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Origin of Pathogenic Determinants of Recombinant Murine Leukemia Viruses: Analysis of *Bxv-1*-Related Xenotropic Viruses from CWD Mice

ANN C. MASSEY,* MICHAEL A. COPPOLA,† AND CHRISTOPHER Y. THOMAS

Departments of Medicine and Microbiology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

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The acquisition of U3 region sequences derived from the endogenous xenotropic provirus *Bxv-1* appears to be an important step in the generation of leukemogenic recombinant viruses in AKR, HRS, C58, and some CWD mice. We report here that each of three CWD lymphomas produced infectious xenotropic murine leukemia virus related to *Bxv-1*. In Southern blot experiments, these proviruses hybridized to probes that were specific for the xenotropic envelope and *Bxv-1* U3 region sequences. Nucleotide sequence analysis of a cloned CWD xenotropic provirus, CWM-S-5X, revealed that the envelope gene was closely related to but distinct from those of other known xenotropic viruses. In addition, the U3 region of CWM-S-5X contained a viral enhancer sequence that was identical to that found in MCF 247, a recombinant AKR virus that is thought to contain the *Bxv-1* enhancer. Finally, restriction enzyme sites in the CWM-S-5X provirus were analogous to those reported within *Bxv-1*. These results establish that the virus progeny of *Bxv-1* have the potential to donate pathogenic enhancer sequences to recombinant polytropic murine leukemia viruses. Interestingly, the three CWD polytropic viruses that were isolated from the same tumor cells that produced the *Bxv-1*-like viruses had not incorporated *Bxv-1* sequences into the U3 region.

The endogenous murine leukemia viruses (MuLVs) found in inbred mouse strains have been classified into families by the type of envelope proteins they encode. These families include the ecotropic, xenotropic, and polytropic viruses (6, 8, 24, 41). In mouse strains with a high incidence of lymphomas, such as AKR, HRS, C58, and CWD, ecotropic viruses are expressed early in life and recombine with the endogenous polytropic and xenotropic sequences to form oncogenic recombinant MuLVs (1, 4, 5, 7, 10, 11, 14, 38, 43, 44, 46–49). The envelope genes are inherited from the endogenous polytropic viruses, but most AKR, HRS, and C58 recombinants inherit U3 region sequences from an endogenous xenotropic provirus (11, 26, 37, 38, 46, 48). Based upon studies by Quint, Hoggan, and co-workers, this provirus is most likely *Bxv-1* (18, 37). The *Bxv-1*-related U3 sequences have been shown to encode functions that contribute to the leukemogenicity of AKR recombinants and facilitate the expression of virus sequences in the target thymocytes (19–21, 51).

Bxv-1 is found on chromosome 1 of most inbred mouse strains, and in at least five strains, it is the only infectious xenotropic provirus (18, 27, 28). This locus can be induced to express an infectious xenotropic MuLV by exposure of mouse fibroblasts to iododeoxyuridine or spleen cells to lipopolysaccharide (12, 18, 29). The other inducible xenotropic viruses that have been described include two independently segregating loci found in NZB mice. One NZB xenotropic virus, NZB-IU-6, has been cloned and sequenced (18, 27, 34). Other xenotropic viruses such as AKR-6, AKR-40, and NFS-1 have been isolated from mouse tumor cells, but it is not clear whether or not they represent the progeny of specific endogenous proviruses (5, 27).

* Corresponding author.
† Present address: Department of Microbiology, Dartmouth Medical School, Hanover, NH 03756.

CWD is a recently described inbred strain that produces recombinant MuLVs and develops nonthymic lymphomas of B- or T-cell origin (1, 44, 45, 47, 49). In the studies described here, we found that three spontaneous CWD lymphomas produced infectious xenotropic viruses. The proviruses hybridized to xenotropic-specific envelope sequences and a *Bxv-1*-specific U3 region probe and shared most restriction sites found in the *Bxv-1* provirus. By DNA sequence analysis, the envelope region of the CWD xenotropic virus was closely related to but distinct from that of the xenotropic virus NZB-IU-6. We also found that the nucleotide sequence of the U3 region of the CWD xenotropic virus was nearly identical to that of the AKR recombinant virus MCF 247, which is believed to be derived from *Bxv-1*. Taken together, these results indicated that the CWD xenotropic viruses had the characteristics expected of a virus progeny of *Bxv-1* and confirmed that this locus is the origin of the U3 region sequences found in many leukemogenic recombinant MuLVs. Although CWD mice carry and express *Bxv-1*, only a minor proportion of the CWD recombinant viruses contain *Bxv-1* markers in the U3 region.

MATERIALS AND METHODS

Mice. CWD mice were obtained from Jackson Laboratories, Bar Harbor, Maine. 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. These mice are available under the designation CWD/Le. Animals were sacrificed and autopsied when ill. Those that died spontaneously were refrigerated and autopsied within 24 h of death.

Viruses. Methods of virus culture using NIH 3T3 mouse fibroblasts and CCL64 mink cells have been described previously (11, 46). The polytropic recombinant proviruses CWM-S-5 and CWM-T-15 were obtained from mink cell lines which had been directly cocultivated with tumor cells

from CWD mice with spontaneous T-cell lymphomas (44). Mink cells chronically infected with CWM-S-101 were obtained by an identical cocultivation protocol by using splenic cells from a mouse with a B-cell lymphoma.

Molecular cloning of CWM-T-15, CWM-S-101, and CWM-S-5X. Chromosomal DNA was extracted from mink cells chronically infected with unpassaged virus stocks. A 500-ng portion of this DNA digested with *Eco*RI was ligated to 1 μg of EMBL 4 bacteriophage DNA. The ligated DNAs were packaged into bacteriophage particles by incubation with Packagene extracts as recommended by the supplier (Promega Biotec). The bacteriophage were plaqued on P2-392 *Escherichia coli*. Approximately 7.5×10^5 plaques were transferred to nylon membranes and screened.

