are represented in the 5' portion of the long terminal repeats (LTRs) that flank the integrated provirus. In the AKR, HRS, and C58 recombinants, the U3 regions are usually inherited from the endogenous xenotropic provirus, *Bxv*-1 (17, 28, 39, 40, 47, 50, 53). The reproducible selection of the *Bxv*-1 sequences is probably related to the fact that the *Bxv*-1 U3 region sequences promote viral thymotropism and pathogenicity as compared with the allelic sequences found in the ecotropic virus parent or the other endogenous viruses (21–23, 57).

The contribution of the U3 regions to the leukemogenicity and target cell specificity of the exogenous Moloney, Friend, and SL3-3 MuLVs has been clearly established (2, 6, 16, 18, 24, 30, 31, 33, 43, 44). As first reported by Speck and Baltimore, the function of the viral enhancer element of the Moloney MuLV is dependent on sequences that are recognized by specific cellular transcription factors (42). Mutations of the binding sites for the transcription factors NF-1, ets-1 (or LfB), and the core-binding protein alter the function of the enhancer and viral pathogenicity (42–44). Similarly, substitution of the enhancer core sequence of the SL3-3 MuLV with those of an endogenous ecotropic virus influences enhancer function and impairs leukemogenesis (2, 32, 33, 37, 55). By analogy, the

pathogenic determinants within the *Bxv*-1 U3 region sequences are probably located within the viral enhancer, a proposition that is supported by the observation that the enhancer element is often duplicated in many recombinant viruses (21, 22, 47). However, it is not known which of the *Bxv*-1 sequences or transcription factors increase viral pathogenicity. A pathogenic sequence located in the U3 region of the AKR recombinant virus MCF13 had been reported to bind a transcription factor, but this determinant is absent in most recombinants that contain *Bxv*-1-related U3 region sequences (58).

Previous studies of the genomes of recombinant viruses from CWD mice suggested that the U3 region sequences were inherited from the endogenous ecotropic virus rather than *Bxv*-1 (48, 51, 54). Why these recombinants had not incorporated the *Bxv*-1 U3 region sequences was unclear since CWD mice carry and express *Bxv*-1 (35). To begin to address this issue, we determined the nucleotide sequence of the U3 regions of two CWD recombinants and characterized the enhancer core region sequences of recombinant viruses and endogenous MuLVs by hybridization of oligonucleotide probes to viral sequences that were amplified by PCR. The results provide new clues about the selection of pathogenic determinants within the U3 region of recombinant MuLVs.

MATERIALS AND METHODS

Mice. CWD mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and are now available under the designation CWD/Le. The CWD breeding stock (*cw d/+ d a/a* and *cw d/cw d a/a*) was maintained by brother-sister matings (*cw/cw* to *cw/+*) at the University of Virginia vivarium. Animals were sacrificed when severely ill, although some animals died spontaneously. Necropsies were performed within 24 h of death.

Phenotype analysis of CWD tumors. At necropsy, tissue samples were fixed in formaldehyde for histopathologic exam-

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ination or snap-frozen in a dry ice-methanol bath and stored at -70°C . Extraction of genomic DNA and Southern blotting of the tumor DNAs were performed as described previously (35). The immunophenotype of the tumors was based on histopathologic testing, the presence or absence of immunoglobulin and T-cell receptor gene rearrangements, and immunocytochemical analysis by techniques detailed in an earlier report (49). The probes for gene rearrangement studies included portions of the J regions of the immunoglobulin heavy chain (J_H) and kappa light chain (J_K) and the constant region of the murine T-cell receptor beta chain. Antibodies for the immunoperoxidase stains included rat anti-mouse Thy 1.2 monoclonal antibody (Becton-Dickinson, Mountain View, Calif.), rabbit anti-mouse immunoglobulin M polyclonal antibody (gift of P. Isakson, University of Virginia), and rat anti-mouse B220 monoclonal antibody (gift of I. Weissman, Stanford University).

Isolation of CWM-T-15 and CWM-S-101 proviruses. Methods of virus culture with NIH 3T3 mouse fibroblasts and CCL64 mink cells have been described previously (17, 50). The polytropic recombinant provirus CWM-T-15 was obtained from mink cell lines which had been cocultivated with tumor cells from a CWD mouse with a spontaneous T-cell lymphoma (48, 51). The CWM-S-101 recombinant virus was isolated by an identical procedure except that the cells were obtained from a spleen that was heavily infiltrated by a spontaneous B-cell lymphoma. The isolation of bacteriophage vectors that contained the 3' ends of the CWM-T-15 and CWM-S-101 proviruses was reported previously (51, 52). The nucleic acid probes used to identify the proviral sequences have been described previously (9, 35, 52).

