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Cas-Br-E Murine Leukemia Virus: Sequencing of the Paralytogenic Region of Its Genome and Derivation of Specific Probes To Study Its Origin and the Structure of Its Recombinant Genomes in Leukemic Tissues

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The ecotropic Cas-Br-E murine leukemia virus (MuLV) and its molecularly cloned derivative pBR-NE-8 MuLV are capable of inducing hind-limb paralysis and leukemia after inoculation into susceptible mice. T_1 oligonucleotide fingerprinting, molecular hybridization, and restriction enzyme analysis previously showed that the *env* gene of Cas-Br-E MuLV diverged the most from that of other ecotropic MuLVs. To analyze proviruses in leukemic tissues, we derived DNA probes specific to Cas-Br-E sequences: two from the *env* region and one from the U3 long terminal repeat. With these probes, we found that this virus induced clonal (or oligoclonal) tumors and we documented the presence of typical mink cell focus-forming-type proviruses in leukemic tissues and the possible presence of other recombinant MuLV proviruses. Since the region harboring the determinant of paralysis was mapped within the *pol-env* region of the virus (L. DesGroseillers, M. Barrette, and P. Jolicoeur, J. Virol. 52:356–363, 1984), we performed the complete nucleotide sequence of this region covering the 3′ end of the genome. We compared the deduced amino acid sequences of the *pol* carboxy-terminal domain and of the *env* gene products with those of other nonparalytogenic, ecotropic, and mink cell focus-forming MuLVs. This amino acid comparison revealed that this part of the *pol* gene product and the p15E diverged very little from homologous proteins of other MuLVs. However, the Cas-Br-E gp70 sequence was found to be quite divergent from that of other MuLVs, and the amino acid changes were distributed all along the protein. Therefore, gp70 remains the best candidate for harboring the determinant of paralysis.

The Cas-Br-E murine leukemia virus (MuLV) is an ecotropic retrovirus that was isolated from the brain of a paralyzed wild mouse (*Mus musculus*) trapped in Lake Casitas, Calif. (for a review, see references 16 and 17). It was shown to induce a progressive form of hind-limb paralysis and leukemia after inoculation into susceptible laboratory mice (16). Initial studies on the molecular structure of the Cas-Br-E MuLV genome revealed that its restriction map (5) and its T_1 oligonucleotide fingerprinting pattern (29) were distinct from those of other ecotropic MuLVs isolated from inbred strains of mice. However, its genome appeared similar to the amphotropic MuLV genome except in the *env* gene, in which they diverged the most (1, 5, 29). Amphotropic MuLVs have also been isolated from wild mice, often from the same mice from which neurotropic MuLVs were isolated, and have been reported to be nonparalytogenic and weakly leukemogenic (16, 18, 36). In an effort to understand the molecular basis of the hind-limb paralysis, we first cloned the genome of Cas-Br-E MuLV and showed that this cloned virus, pBR-NE-8 MuLV, had retained the paralysis-inducing and leukemogenic potential of the parental MuLV (25). Using this cloned viral genome, we could subsequently map the primary paralysis-inducing determinant of pBR-NE-8 MuLV within a 3.9-kilobase-pair (kbp) *pol-env* fragment (7). We also reported that its long terminal repeat (LTR) region harbored sequences influencing the incidence and clinical manifestation of the neurological disease (11). Later, studying the leukemogenic potential of this virus, we found that several determinants of leukemia seemed to be distributed along its genome (24).

To monitor the fate of Cas-Br-E MuLV nucleic acids in infected tissues and to analyze its proviruses in infiltrated leukemic organs, we isolated probes which would not hybridize to mouse endogenous viral sequences and which are specific to Cas-Br-E MuLV sequences. Moreover, to get a better understanding of the specific molecular alteration(s) within the *pol-env* region that are responsible for the paralytogenic phenotype of Cas-Br-E MuLV, we sequenced the viral DNA fragment harboring the determinant of paralysis. The present paper reports the identification and characterization of Cas-Br-E MuLV-specific probes and the DNA sequence of the 3′ end of Cas-Br-E MuLV genome.

MATERIALS AND METHODS

Viral DNA clones. The structure and characterization of the infectious viral DNA genome pBR-NE-8 were described previously (25). The cloned viral genomes from amphotropic 4070-A (5), Moloney (11), BALB/c endogenous ecotropic B-Cl-11 (8, 37), and BALB/c endogenous nonecotropic BA-14 (38) MuLVs and from BL/VL_3 V-13 (40) and G_6T_2 (40) radiation leukemia viruses were described previously.