CWM-T-15, CWM-S-101, and CWM-S-5X were cloned as two DNA fragments 5′ and 3′ of the *Eco*RI site present in the envelope region. The SX *pol* probe was used to identify clones that contained the 5′ fragment, and the T25PB probe was used to detect phages that carried the 3′ fragment.

Hybridization probes. The SX *pol* probe was an 800-bp *Sac*I-*Xho*I fragment from the Akv 623 provirus clone (30). The T25PB probe was the 162-bp *Pst*I-*Bgl*II fragment from the p15e region of the polytropic recombinant CWN-T-25 (9). The P1LTR-GAG probe contained ecotropic virus-related sequences from the 1,800-bp *Pst*I-*Bam*HI fragment from the 5′ end of the HRS recombinant virus PTV-1 (C. Thomas, unpublished observations). These probes were excised from their plasmid vectors and labeled with [^{32}P]dATP or [^{32}P]dCTP by random-primer extension (Boehringer Mannheim).

The JS 21 oligonucleotide probe was a 27-mer beginning 80 bases 3′ of the *Pst*I site in the MCF 247 long terminal repeat (LTR) and was a gift of Jonathan Stoye and John Coffin. This probe was labeled by using T4 polynucleotide kinase (New England BioLabs) and [^{32}P]dATP as previously described (42).

The xeno *env* probe represented 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 (35). The single-stranded probe was generated from M13 bacteriophage DNA that contained the xenotropic sequence. A 2-μl portion of universal forward primer (2.5 ng/μl) and 9 μl of 5× buffer from the Sequenase kit (New England Nuclear Corp.) were annealed to 10 μl of probe template DNA (approximately 200 ng) in a total volume of 30 μl. After cooling, 3 μl of 0.1 mM dithiothreitol, 4.5 μl of nucleotides (0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 50 mM NaCL), and approximately 50 μCi of [^{32}P]dATP were added to the reaction mix for a total volume of 45 μl. This mixture was incubated at 37°C for 30 min. A 1-μl portion of 2 mM dATP was added for another 20 minutes at 37°C. The DNA was precipitated with ethyl alcohol, suspended in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), and digested with *Hin*dIII and *Eco*RI at 37°C for 2 h. The radioactive reaction mixture was then electrophoresed on a 1.8% low-melting-point agarose gel. The 128-bp fragment (112-bp probe with short M13mp8 flanking sequence) was diluted in an equal volume of 10:1 TE, melted at 65°C, and added to the Southern blot hybridization solution.

Southern blotting and hybridization. DNA was extracted from tumor tissues after they had been rapidly thawed. Tissues were added to DNA isolation buffer (20 mM Tris hydrochloride [pH 7.5], 20 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate) and homogenized in glass grinders. They were then digested with proteinase K, extracted with phenol-chloroform, and precipitated as previously described (32, 44). Five to ten micrograms of DNA was digested with the appropriate restriction enzymes, electrophoresed in 0.7% agarose gels, and blotted to nylon membranes as previously described (16, 43). The membranes were incubated at 65°C for a minimum of 2 h in bags which contained prehybridization solution (4× SSCP [1× SSCP = 120 mM NaCl, 15 mM sodium citrate, 15.4 mM dibasic sodium phosphate, 5.3 mM monobasic sodium phosphate {pH 7.0}]–1× BFP [1× BFP = 0.2 mg of bovine serum albumin per ml, 0.2 mg of Ficoll type 200 per ml, 0.2 mg of polyvinylpyrrolidone per ml]–1% sodium dodecyl sulfate–250 μg of denatured salmon sperm DNA per ml). They were then hybridized at 65°C for 18 to 24 h in hybridization solution (4× SSCP, 1× BFP, 1% sodium dodecyl sulfate, 10% dextran sulfate) to which 0.5×10^8 to 10×10^8 cpm had been added. The membranes were then washed to a final stringency of 0.1× SSCP at 65°C. Kodak XRP-5 film was exposed to the filters with intensifier screens at −70°C for 1 to 10 days. The xenotropic envelope probe required longer autoradiography due to a lower specific activity (approximately 2×10^7 to 5×10^7 cpm).

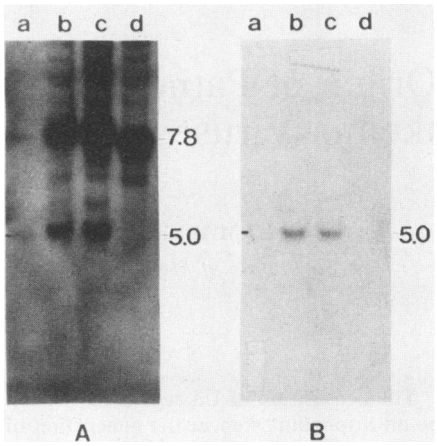

FIG. 1. Southern blot to identify proviral sequences in DNAs of mink cells cocultivated with CWD tumor cells. The DNAs were digested with *Eco*RV and hybridized to the SX *pol* probe which hybridizes to most MuLVs (A) and the xenotropic-specific *env* probe, xeno *env* (B). The dash in lane a marks a faint 5.0-kb band in panels A and B. The size of the bands in kilobase pairs is listed at the right of the figure. Lanes: a, CWM-S-5; b, CWM-S-101; c, CWM-T-15; d, control DNA from mink cells infected with the HRS polytropic virus PTV-1.