Amplification of endogenous and recombinant proviruses by PCR. The oligonucleotide primers were selected to amplify segments of specific endogenous proviruses, recombinant proviruses, or both (see Fig. 3). The 5' primer POLYENV5 contained polytropic-specific envelope sequence found within the central portion of the gp70 gene 1,164 bp 5' of the start of the U3 region (or positions 798 to 817 from the mutated ATG start codon of the HRS/J endogenous polytropic provirus, MX27 [45]). The PXENV5 5' primer corresponded to sequences in the extreme 3' end of the p15E gene of polytropic and xenotropic viruses that begin 74 bp from the start of the U3 region. ECOLTR5 contained ecotropic virus-specific sequences located between positions 93 and 110 (see Fig. 2). The 3' primers, LTR3GEN, ECOLTR3, and XENOLTR3, corresponded to sequences found in the 3' portion of the U3 region. LTR3GEN was considered a "generic" primer and was complementary to the sequence between positions 310 and 329 that is common to each of the three major classes of endogenous MuLVs. ECOLTR3 and XENOLTR3 contained sequences complementary to those found between positions 220 and 241 and positions 233 and 256 of the ecotropic and xenotropic LTR sequences, respectively (see Fig. 2 and 3). The oligonucleotide primers were synthesized at the University of Virginia Nucleic Acid and Protein Sequence Center. The 5' primers, with the viral sequences underlined, were POLYENV5 (5'-GCGAAT TCTCTATAGTCCCTGAGACTG), PXENV5 (5'-CTCAAA TCAATAGATCCAGA), and ECOLTR5 (5'-AAACAAGA ACAAGGAAGT). The 3' primers were LTR3GEN (5'-TA CAGAAGCGAGAAGCGAGC), ECOLTR3 (5'-TTCCCGG GTCTCTTGAAACTGTTGTTG), and XENOLTR3 (5'-CT TGAAACTGAGGTGGGACTTTCCA). The 5' ends of the POLYENV5 and ECOLTR3 primers contained additional nucleotides that created an *EcoRI* and a *SmaI* restriction site, respectively.

Each 50- μl PCR mixture contained 500 ng of genomic DNA

or 2.5 ng of control plasmid DNA; 0.4 μM each primer; 200 μM each dATP, dCTP, dGTP, and dTTP; 50 mM KCl; 10 mM Tris (pH 8.3); 1.5 mM MgCl_2 ; 0.001% gelatin; and 2 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.). 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 PCRs were performed in a Perkin-Elmer Cetus Cycler for 30 cycles, each consisting of denaturation for 30 s at 94°C , annealing for 30 s at 50°C , and extension for 1 min at 72°C , with a final extension for 5 min.

Probes for DNA hybridizations. The JS21 oligonucleotide probe was a 27-mer beginning 47 bp 3' of the conserved *PstI* site in the MCF247 LTR and was a gift of Jonathan Stoye and John Coffin (28, 47) (see Fig. 2). It differs from the endogenous xenotropic *Bxv*-1 proviral sequence by one base substitution (A to G at position 82; see Fig. 2) (35, 47). The 5' end was labeled by incubation of the oligonucleotides with [γ - ^{32}P]dATP in the presence of T4 polynucleotide kinase (New England BioLabs) as previously described (46). The xeno *env* probe contained the 112-bp *Bst*NI-*Xma*I fragment of the NZB-IU-6 xenotropic virus envelope gene and was a gift of R. O'Neill (38). This single-stranded probe was generated from M13 bacteriophage DNA by primer extension as described previously (35).

The ecotropic-specific U3ECO probe is a 153-bp fragment generated by the asymmetric PCR technique with the ECOLTR5 and ECOLTR3 primers (see Fig. 2 and 3) (12). The p15EGEN probe was the 507-bp *Xba*I-*Pst*I *env*-LTR fragment isolated from a proviral clone of AKV 623. This probe includes envelope sequences that are conserved among MuLVs of different host ranges (19, 34).

The enhancer core region probes were 18-bp single-stranded oligonucleotides that were complementary to the core and the 3'-flanking sequences (see Fig. 5C). The five probes were ECO CORE, the endogenous ecotropic core region sequences; SL3-3 CORE, the SL3-3 virus core with a single C-to-T change within the enhancer core sequences; T15 CORE, the CWM-T-15 core region; XENO CORE, the *Bxv*-1 xenotropic core region; and POLY CORE, the endogenous polytropic core region. For hybridization assays, the 5' residues of the oligonucleotides were labeled with [γ - ^{32}P]dATP by using T4 polynucleotide kinase as described previously (46).

Southern blotting and hybridization. The procedures for isolation of genomic DNAs and Southern blot assays were presented previously (35). The protocol for analysis of the PCR products was modified to eliminate the acid treatment prior to denaturation with NaOH. Hybridization conditions with the xeno *env*, U3ECO, and p15EGEN probes have been reported in detail elsewhere (35, 38); the incubation temperature was 65°C , and the final wash of the blot was in $0.1\times$ SSC.

Hybridization conditions for the JS21 oligonucleotide probe have been described previously (35, 47); in these experiments the blot or gel wafer and the probe were incubated at 56°C and the final wash contained $1\times$ SSPE. The hybridization solutions for each of the oligonucleotide CORE probes were the same as for the JS21 probe, but the incubation temperature was 50°C with a final wash in $0.5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min.

DNA sequencing. Appropriate restriction fragments of the U3 regions of CWM-T-15 and CWM-S-101 proviruses were propagated in the pUC13 plasmid or M13 bacteriophage vector. The DNA sequence of the inserts was determined by the dideoxynucleotide termination method with Sequenase (United States Biochemical) and ^{35}S -labeled dATP (41). The U3 region sequences of CWM-T-15 located 3' of the *PstI* site have been reported earlier (51).

FIG. 1. Southern blot of CWD tumor DNAs to determine the origin of the U3 regions of recombinant proviruses. (A) The DNAs were digested with *Hpa*I and hybridized to the JS21 oligonucleotide probe (see Fig. 2). The arrows in lanes CW6 and CW19 denote acquired proviruses with the *Bxv*-1-derived U3 region sequence. (B) The same blot was rehybridized to a xenotropic-specific envelope (*xeno env*) probe. (C) The same tumor DNAs were digested with *Eco*RI and hybridized to a 153-bp ecotropic-specific U3 region probe (U3ECO). Lanes: CW5 to CW20, CWD tumor DNAs; CWD/E, CWD embryo DNA.