Construction of specific probes. The probe NE-8A, derived from the *env* region of pBR-NE-8, corresponded to the 595-base-pair (bp) *Xba*I-*Bam*HI fragment subcloned in the Sp64 vector (Fig. 1). The probe NE-8B, also from the *env* region of pBR-NE-8, corresponded to the 1,050-bp *Taq*I-*Bam*HI fragment subcloned in *Acc*I-*Bam*HI sites of the

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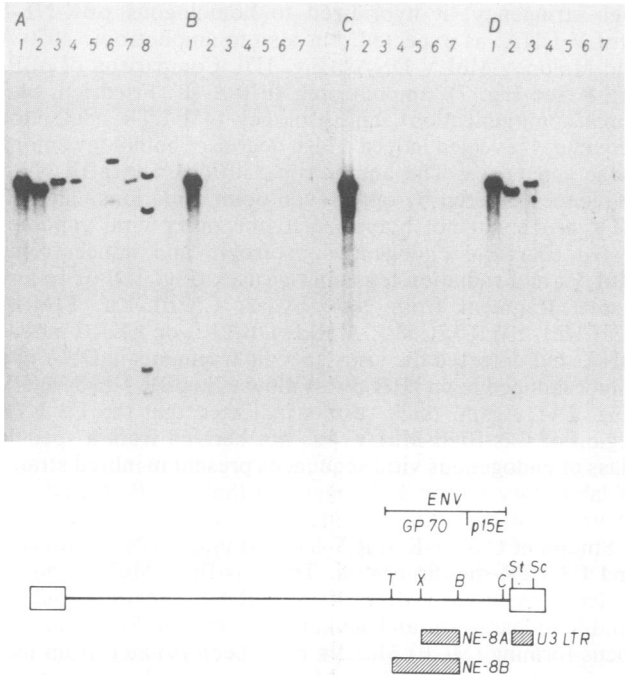

FIG. 1. Hybridization of various MuLV DNA genomes with Cas-Br-E MuLV-specific probes. Equal amounts of cloned DNA of Cas-Br-E (pBR-NE-8) (lane 1), ampho 4070-A (lane 2), Moloney (lane 3), BALB/c endogenous B-Cl-11 (lane 4), and BALB/c endogenous nonecotropic (lane 5) MuLVs and of BL/VL_3 V-13 (lane 6) and G_6T_2 (lane 7) radiation leukemia viruses were digested with the appropriate restriction endonucleases (to excise the full-length viral genome from pBR322), fractionated by electrophoresis on a 1% agarose gel in duplicate, and transferred by double transfer onto two nitrocellulose membranes. The four membranes were then hybridized with ^{32}P-labeled representative Cas-Br-E MuLV probe (A), or NE-8A (B), NE-8B (C), or U3 LTR (D) Cas-Br-E MuLV probe. The 8.2- or 8.8-kbp fragments contain the full-length viral genomes. In lane 6, the 7-kbp fragment contains pBR322 and cellular sequences and the 12-kbp fragment contains V-13 viral and cellular sequences. *Hin*dIII-digested λ DNA was used as marker (lane 8). Bottom. The region of the viral genome from which these probes were derived. Only relevant restriction sites are illustrated. Open boxes represent the LTR. Restriction sites: B, *Bam*HI; C, *Cla*I; Sc, *Sac*I; St, *Stu*I; T, *Taq*I, X, *Xba*I.

pEMBL18 vector (Fig. 1). The U3 LTR probe was constructed from a purified *Sac*I-permuted LTR-containing DNA fragment. The fragment was cleaved with *Stu*I, and *Eco*RI linkers were added by using T4 DNA ligase. After ligation, the fragment was further cleaved with *Eco*RI and *Sac*I, and the 280-bp *Eco*RI-*Sac*I U3 fragment was subcloned into the Sp64 vector (Fig. 1). To be used as a probe, each DNA fragment was excised from the vector with appropriate restriction endonucleases and purified by polyacrylamide (33) (U3 LTR fragment) or agarose (11) (fragments NE-8A and NE-8B) gel electrophoresis prior to being labeled and used for hybridization (7, 25).

Mice, tumors, and DNA extraction. AKR/J, DBA/2J, C3H/HeJ, SWR/J, C58/J, and A/HeJ mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. C57BL/Ka mice were initially obtained from H. S. Kaplan, Stanford University, Stanford, Calif., and NIH Swiss mice were obtained from the Small Animal Resources, National Institutes of Health, Bethesda, Md. Newborn NIH Swiss mice (<48 h old) were used for induction of tumors. They were inoculated intraperitoneally with 0.15 ml of filtered pBR-NE-8 virus suspension, as described previously (25). DNA extraction of infiltrated leukemic organs or of mouse livers was performed as described previously (39).