Hybridization with the JS 21 oligonucleotide probe. Nylon membranes were placed in a bag with prehybridization solution (6× NET [1× NET = 0.15 M NaCl, 0.03 M Tris hydrochloride {pH 8.0}, 1 mM EDTA]), 10× BFP, 1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 100 μg of denatured salmon sperm DNA per ml, 50 μg of denatured yeast RNA per ml) at 56°C for at least 2 h. They were then hybridized at 56°C for 18 to 24 h in hybridization solution (same as prehybridization mix without the DNA or RNA carrier) to which approximately 5×10^6 cpm/ml had been added. The gels were washed to a final stringency of 1× SSPE (10 mM Na phosphate [pH 7.0], 0.18 M NaCl, 1 mM EDTA). Kodak XRP-5 film was exposed to the gel with intensifier screens for 7 to 23 days.

DNA sequencing. Appropriate restriction fragments of the proviruses were subcloned into pUC13 plasmid or M13 bacteriophage. These fragments were sequenced by the

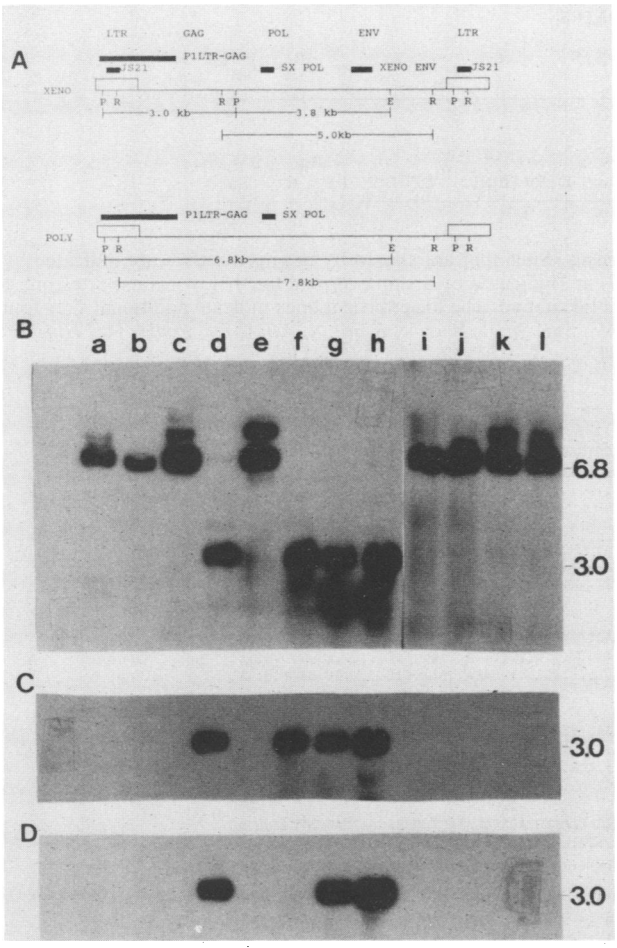

FIG. 2. Identification of cloned CWD xenotropic and polytropic proviruses. (A) Predicted restriction enzyme sites in both xenotropic (XENO) and polytropic (POLY) proviruses are shown relative to the locations of the SX *pol*, P1LTR-GAG, xenotropic envelope (xeno *env*), and xenotropic LTR (JS 21) probes. Predicted size of the fragments are given in kilobase pairs. P, *Pst*I; E, *Eco*RI; R, *Eco*RV. (B, C, and D) Southern blot analysis of phage DNAs digested with *Pst*I and *Eco*RI. The blot was hybridized to the P1LTR-GAG probe (B), xeno *env* probe (C), and JS 21 probe (D). The size of the bands in kilobases is indicated on the right side of the figure. Proviruses cloned from mink cells cocultivated with tumor cells are shown in the following lanes: a, e, and h, CW-15; b, d, and i through l, CW-101; f and g, CW-5. Lane c is a provirus from mink cells infected with the HRS recombinant PTV-1.

dideoxynucleotide termination method by using Sequenase (United States Biochemical) and ^{35}S-labeled dATP (39).

RESULTS

Xenotropic MuLVs were readily recovered from spontaneous CWD tumors. Tumor cells from the CWD lymphomas CW5, CW15, and CW101 were originally cocultivated with CCL64 mink lung fibroblasts to isolate polytropic recombinant viruses (44). The three mink cell lines derived from these experiments, CWM-S-5, CWM-T-15, and CWM-S-101, were shown to produce polytropic recombinant viruses by T1 oligonucleotide fingerprints of viral RNA and Southern blot analysis of the cellular DNA (44). However, proviruses that were distinct from those of the polytropic recombinants were also detected in these experiments. Shown in

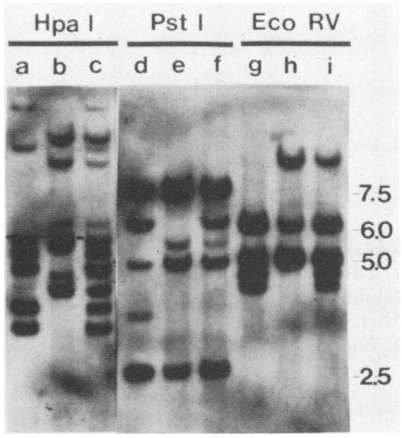

FIG. 3. Southern blot analysis of endogenous xenotropic viruses of CWD and HRS mice. The DNAs were digested with *Hpa*I (lanes a to c), *Pst*I (lanes d to f), and *Eco*RV (lanes g to i) and hybridized to the xenotropic envelope (xeno *env*) probe. The dashes indicate a shared band. Lanes a, d, and g, CWD embryo DNA; lanes b, e, and h, liver DNA from a normal HRS mouse, HR51; lanes c, f, and i, liver DNA from a normal CWD × HRS F_1 mouse, CH37.

Fig. 1A is a Southern blot of mink cell DNAs that were digested with *Eco*RV and hybridized to the SX probe, which hybridizes to the *pol* gene of most MuLV proviruses (43, 44). The DNA of each cell line contained the 7.8-kb band of the polytropic provirus. This internal proviral fragment spanned the *Eco*RV site in the 5′ LTR to the *Eco*RV site in p15E. However, the probe also detected a 5.0-kb proviral fragment that indicated that the cells contained a second type of MuLV provirus.