RESULTS

Analysis of U3 region sequences of acquired proviruses in CWD lymphomas. CWD tumors that had been previously shown to contain polytropic recombinant proviruses were digested with *Hpa*I or *Eco*RI and subjected to Southern blot analysis (48). To characterize the U3 region sequences of acquired proviruses, we hybridized the blots to probes for two different types of U3 region sequences. As shown in Fig. 1A, each of the DNAs contained 9.0- and 6.8-kb *Hpa*I fragments that hybridized JS21, an oligonucleotide probe that is specific for the *Bxv*-1-derived LTR of mink cell focus-forming viruses (47). These bands represented the 3' and 5' ends, respectively, of the endogenous *Bxv*-1 provirus (20, 35, 39). Of the 10 CWD tumors, 2 had acquired proviruses with *Bxv*-1-related U3 region sequences (Fig. 1A, lanes CW6 and CW19). The faint band above the endogenous bands in CW13 was not seen

reproducibly with this sample, and no new bands were seen on close inspection of CW20. The acquired bands in CW6 and CW19 did not represent intact *Bxv*-1 proviruses, because only the endogenous xenotropic viruses were detected when the tumor DNAs were hybridized to a xenotropic envelope gene probe (Fig. 1B). This implied that CW6 and CW19 tumor DNAs contained recombinant proviruses that had incorporated the U3 region sequences of *Bxv*-1.

Southern blots of the *Eco*RI-cleaved DNAs from control tissues and from CWD tumors were hybridized to a probe for ecotropic virus U3 region sequences (U3ECO; see Materials and Methods). As seen in Fig. 1C, the normal control DNA (CWD/E) generated two high-molecular-weight bands that contained the 5' and 3' LTRs of the two endogenous ecotropic proviruses found in CWD mice, *Emv*-1 and *Emv*-3 (25, 26, 48, 54). New proviruses with ecotropic virus-related U3 region sequences were seen in seven of the tumor DNAs but not in those that had acquired proviruses with xenotropic U3 region sequences (CW6 and CW19). The bands of greater than 9 kb may have represented acquired ecotropic or polytropic recombinant proviruses. However, the smaller bands were most likely to be fragments that carried the 5' or 3' end of recombinant proviruses. These proviruses contain an *Eco*RI site within *env*, whereas the ecotropic proviruses do not and therefore must be found on fragments larger than 8.8 kb (8). However, the studies did not exclude the possibility that some of the smaller bands represented ecotropic proviruses with large internal deletions or viruses that had acquired an anomalous *Eco*RI restriction site.

Sequence analysis of the U3 regions of CWD recombinants. We next determined the nucleotide sequences of the U3 regions of two CWD recombinant viruses that lacked markers of *Bxv*-1 (48). CWM-S-101 was recovered from a spontaneous splenic B-cell lymphoma, and CWM-T-15 was originally isolated from a spontaneous T-cell lymphoma. Molecular clones of both proviruses were obtained from a bacteriophage lambda library of *Eco*RI DNA fragments from infected mink cell lines (48, 51, 52, 54). The sequence of the CWM-T-15 U3 region 3' of the *Pst*I site was included in a previous report (51).

Figure 2 compares the U3 region sequences of CWM-T-15 and CWM-S-101 with those of select endogenous ecotropic and polytropic viruses and with xenotropic *Bxv*-1 U3 sequences found in the AKR recombinant mink cell focus-forming virus MCF247 (19, 25, 26, 28, 29, 45, 51). The U3 regions of both CWM-T-15 and CWM-S-101 were apparently derived from the endogenous ecotropic parent viruses *Emv*-1 and/or *Emv*-3, given the high degree of homology throughout the U3 region and the fact that they contain the same unique substitution at position 51. At positions 182 and 188, both CWM-S-101 and CWM-T-15 share the same bases with *Emv*-1 but not with *Emv*-3, which suggests that they were derived from *Emv*-1, at least within this portion of the U3 region. However, the CWM-T-15 and CWM-S-101 sequences differed from that of *Emv*-1 at five and seven nucleotides, respectively. Three of the four shared substitutions, as compared with *Emv*-1, were clustered immediately 3' of the enhancer core (positions 165, 169, and 170). One of these, position 165, was unique to the two CWD recombinant viruses. The other two substitutions, positions 169 and 170, were identical to those found in the allelic region of the noncancerous endogenous viruses including *Bxv*-1. The same was true for three of the other substitutions (position 29 in CWM-T-15 and positions 14 and 181 in CWM-S-101). Taken together, the sequence studies suggested that the three substitutions in the enhancer core region were not random and that the U3 regions of two CWD viruses were

FIG. 2. Nucleotide sequence of the U3 regions of endogenous and recombinant MuLVs. AKV623 is an ecotropic AKR MuLV clone (19, 34); *Emv*-1 and *Emv*-3 are endogenous ecotropic viruses present in CWD mice (25, 26, 48); CWM-T-15 and CWM-S-101 are the CWD recombinant viruses; MCF247 is an AKR recombinant virus that contains U3 region sequences derived from *Bxv*-1 (28, 35, 47); MX27 and MX33 are endogenous polytropic and modified polytropic viruses, respectively (45). The direct repeats of the enhancer sequences found in AKV623 and MCF247 are not shown (the repeated segment in AKV623 is flanked by two asterisks). The arrows indicate the location of insertion sites or repeats present in MX27 and MX33; the sequence of the 190-bp insert is not shown. The underlined sequences are those that are recognized by the indicated probes, primers, or transcription factors. The transcription factor-binding sites are labeled at the top of each set of sequences: CTF/NF-1, CCAAT-binding transcription factor or nuclear factor 1 (27, 42); CORE, enhancer core-binding proteins (42, 56); Lvb/ets, Moloney MuLV binding site known to bind Lvb and/or ets-1 protein (42); GRE, glucocorticoid response element (3, 4, 42). The inverted repeat (IR), CCAAT box, and TATA box are also noted. JS21 is a 27-bp oligonucleotide derived from MCF247. ECOLTR3, ECOLTR5, XENOLTR3, LTR3GEN, and T15CORE indicate sequences recognized by the oligonucleotide probes described in Materials and Methods.