To determine whether the mink cell DNAs contained xenotropic as well as polytropic proviruses, the blot was rehybridized to a probe that is specific for xenotropic envelope sequences (35). As shown in Fig. 1B, xenotropic proviruses were detected in each of the three mink cell lines. Xenotropic proviruses were not found in mink cells infected with the HRS/J polytropic virus PTV-1 that had first been purified in vitro by six rounds of endpoint dilution (Fig. 1B, lane d). Presumably, xenotropic MuLVs produced by the CWD tumor cells had infected the mink cells during the recovery of the polytropic MuLVs.

Bacteriophage DNA libraries were then constructed from the mink cell DNAs, and individual plaques were screened for the insertion of MuLV-related sequences. Seven of the clones from the three different cell lines contained proviral fragments that lacked restriction sites characteristically found in the recombinant polytropic proviruses. The DNAs from selected phages were digested with *Pst*I and *Eco*RI, blotted, and hybridized to a MuLV LTR-*gag* probe, P1LTR-GAG. As shown in Fig. 2A and B, most of the clones contained the expected 6.8-kb *Pst*I-*Eco*RI band that represented the 5′ end of polytropic proviruses. However, four clones had 3.0-kb *Pst*I-*Pst*I LTR-*gag* fragments similar in size to those found in some xenotropic viruses (5; Fig. 2B, lanes d and f through h). The xenotropic origin of the four clones was confirmed by hybridization of the phage DNAs to the xenotropic envelope probe, as shown in Fig. 2C.

In order to determine the origin of the U3 region of the xenotropic proviruses, the blot was rehybridized to the xenotropic-specific U3 region probe, JS 21. This probe contains nonecotropic sequences found in the 5′ portion of the U3 region of the AKR recombinant MCF 247 (26; see

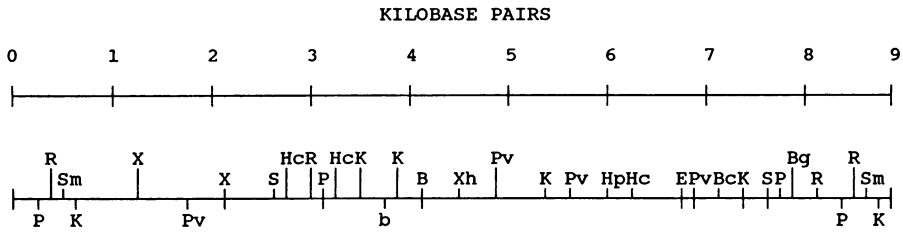

FIG. 4. Restriction endonuclease map of CWM-S-5X and *Bxv-1* proviruses. Sites that are shared by both proviruses are indicated by a capital letter. Those sites present in the proviral map from Quint et al. (37) are indicated by a short vertical line below. Those sites present in the proviral map from Hoggan et al. (18) are indicated by a short vertical line above. The long vertical lines indicate additional sites found in CWM-S-5X that may or may not be present in *Bxv-1*. P, *Pst*I; R, *Eco*RV; Sm, *Sma*I; K or k, *Kpn*I; X, *Xba*I; Pv, *Pvu*II; Hc, *Hinc*II; S, *Sac*I; B or b, *Bam*HI; Xh, *Xho*I; Hp, *Hpa*I; E, *Eco*RI; Bc, *Bcl*I; Bg, *Bgl*II.