created by a recombination between the U3 region sequences of *Emv*-1 and an endogenous nonecotropic provirus.

A comparison of the adjacent 3' *env* sequences of CWM-S-101 with those of the endogenous ecotropic viruses suggests that the substitution by the polytropic *env*-U3 sequences extends to at least position 15 within the U3 region (52). In contrast, the 5'-most site for the crossover from polytropic to ecotropic virus sequences in the genome of CWM-T-15 is

located 14 bases before the beginning of the U3 region (reference 52 and data not shown). Because of the high degree of homology between the ecotropic and polytropic virus sequences in this region, however, the 3' end of the polytropic virus sequences could not be determined with certainty.

Analysis of the U3 region of endogenous and recombinant proviruses amplified by PCR. As a first step to determining the origin of the enhancer core regions of other CWD recomb-

FIG. 3. Predicted products of proviral sequences amplified by PCR. The relative location of each set of primer pairs is shown, and the degree of shading indicates the specificity of the primers for polytropic (■), ecotropic (▨), or xenotropic (▩) virus sequences (□, amplifies all of the above). The predicted lengths are based on the assumption that there are no acquired insertions or duplications within the U3 region. The composition of the LTR3GEN, ECOLTR3, and XENOLTR3 primers within the U3 region is shown in Fig. 2. POLYENV5 and PXENV5 contain noncancerous gp70 and p15E envelope gene sequences, respectively (see Materials and Methods). For ease of illustration, the relative size of the LTRs is exaggerated.

nant viruses, we developed a PCR technique to amplify the 3' ends of the different types of MuLV proviruses. As illustrated in Fig. 3, the 3' portions of certain proviruses or groups of proviruses were amplified with different pairs of specific or nonspecific oligonucleotide primers. The size of the predicted PCR fragments would depend on the primers used and the length of the U3 region. Variation in the size of the U3 region sequences results from deletions or insertions in the endogenous sequences, such as the 190-bp insert found in the endogenous polytropic proviruses, or from amplification or rearrangements of the enhancer sequences that are acquired during virus replication (10, 45). Because of the high degree of homology between the *env* and U3 region sequences of the different endogenous and acquired proviruses, the primers and conditions of the reaction must be chosen carefully to maintain the desired specificity. As described in Materials and Methods and below, the optimal conditions for the assays were determined empirically and confirmed in a series of control experiments.

The POLYENV5 and LTR3GEN primers were selected to amplify both the endogenous polytropic and the acquired recombinant polytropic proviruses. As shown in Fig. 4A, these primers directed the synthesis of the predicted 1.5-kb *env*-U3 fragments from the CWM-T-15 provirus and a 1.7-kb PCR product from the endogenous polytropic provirus MX27, which contained the 190-bp insert in U3 (45). The Southern blot in Fig. 4B shows that these amplified proviral fragments hybridized to an internal envelope gene probe. However, as shown in Fig. 4C, the two PCR products could be distinguished from one another by rehybridization of the blot to an oligonucleotide probe that recognizes the CWM-T-15 core region sequences (T15 CORE). The absence of products in PCRs that contained DNAs of the endogenous ecotropic virus AKV623 or the endogenous xenotropic virus S5X (*Bxv*-1) confirmed the specificity of the POLYENV5 primer for polytropic *env* sequences. However, both AKV623 and S5X were suitable substrates in PCRs that contained appropriate primers (ECOLTR5 + ECOLTR3 or PXENV5 + LTR3GEN, respectively).

Another concern in the PCR experiments was that the reaction itself may generate recombinant DNA molecules. This results when the products from different templates hybridize to one another and become substrates for amplification by the *Taq*I polymerase. Such artifactual templates could be formed during the amplification of MuLV sequences since portions of *env* and the U3 regions of the different types of endogenous proviruses are highly homologous. If these templates are amplified during PCR, the products would be difficult to distinguish from those that represented the 3' ends of integrated proviruses. To test for such an artifact, we used the POLYENV5 and LTR3GEN primers to amplify the 3' end of the MX27 endogenous polytropic virus in the presence of the ecotropic virus AKV623. Although the AKV623 *env*-U3 region fragment should not be amplified, since this virus lacks a binding site for the POLYENV5 primer, the LTR3GEN oligonucleotide should anneal to the U3 region sequences of AKV623 and lead to the synthesis of single-stranded molecules that could anneal to the products of the MX27 provirus. Some of these hybrid molecules would, in theory, contain the MX27 *env* and AKV623 U3 region sequences and would result in the synthesis of a 1.6-kb PCR product. However, as shown in lane MX27 + AKV623 (coPCR) of Fig. 4A, the reaction produced only the predicted 1.7-kb *env*-U3 product of the MX27 provirus. In another control experiment, we amplified the 3' ends of the MX27 and CWM-T-15 proviruses in the same PCR [lane MX27 + T15 (coPCR)]. Despite a high degree of sequence homology between these two proviruses, we found no evidence that the enhancer core region sequences of CWM-T-15 were transferred to the larger MX27 proviral fragments by PCR-mediated recombination (Fig. 4B). These experiments verified that under the conditions described here, the oligonucleotide primers exhibited the desired degree of sequence specificity and that the PCRs themselves did not generate detectable amounts of recombinant molecules that could be confused with the products generated from endogenous or recombinant proviruses.