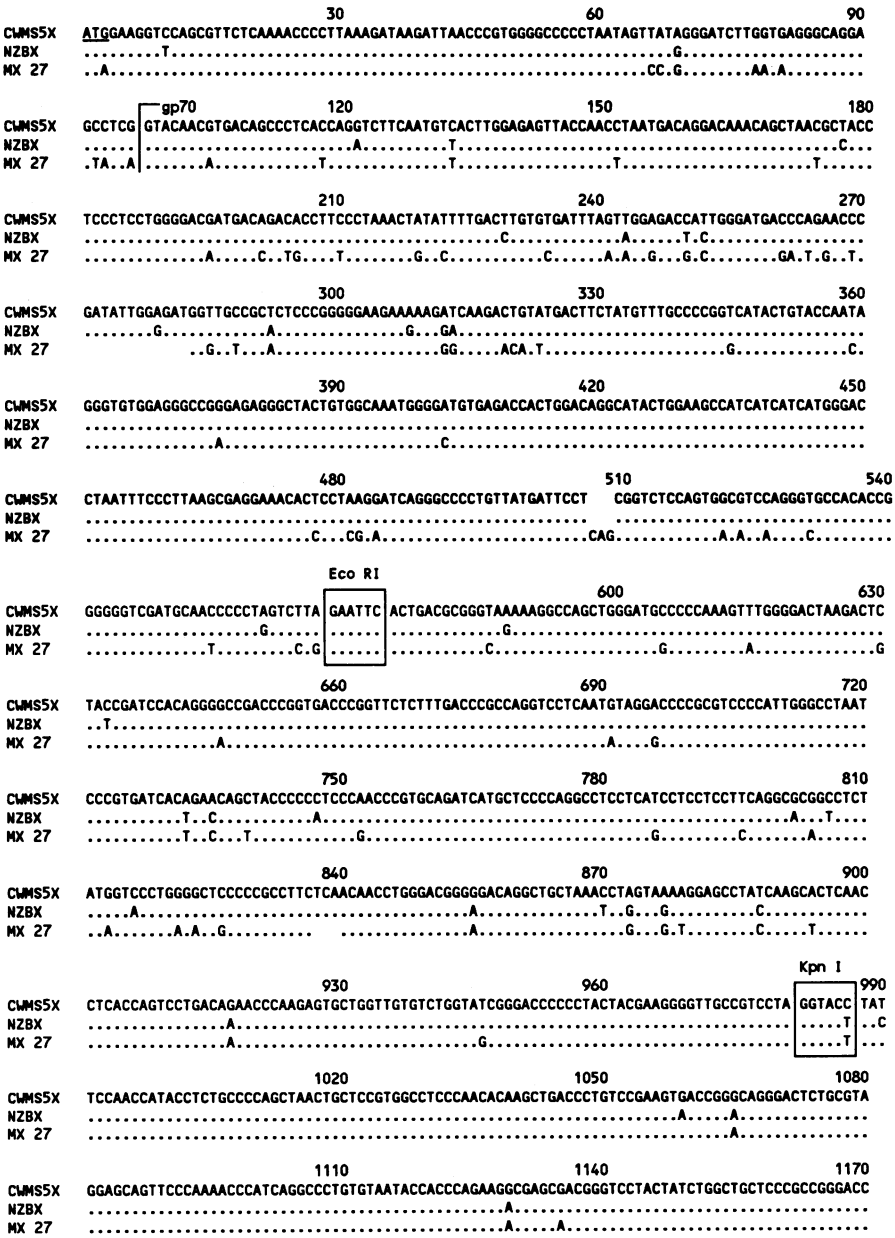

FIG. 5. Comparison of the nucleotide sequence of the envelope regions of CWM-S-5X with the xenotropic virus NZB-IU-6 (NZBX) and the endogenous polytropic virus MX 27 (34, 41). Nucleotides that are identical are indicated by a dot. Absence of a nucleotide is noted by a blank space, while substitutions are indicated by the insertion of the appropriate base symbol.

Fig. 7). As seen in Fig. 2D, three of the four clones with xenotropic envelope sequences hybridized to the JS 21 probe. Importantly, the 6.8-kb polytropic viral fragments did not hybridize to this oligonucleotide and therefore lacked these *Bxv-1*-specific sequences. This suggested that the xenotropic viruses were not themselves recombinants that had acquired the U3 region sequences from the CWD polytropic recombinant MuLVs.

The one xenotropic provirus that did not hybridize to the JS 21 probe (Fig. 2C and D, lane f) was later found to have a *Bxv-1*-like enhancer in the U3 region. Remarkably, this provirus contained a small 24-bp deletion in the 5′ end of the U3 region that included sequences that are recognized by the JS 21 probe (data not shown).

Analysis of the endogenous xenotropic viruses of CWD mice. To determine the origin of the CWD xenotropic viruses, we characterized the endogenous xenotropic proviruses of CWD mice. CWD embryo and control DNAs were digested with *Hpa*I. Southern blots of these DNAs were then hybridized to the xenotropic envelope probe, xeno *env* (35). As shown in the Southern blot in Fig. 3 (lane a), seven distinct bands were seen in the CWD genome. Since *Hpa*I is present once in known xenotropic proviruses, each band presumably represented a distinct endogenous xenotropic provirus (5; Fig. 4). As seen in lane b of Fig. 3, HRS liver DNA also contained seven xenotropic-specific bands, although only one of these was shared with CWD. This was confirmed by the pattern seen in DNA from a CWD × HRS F_1 mouse (see Fig. 7, lane c). The shared band most likely represented *Bxv-1*, since both strains carry this locus (A. Khan and J. Stoye, personal communications).

To further characterize the endogenous xenotropic viruses, the same DNAs were digested with *Pst*I or *Eco*RV restriction enzyme. Both enzymes cleave at more than one site within the provirus. As judged by the size of the internal *Pst*I proviral fragments (Fig. 3, lanes d through f), CWD and HRS shared three families of xenotropic proviruses. The 5.0-kb *Pst*I-*Pst*I fragments of proviruses of the *Bxv-1* family were seen (as shown in Fig. 4) (5, 17, 18). In addition, the DNAs contained the 7.5-kb fragment characteristic of the NZB xenotropic virus family (5, 17). The common 2.5-kb fragment in HRS and CWD mice suggested that there was another family that contained a *Pst*I site in the 3′ end of the *gag* region, although deletions within the proviruses could yield the same pattern. The *Eco*RV digests of the DNAs (Fig. 3, lanes g through i) demonstrated that the HRS and CWD mice shared two xenotropic proviral fragments. A 5.0-kb *Eco*RV-*Eco*RV band was evident, similar in size to that found in the mink cells infected with CWD xenotropic viruses. The DNAs of both strains also contained a 6.0-kb proviral band, while two smaller proviral fragments were detected in CWD embryo DNA but not HRS DNA. Taken together, the data supported the conclusion that the xenotropic MuLVs recovered from CWD tumors represented the progeny of a particular subset of the endogenous xenotropic viruses. These proviruses shared an internal 5.0-kb *Eco*RV fragment and a *Pst*I site within the *pol* region.

The CWD xenotropic virus CWM-S-5X most likely represents the progeny of the *Bxv-1* locus. One of the cloned xenotropic proviruses that was derived from the CWM-S-5 mink cell DNA was selected for further study. The provirus, CWM-S-5X, was cloned as two DNA fragments, one 5′ and

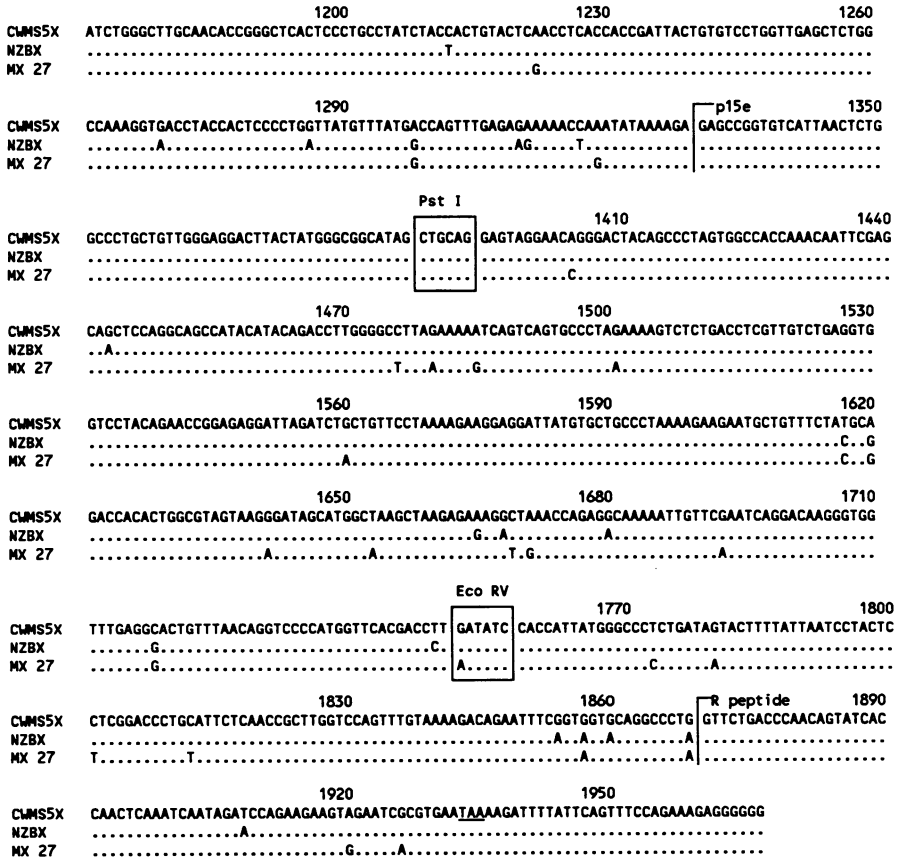

FIG. 5—*Continued.*

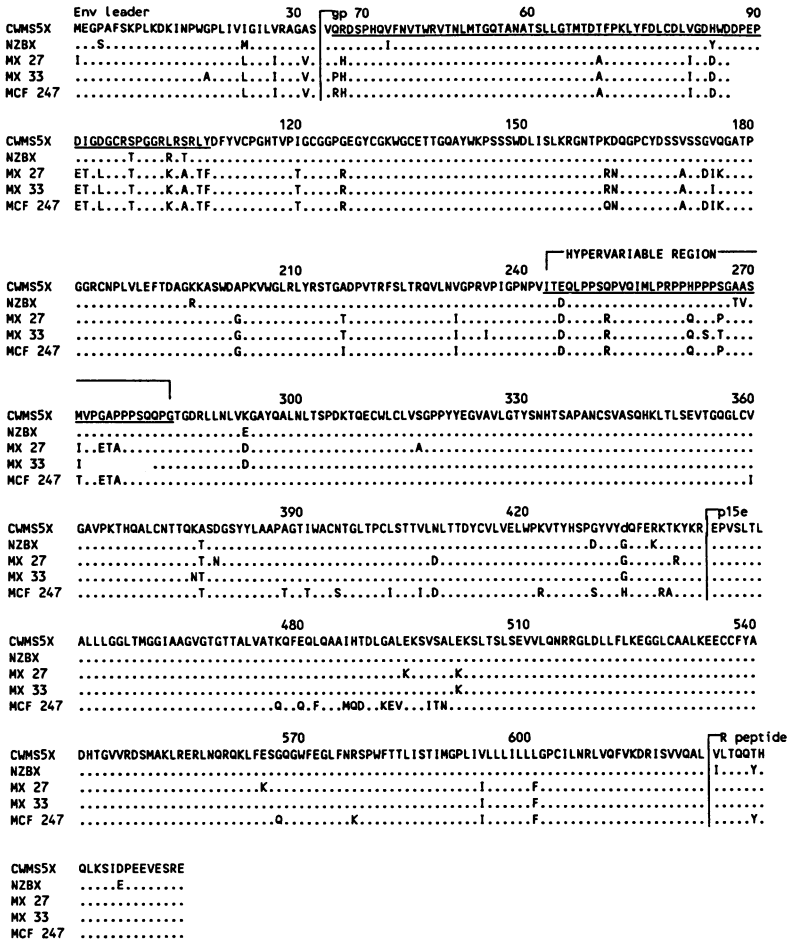

FIG. 6. The predicted amino acid sequence of the CWM-S-5X envelope region compared with the xenotropic virus NZB-IU-6 (NZBX), the endogenous polytropic viruses MX 27 and MX 33, and the AKR recombinant MCF 247 (22, 26, 34, 41). The two regions with the most marked differences between the xenotropic and polytropic amino acid sequences are underlined: (i) 75 residues at the extreme 5′ end of gp70 and (ii) the proline-rich "hypervariable region" (36).

one 3′ of the *Eco*RI site located in *env*. The 3′ CWM-S-5X clone was originally thought to represent the 3′ end of the polytropic virus but was found to contain xenotropiclike sequences in the envelope gene and U3 region (see below).

The restriction map of the CWM-S-5X provirus was deduced from analyses of the 5′ and 3′ clones and is shown in Fig. 4. The map revealed that the CWM-S-5X provirus was related to one of the two families of endogenous xenotropic viruses we had detected and to xenotropic viruses described by Chattopadhyay et al. (5). CWM-S-5X contained the *Pst*I site at 3.0 kb and the *Kpn*I site at 5.5 kb, which are thought to be unique to the xenotropic family that includes *Bxv-1*. The *Pvu*II site at 4.8 kb and the *Hinc*II site at 6.2 kb of the CWM-S-5X provirus are not found in members of the other families of xenotropic viruses. Conversely, CWM-S-5X lacked the characteristic *Sac*I and *Sma*I sites of the NZB and NFS xenotropic viruses and the *Bam*HI site found in the envelope genes of most polytropic viruses (5, 17, 34).