Analysis of enhancer core region sequences in recombinant proviruses from CWD and HRS/J tumors. The 3' ends of

FIG. 4. Specificity of the PCRs. The primers for each PCR are listed above the horizontal bar or in parentheses. (A) Ethidium bromide stain of products from control PCR with plasmid DNAs. The arrow represents a nonspecific PCR product. (B) Southern blot of PCR products hybridized to a MuLV nonspecific envelope gene probe, p15EGEN. (C) Hybridization of the same blot to the T15 CORE U3 region probe (Fig. 2 and 5C). Lanes: AKV623, ecotropic AKR MuLV clone; S5X, viral clone of the endogenous xenotropic provirus *Bxv-1*; MX27, endogenous polytropic provirus; T15, CWM-T-15 tumor recombinant provirus; MX27 + T15 (coPCR), PCR mixture contained both proviruses; MX27 + T15 (mix), the proviral DNAs were amplified separately and the PCR products were mixed prior to loading in the agarose gel; MX27 + AKV623 (coPCR), PCR mixture contained both proviruses.

recombinant and endogenous polytropic proviruses from 37 spontaneous CWD tumor DNAs and 10 spontaneous HRS/J tumors were amplified in PCRs that contained the POLY ENV5 and LTR3GEN primers. The products were characterized by Southern blots that were sequentially hybridized to different oligonucleotide probes. As shown in Fig. 2 and 5C, these 18-mer probes corresponded to the enhancer core regions of the endogenous ecotropic, xenotropic, or polytropic proviruses or those found in the CWM-T-15 and SL3-3 MuLVs (see Materials and Methods). Under the conditions used in these experiments, these probes could distinguish one enhancer core region sequence from another, although the ECO CORE and SL3-3 CORE probes, which differed by a single nucleotide, occasionally exhibited a slight degree of cross-hybridization.

Hybridization of the amplified proviral DNAs to the probe for the core region sequences of the endogenous polytropic proviruses detected a 1.7-kb band in each of the PCRs (Fig. 5A, panel P). This confirmed that the PCR products contained the amplified fragments from the endogenous polytropic proviruses. There was no evidence of a smaller (1.5-kb) band, which would have been expected if an endogenous or recom-

FIG. 5. Southern blot analysis of recombinant and endogenous polytropic proviruses from CWD and HRS/J tumor DNAs that were amplified by PCR and hybridized sequentially to oligonucleotide probes. The probes are listed to the left of each autoradiograph by the single-letter abbreviations as shown in panel C. (A) CWD tumors. Lanes: 1, CW5; 2, CW6; 3, CW7; 4, CW9; 5, CW15; 6, CW19; 7, CW199; 8, CW202; 9, CW210; 10, CW211; 11, CW222; 12, CW229; 13, CW235; 14, CW189; 15, CW201; 16, CW14; 17, CW20; 18, CW101; 19, CW206; 20, CW209; 21, CW212; 22, CW216; 23, CW218; 24, CW223; 25, CW228; 26, CW230; 27, CW233; 28, CW234; 29, CW236; 30, CW237; 31, CW238; 32, CW239; 33, CW240; 34, CW241; 35, CW243; 36, CW194; 37, CW197. Immunophenotype of tumors: T cell, lanes 1 to 13 and 36; pre-B cell, lanes 14 and 15; B cell, lanes 16 to 35. (B) Lanes: 1 to 10, HRS/J tumor DNAs (all T-cell lymphomas); N, DNA from normal HRS/J liver; E, S, X, and T, products from PCR controls that contain different U3 region sequences (E, ecotropic U3 region sequence from AKV623; S, SL3-3 core region amplified from PTV-1 provirus; X, xenotropic U3 region sequence from *Bxv-1*; T, T15 U3 region sequence from CWM-T-15 provirus). (C) Nucleotide sequences that are recognized by the different oligonucleotide probes (complementary to the sequence shown): E, ecotropic core region (ECO CORE); S, SL3-3 core region (SL3-3 CORE); T, CWM-T-15 core region (T15 CORE); X, *Bxv-1* core region (XENO CORE); P, polytropic core region (POLY CORE). Boxed sequences represent known or potential protein-binding sites.

binant proviruses contained the polytropic core region sequences in the absence of the 190-bp insertion in the U3 region (a nonspecific partial degradation of the DNAs was responsible for the signal from the lower-molecular-weight DNAs in lanes 11 and 12). As described below, all but nine of the PCR products hybridized to one of the other oligonucleotide

probes. The nine that did not may have represented tumors that lacked recombinant proviruses. Alternatively, the *env*-U3 portions of the recombinant proviruses in these tumors may have failed to amplify, were indistinguishable from those of the endogenous polytropic proviruses, or contained enhancer core region sequences that were not represented in the probes used in these experiments.