Comparison of the restriction maps of CWM-S-5X and the endogenous *Bxv-1* xenotropic proviruses revealed marked similarities (18, 37; Fig. 4). We could not detect the *Bam*HI restriction enzyme site found at 3.8 kb in the *Bxv-1* proviral map of Quint et al. (37). This *Bam*HI site is located less than 300 bp from another *Bam*HI site further 3′ and may not have been detected in our gels. We also found additional restriction enzyme sites in the CWM-S-5X provirus that had not been detected in *Bxv-1*. However, the Southern blots that were used in the previous studies were hybridized to probes for the 3′ end of the *Bxv-1* provirus (18, 37). Thus, the blots would identify only the most 3′ site for each of the restriction enzymes and may have missed the 5′ sites found in our clone.

Sequence analysis of the envelope and U3 regions of CWM-S-5X. Provirus fragments that contained the envelope gene and U3 regions of CWM-S-5X were subcloned into the pUC13 plasmid for DNA sequence analysis. The nucleotide sequence of the CWM-S-5X envelope region is presented in Fig. 5 and is compared with the *env* regions of the xenotropic virus NZB-IU-6 (NZBX) and the endogenous polytropic virus MX27 (34, 41). The entire CWM-S-5X *env* sequence had 98% identity with the NZB xenotropic virus, 93% identity with the endogenous polytropic virus MX 27, but less than 40% identity with ecotropic virus sequence (15; ecotropic sequence not shown). The region that reportedly distinguishes between the envelope genes of the xenotropic and polytropic viruses is located within the first 220 bases of *env*, which encode the amino-terminal portion of gp70. The

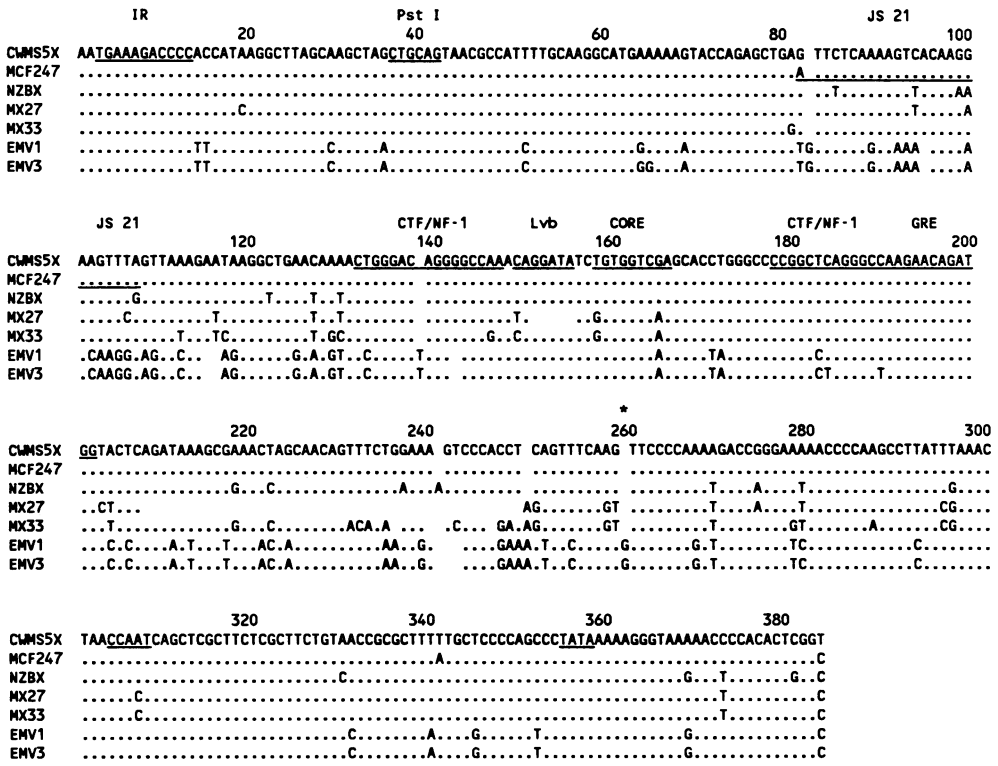

FIG. 7. The sequence of the U3 region of the xenotropic provirus CWM-S-5X is compared with those of the AKR recombinant virus MCF 247, the xenotropic virus NZB-IU-6 (NXBX), the endogenous polytropic viruses MX 27 and MX 33, and the endogenous ecotropic viruses *Emv-1* and *Emv-3* (23, 26, 34, 41). The location of consensus sequences that are likely recognized by certain DNA-binding proteins are also shown. CTF-1/NF-1, CCAAT-binding transcription factors or nuclear factor 1 (25, 33); CORE, enhancer core-binding proteins (50); Lvb, Moloney leukemia virus-binding protein b (40); and GRE, glucocorticoid response element (3). JS 21 is the 27-bp oligonucleotide xenotropic-specific probe derived from MCF 247 (courtesy of Jonathan Stoye and John Coffin). The inverted repeat (IR), CAT box, and TATA box are also noted. The asterisk indicates the location of the 190-bp insert present in MX 27 and MX 33 (41; insert sequence not shown).

CWM-S-5X sequence had 95% identity with NZBX within this small segment, as compared to 80% with the polytropic virus-related sequences found in the MX 27 provirus.

In Fig. 6, the deduced amino acid sequences for the envelope protein of CWM-S-5X are compared with those of the xenotropic virus NZB-IU-6, the endogenous polytropic virus MX 27, the endogenous modified polytropic virus MX 33, and the AKR class I recombinant MCF 247 (22, 26, 34, 41). At the amino acid level, the differences between the polytropic and xenotropic viruses in the amino-terminal portion of the major envelope protein were evident. The CWM-S5X and NZBX envelope proteins also exhibited striking homology in the previously described proline-rich "hypervariable region" (residues 243 to 283) (36). Taken together, the homology patterns suggested that CWM-S-5X is more closely related to the NZBX xenotropic virus than to the polytropic viruses.