When the blot was rehybridized to the CWM-T-15 core region probe (Fig. 5A, panel T), a slightly smaller PCR product was detected in 12 of the 37 tumors. This included the product from the tumor that produced the CWM-T-15 virus (lane 5). The bands most probably represented the 3' fragments of recombinant polytropic recombinants with CWM-T-15 enhancer core region sequences. Although not readily seen in Fig. 5A, each lane contained a faint high-molecular-weight band that comigrated with the fragments that were derived from the endogenous proviruses. This suggested that one or more of the endogenous polytropic proviruses contained a core region sequence that was similar or identical to that found in CWM-T-15 (see below). Nine of the PCR products from the CWD tumors hybridized to the probe for the *Bxv*-1 enhancer core region sequences (Fig. 5A, panels X). Prominent bands were seen in lanes 2 and 6, which represented the products from tumors CW6 and CW19, respectively. This agreed with the Southern blot assays (Fig. 1) that had detected proviruses with xenotropic U3 region sequences in these tumors. Of interest, the size of the fragments that hybridized to the *Bxv*-1 and CWM-T-15 core region probes was that expected for proviruses with a single enhancer element. This finding contrasts with those of studies of AKR recombinants, which revealed frequent duplications of the acquired *Bxv*-1 enhancer sequences (22, 47).

The products from three of the tumors hybridized to the probe for the core region sequences of the highly leukemogenic SL3-3 and Gross passage A MuLVs. The enhancer core of these viruses differs by only a single base from that found in the endogenous ecotropic viruses such as *Emv*-1 (10, 25, 30). Presumably the SL3-3 MuLV-specific substitution in the enhancer core resulted from a spontaneous mutation, since endogenous proviruses are not known to contain this substitution. The PCR products from 13 of the tumors hybridized to the probe for the ecotropic core region sequences; in 2 the signal was faint, and in 6 the DNAs also hybridized to one of the other oligonucleotides. Overall, 9 of the 37 tumor DNAs hybridized to more than one nonpolytropic core sequence. This included the products from tumor CW101 (lane 18), which had produced the CWM-S-101 virus. Presumably, this pattern of hybridization was indicative of a mixture of recombinant proviruses that contained distinct enhancer core region sequences. The different proviruses may have been present in the DNA of the tumor cells or perhaps in nonneoplastic cells that contaminated the tumor tissues. Other possible explanations include DNA contamination of the PCRs or, less likely, the amplification of an uncharacterized enhancer sequence that was capable of hybridization to more than one of the oligonucleotide probes.

In contrast to the results seen with the CWD tumors, Southern blots of the PCR products from the DNA of 10 spontaneous HRS/J tumors (kindly supplied by Neal Copeland and Nancy Jenkins) failed to demonstrate recombinant proviruses with CWM-T-15 enhancer core region sequences (Fig. 5B). However, 7 of the 10 PCR products hybridized to the *Bxv*-1 enhancer core region probe. These results are consistent with earlier studies that have shown that most HRS/J recombinant viruses acquire *Bxv*-1 U3 sequences (17, 53). Importantly, the HRS/J experiments support the validity of the PCR

TABLE 1. Results of hybridization of enhancer-specific oligonucleotides to PCR products from CWD and HRS/J tumors

Oligonucleotide probe	Specific enhancer	No. of CWD tumors		No. of HRS/J tumors (all T cell)
		B cell	T cell	
P	Polytropic	All (14)	All (23)	All (10)
T	T15	4	8	0
X	Xenotropic (<i>Bxv</i> -1)	4	5	7
S	SL3-3	3	0	0
E	Ecotropic (<i>Emv</i> -1)	11	2	3
	Polytropic only	7	2	2
	More than one nonpolytropic detected	6	3	2

blot assay for determining the origin of the U3 region sequences of recombinant proviruses and the proposition that recombinant MuLVs from highly leukemic strains other than CWD do not contain CWM-T-15 enhancer core sequences.

The results of the Southern blot assays of the PCR products from both the HRS/J and CWD tumors are summarized in Table 1. Recombinant proviruses were detected in 28 of the 37 CWD tumors and 8 of the 10 HRS/J tumors. Of the 28 CWD recombinants and 8 HRS/J recombinants, 23 and 7, respectively, contained viruses with noncotropic enhancer core region sequences. Taken together, these observations support the hypothesis that recombinants with certain noncotropic enhancer sequences enjoy a selective advantage in vivo compared with viruses that retain the allelic sequences of the endogenous ecotropic parent.

Comparison of the enhancer core region sequences of the recombinant MuLVs with the tumor immunophenotype. The enhancer elements of the exogenous Moloney and Friend MuLVs are known to influence target cell specificity (16, 31, 43, 44). In addition, T-cell tropism has been associated with the *Bxv*-1 U3 region sequences that are found in AKR recombinant MuLVs and with the enhancer core region sequences of the SL3-3 MuLV (2, 21, 22, 33, 57). Therefore, it was of interest to determine if there was a correlation between enhancer core region sequences in the CWD recombinant viruses and the immunophenotype of the lymphoma. As shown in Table 1, recombinant viruses with the enhancer core region sequences of CWM-T-15, *Bxv*-1, or *Emv*-1 were detected in both B- and T-cell CWD lymphomas. A somewhat higher proportion of T-cell tumors was seen in animals with recombinant viruses that contained the CWM-T-15 enhancer sequences, but this association was not of statistical significance. This indicated that none of these enhancer core region sequences alone conferred T- or B-cell tropism. The three CWD tumors that contained viruses with enhancer sequences that hybridized only to the SL3-3 core probe were B-cell lymphomas. Because of the small number of tumors, we could not conclude that CWD recombinants with SL3-3 core region sequences are exclusively associated with B-cell tumors; nonetheless, the results clearly indicated that the core sequence alone did not confer T-cell tropism to these CWD viruses.

Evidence that the CWM-T-15 core region sequences are derived from an endogenous polytropic provirus. We next asked if the endogenous polytropic proviruses of CWD mice had the potential to donate the three 1-bp substitutions that together are unique to the CWM-T-15 enhancer region. In the initial experiment, the POLYENV5 and LTR3GEN oligonucleotide primers and PCR were used to amplify the endoge-

nous polytropic proviruses from normal tissues of CWD, HRS, and a (CWD $\times$ HRS) F_1 mouse. As a positive control, the endogenous and recombinant proviruses in CWD tumor CW-15 were amplified by PCR under the same conditions. The products were analyzed by Southern blot hybridization to the T15 CORE oligonucleotide (probe for the CWM-T-15 core region sequence). As expected, the products from the CW-15 tumor DNA generated an intense 1.5-kb band that corresponded to the 3' end of the integrated CWM-T-15 provirus. Another faint high-molecular-weight band may have represented a population of recombinant proviruses with an expanded U3 region. A third band of about 1.7 kb was also seen in the PCR products from CWD (CW40D) and (CWD $\times$ HRS) F_1 (CH37L) mice but not in those from the HRS/J (HR51L) animal. Since this band comigrates with the products of the endogenous polytropic proviruses, the data suggest that one or more of these viruses contain sequences that are identical or closely related to those of the CWM-T-15 core region. To determine if other endogenous virus elements contain the CWM-T-15 core region sequences, different sets of real and hypothetical "hybrid" endogenous proviruses were amplified by PCR, blotted to nylon membranes, and hybridized to the T15 CORE probe. The primer pairs for the reactions are listed above the bars in Fig. 6, and the predicted products are shown schematically in Fig. 3. However, either the germ line DNAs did not generate a product or the products failed to hybridize to the T15 CORE probe. Thus, the latter PCR assays failed to detect CWM-T-15 core sequences in the endogenous xenotropic proviruses, ecotropic virus-related LTRs, or hypothetical hybrid proviruses with noncotropic *env* and ecotropic virus-related 3' U3 region sequences.

In the next experiment, the 3' ends of endogenous polytropic proviruses from 12 additional inbred strains were amplified by PCR. As shown in Fig. 7, the Southern blot assay of the PCR products revealed that the proviral fragments from each strain hybridized to the probe for the endogenous core region sequences, POLY CORE, as expected. The blot was rehybridized to the CWM-T-15 core region probe, and bands were seen with the PCR products from strains CWD, C57BL/6J, C57L/J, C3H/HeJ, DBA/2J, and 129/J but not those from the other strains. This pattern of hybridization was compared with the presence or absence of specific endogenous polytropic viruses in each of the strains as reported by Frankel et al. (15). This comparison suggested that the CWM-T-15 core region sequences are found on more than one endogenous polytropic provirus since a single locus would not have produced the observed results. In the CWD mice, the candidates include the endogenous polytropic proviruses *Pmv*-22 and *Pmv*-40 and an unnumbered provirus that is unique to this strain (14a, 15).

DISCUSSION

The results of these studies suggest that recombinant MuLVs from CWD mice can acquire pathogenic U3 region sequences by recombination with one of at least two endogenous noncotropic viruses. As shown here, recombinant MuLVs from about one-fourth of the spontaneous CWD lymphomas had inherited the U3 region sequences from the endogenous xenotropic virus *Bxv*-1. This locus is also the donor of the U3 region sequences found in the majority of recombinants from the other highly leukemic mouse strains, HRS/J, AKR, and C58 (17, 28, 39, 40, 47, 50, 53). In about one-third of the CWD tumors, the recombinants had acquired a distinct enhancer core region sequence that was first noted in the U3 regions of two spontaneous CWD recombinants, CWM-T-15 and CWM-S-101. This sequence, referred to as the CWM-T-15

FIG. 6. Analysis of PCR products from normal HRS, CWD, and (HRS $\times$ CWD) F_1 DNAs and CW-15 tumor DNA. The primers for each reaction are listed above the horizontal bar. (Upper panel) DNA products separated in an agarose gel and stained with ethidium bromide. (Lower panel) Autoradiograph of Southern blot of the DNA products after hybridization of the T15 CORE probe. Lanes: CW40D, normal CWD embryo DNA; HR51L, normal HRS liver DNA; CH37L, (CWD $\times$ HRS) F_1 liver DNA; CW15, tumor DNA which contains the CWM-T-15 provirus. Control DNAs are shown in the lower right corner of the figure: MX27, polytropic core sequence; S5X, xenotropic core sequence; PTV-1, HRS/J recombinant virus with SL3-3 MuLV core sequence; T15, T15 enhancer core sequence; AKV623, endogenous ecotropic MuLV core sequence.

enhancer core region, differed from that of *Bxv*-1 by only a single nucleotide. A few of the tumor-associated CWD recombinants may have acquired a pathogenic sequence in the enhancer core as a result of spontaneous mutation rather than recombination.