In Fig. 7, the U3 region sequence of CWM-S-5X is compared with that of MCF 247, NZB-IU-6, the endogenous polytropic viruses MX 27 and MX 33, and the CWD endogenous ecotropic viruses *Emv-1* and *Emv-3* (23, 26, 34, 41). As can be seen, the enhancer elements of CWM-S-5X and the leukemogenic recombinant AKR virus MCF 247 are virtually identical and the entire U3 regions differ by only three bases (26; Fig. 7). The CWM-S-5X U3 region was more homologous to that of the xenotropic virus NZBX, as compared to the U3 regions of the endogenous polytropic, modified polytropic, or ecotropic viruses.

Thus, the sequence data confirmed that CWM-S-5X contained xenotropic virus-related envelope sequences and U3 region sequences that are specifically associated with the *Bxv-1* locus. The virus contained a single copy of the enhancer element, as is seen in *Bxv-1*, rather than a duplicated enhancer that is usually found in recombinant polytropic viruses (20). We have also sequenced portions of the CWM-S-5X *gag* region adjacent to the *Pst*I site (data not shown). There are a number of base substitutions compared with the analogous regions of the endogenous ecotropic and polytropic viruses. This provides additional evidence that CWM-S-5X was not a recombinant between xenotropic and ecotropic or polytropic viruses.

DISCUSSION

In these experiments, we found that tumor cells from spontaneous CWD lymphomas frequently released xenotropic as well as polytropic MuLVs. The xenotropic viruses most likely represented the progeny of the endogenous virus *Bxv-1*. Several observations supported this conclusion: (i) the restriction enzyme map of one of the CWD xenotropic proviruses, CWM-S-5X, was virtually identical to that of the endogenous *Bxv-1* provirus (18, 37); (ii) the sequence of the U3 region differed by only 3 bp from the *Bxv-1*-related sequences found in the AKR recombinant MCF 247 (26); (iii) the envelope gene was closely related but distinct from that of other known xenotropic viruses, such as NZB-IU-6 (34);

(iv) Southern blots of proviral DNAs, restriction maps, and sequence analysis of the cloned CWM-S-5X provirus provided strong evidence that the CWD xenotropic viruses were not recombinants that had been generated in vivo or in vitro.

The acquisition of *Bxv-1* U3 region sequences which include the viral enhancer element is an important step in the generation of leukemogenic recombinant viruses in AKR, HRS, C58, and some CWD mice (11, 14, 16, 26, 37, 38, 46–49; A. Massey and C. Thomas, unpublished observations). The CWD xenotropic virus we have characterized, CWM-S-5X, had the predicted features for the donor of the *Bxv-1* U3 region sequences. The genomic RNA of this virus could be packaged into particles with ecotropic or polytropic virus genomes. Upon infection, recombinant proviruses could be generated during reverse transcription by a switch of templates by the viral polymerase or by some other mechanism (2, 13). Once recombinants that contained *Bxv-1* U3 sequences were generated, additional rounds of replication and infection select for viruses that have also acquired polytropic envelope gene sequences and perhaps a duplication of the *Bxv-1* enhancer (20, 46).

Some laboratories, including our own, have suggested that *Bxv-1* might also donate p15E gene sequences to the 3′ end of the recombinant virus genomes (31, 46, 48). Because the xenotropic and polytropic viruses share extensive homology within the p15E gene, it is difficult to determine the precise origin of the p15E sequences. However, the 3′ p15E gene of the AKR MCF 247 recombinant contains two codons not found in *Bxv-1* that are present in the endogenous polytropic viruses (26; Fig. 6, positions 594 and 601). This suggests that the p15E gene sequences of the recombinant viruses may be acquired from endogenous polytropic sequences rather than *Bxv-1* and that the selection for the nonecotropic p15E gene sequence is not directly related to the process that selects for the *Bxv-1* U3 region sequences.

Our studies also revealed that the viral enhancer of *Bxv-1* may function in non-T cells. In transient expression assays, *Bxv-1*-related enhancers appear to function more efficiently in T-cell lines than other cell types (51). However, as shown here, viruses with *Bxv-1*-related U3 sequences were readily recovered from a CWD B-cell tumor, CW101. Although it is possible that the CW101 xenotropic viruses were produced by non-B cells in the tumor cell preparation, our observations suggest that the association of recombinant viruses that contain the *Bxv-1* enhancer with T-cell lymphomas may result from subtle differences in enhancer function in the different cell types or perhaps involve unidentified mechanisms.

Although the frequent isolation of *Bxv-1*-encoded xenotropic viruses from mouse strains with a high incidence of leukemia might be anticipated, we did not expect this to be the case with CWD mice. The majority of CWD recombinants do not contain *Bxv-1*-like sequences in the U3 region, although such sequences were detected in the acquired proviruses of two spontaneous CWD T-cell tumors (44; A. Massey and C. Thomas, unpublished observations). In this regard, it is remarkable that the genomes of the three CWD polytropic viruses that were isolated from the same tumor cells that produced the *Bxv-1*-like viruses had not incorporated *Bxv-1* sequences into the U3 region. One explanation is that there is less of a selective advantage for recombinants with the *Bxv-1* enhancer in CWD mice. Alternatively, CWD mice may express another endogenous virus that donates pathogenic U3 region sequences to the recombinant viruses. Preliminary sequence analysis of the U3 regions of CWD recombinant viruses is consistent with the latter possibility (A. Massey and C. Thomas, unpublished observations). Since recombinant viruses with the *Bxv-1* enhancer are associated with T-cell lymphomas, the absence of the *Bxv-1* enhancer in the CWD recombinants may explain the relatively high frequency of B-cell lymphomas in this strain.

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