Several observations suggested that enhancer core region sequences of CWM-T-15 were derived from a CWD endogenous polytropic provirus. For instance, this sequence was detected in two recombinants that were isolated independently and in a significant proportion of recombinant MuLVs that were associated with CWD lymphomas. This argues against the possibility that the noncotropic substitutions in the CWM-T-15 core region sequence arose through the accumulation of spontaneous mutations. The nucleotide sequences of the U3 regions of the two CWD recombinants were nearly identical to that of the endogenous ecotropic virus parent *Emv*-1, but the two recombinants contained a total of seven noncotropic base substitutions. Five of the substitutions were shared with the endogenous *Bxv*-1 and polytropic and modified polytropic viruses. Thus, the substitutions in the CWM-T-15 enhancer sequences appear to be derived from a donor that is closely

FIG. 7. Southern blot of amplified *env*-U3 fragments of the endogenous polytropic proviruses of selected inbred mouse strains. The same blot was exposed to oligonucleotide probes for endogenous polytropic enhancer core region or the T15 enhancer core region.

related but not identical to the known nonectropic endogenous proviruses. The donor could be distinguished from the other viruses by the unique adenosine substitution at position 165 (Fig. 2). This putative donor(s) was detected in the endogenous polytropic proviruses of CWD and several other inbred mouse strains by oligonucleotide hybridization studies of proviral fragments that had been amplified by PCR. Potential donors were not seen in the DNAs from the other highly leukemic strains, which may explain why these animals are not known to produce recombinant MuLVs with the CWM-T-15-related enhancer sequences. Certainly, confirmation that these sequences are of endogenous origin will require molecular cloning and DNA sequence analysis of the relevant CWD provirus. As discussed previously, the PCR assays suggested that the best candidates are the endogenous polytropic proviruses *Pmv-22*, *Pmv-40*, or an uncharacterized provirus unique to the CWD strain (14a, 15).

The genomes of the recombinant viruses probably incorporate the CWM-T-15 enhancer core or the *Bxv-1* U3 region sequences through a similar process. However, the U3 regions of the two CWD viruses with the CWM-T-15 core region sequences contained only a limited number of substitutions by the nonectropic sequences. Since these substitutions were clustered just 3' of the enhancer core, these U3 regions were probably generated by substitution of a small segment of nonectropic sequences into the enhancer of the *Emv-1* U3 region. In contrast, recombinant MuLVs appear to incorporate the *Bxv-1* U3 region sequences en bloc such that most or all of the U3 region is derived from this nonectropic virus (21, 23, 35, 47). The different patterns of substitution are probably unrelated to differences in the recombination process itself. Perhaps a more plausible explanation is that, unlike *Bxv-1*, the donor of the CWM-T-15 enhancer core region sequences contains defective or inhibitory U3 region sequences that flank the boundaries of the enhancer core region. In support of this hypothesis are reports from other investigators that the endogenous polytropic proviruses (which appear to be closely related to the donor of the CWM-T-15 enhancer sequences) contain substitutions, deletions, insertions, and negative regulatory elements in the U3 region that are absent in the allelic sequences of replication-competent MuLVs (5, 14, 42, 43, 45). Thus, viruses that acquire a large segment of the U3 region of the CWM-T-15 donor and the associated defective sequences may be subject to a negative selection. Conversely, this would result in a positive selection for viruses, such as CWM-T-15 and CWM-S-101, that retain the nondefective ecotropic virus U3 region sequences that are located outside the enhancer core region. Knowledge of the nucleotide sequence and the

functional characteristics of the U3 region of the endogenous nonectropic donor would help to confirm or refute this hypothesis.

Our studies failed to demonstrate a correlation between the immunophenotype of a specific CWD tumor and the enhancer core region sequence of the recombinant viruses found in the tumor DNA. This was somewhat unexpected since the *Bxv-1* U3 region and the enhancer core of the SL3-3 MuLV have been reported to contribute to the T-cell tropism of these viruses (2, 22, 23, 32, 33, 55, 57). The few CWD tumors that contained viruses with the SL3-3 MuLV-related core region sequences were of B-cell rather than T-cell origin. Thus, in the context of CWD recombinant viruses, the *Bxv-1* and SL3-3 MuLV core region sequences alone did not confer T-cell specificity. It is likely that the T-cell tropism of the SL3-3 MuLV requires sequences that are located outside the enhancer core, since this virus readily induces T-cell lymphomas when injected into CWD mice (54). However, we cannot exclude the possibility that host factors expressed by CWD mice influenced the immunophenotype of the lymphomas and obscured a modest influence of the enhancer core region sequences on viral tropism.

On the basis of previous studies and the work presented here, we propose that CWD recombinants acquire certain nonectropic sequences in the enhancer that augment viral pathogenicity. These include the CWM-T-15 core region sequences, the U3 region of *Bxv-1*, and perhaps specific substitutions within the enhancer core. Since the U3 region of the CWD recombinant CWM-T-15 contains only five substitutions relative to the endogenous ecotropic virus parent, the number of sites that may constitute a pathogenic determinant(s) is limited. The best candidates would be the substitutions that are clustered near the enhancer core, since these have the potential to influence viral pathogenicity by their effects on enhancer function (2, 32, 36, 43, 44). In this regard, two of the three substitutions in the enhancer are identical to those seen in the pathogenic *Bxv-1* U3 region, although the latter contains many other nonhomologous substitutions. The two shared substitutions do not alter the adjacent binding sites for the core-binding protein and NF-1 transcription factors or other known protein recognition sites (42, 43). However, the substitutions create a sequence motif known as an E box (CACCTGG [fig. 5C]) that is recognized by members of the basic helix-loop-helix family of transcription factors and appears to influence the function of the immunoglobulin heavy-chain gene enhancer (11). Thus, it is tempting to speculate that basic helix-loop-helix transcription factors such as E47 and E12 that are expressed in T-cell or B-cell precursors bind to the CWM-T-15 and *Bxv-1* enhancers to promote viral replication or transformation of the target cells. Experiments to test this hypothesis are currently in progress.

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