DNA sequence analysis. For DNA sequencing, the infectious pBR-NE-8 DNA molecule, subcloned in pBR322, was used (25). Appropriate restriction endonuclease fragments were treated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Montreal, Canada) and labeled at the 5′ end with 600 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and 20 U of polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) as already described (33, 40). The same fragments were also labeled by filling out the 3′ ends with 50 μCi of the appropriate α-^{32}P-labeled deoxynucleoside triphosphate (3,000 Ci/mmol; New England Nuclear) and 5 U of the Klenow fragment of DNA polymerase 1 (40). The reaction was carried out in the restriction enzyme buffer, supplemented with 10 mM dithiothreitol, for 20 min at 20°C. End-labeled DNA fragments were further cleaved with appropriate restriction endonucleases and then isolated by electrophoresis on a 5% polyacrylamide gel (33). The nucleotide sequence was determined by the procedure of Maxam and Gilbert (33). Nucleotide sequences were analyzed with a VAX 11-750 computer and programs developed by Mamdouh Mikhail from our Institute and the DB program of Roger Staden (Medical Research Cambridge Center).

Hybridization procedure. Cellular DNA fragments transferred to nitrocellulose membranes were detected by hybridization with ^{32}P-labeled DNA fragments subcloned from pBR-NE-8 DNA (25, 46). Probes were labeled by nick translation as described previously (41). After being annealed (50% formamide, 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], Denhardt solution) at 42°C, filters were washed sequentially in 2× SSC for 20 min at room temperature, in 0.1× SSC–0.1% sodium dodecyl sulfate for 1 h at 60°C, and then in 0.1× SSC for 2 min at room temperature. Membranes were then dried and exposed at −70°C to RP Royal X-Omat film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning Plus intensifying screen (Du Pont Co., Wilmington, Del.).

RESULTS

Isolation and characterization of *env*-specific probes from Cas-Br-E MuLV. Probes specific for the *env* portion of AKR ecotropic endogenous MuLV were derived previously (20, 21). Because of the presence of numerous endogenous retroviral sequences in the mouse genome, these probes have been instrumental in the study of different aspects of the cycle of these ecotropic MuLVs, both in normal and malignant cells. Despite the fact that the neurotropic Cas-Br-E MuLV (or its molecularly cloned derivative pBR-NE-8) is an ecotropic MuLV, its genome does not hybridize significantly at high stringency with gp70-related AKV-3 or with p15E-related AKV-5 or AKV-6 probes derived from the endogenous AKR ecotropic MuLV genome (20, 21; our unpublished results), and these probes could not be used to detect Cas-Br-E MuLV sequences. Therefore, to determine the origin of the gp70 neurotropic sequences and to detect neurotropic *env* mRNA or DNA sequences without detecting other homologous sequences from the endogenous mouse viral sequences, we derived probes specific for pBR-NE-8 MuLV *env* sequences.

The 3′ end 3.9-kbp *Sal*I-*Cla*I fragment was first cleaved with *Sau*3A, and the resulting fragments were subcloned at

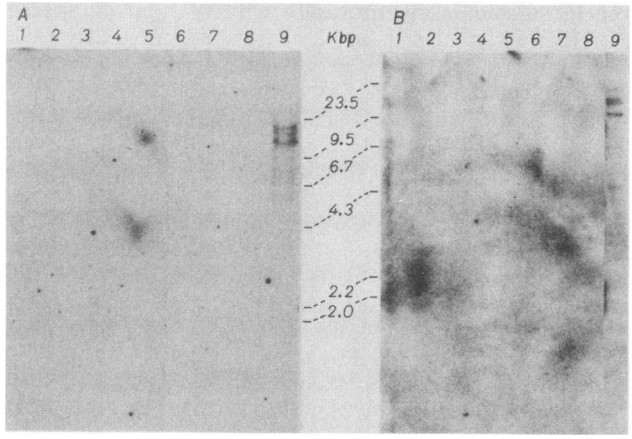

FIG. 2. Hybridization analysis of DNA from different mouse strains with Cas-Br-E MuLV-derived probes. Liver DNA from A/HeJ (lane 1), C58/J (lane 2), AKR/J (lane 3), DBA/2J (lane 4), C3H/HeJ (lane 5), SWR/J (lane 6), C57BL/Ka (lane 7), and SIM.R (lane 8) mouse DNA and control DNA from a Cas-Br-E MuLV-induced tumor in an NIH Swiss mouse (lane 9) were digested with *Eco*RI, phenol extracted, and fractionated by electrophoresis on a 1% agarose gel. Fragments were transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled U3 LTR (A) or NE-8B (B) Cas-Br-E MuLV probes.

the *Bam*HI site of pBR322. Each subclone obtained was tested for its inability to hybridize to a variety of MuLV DNA genomes. Four clones with this characteristic were identified. They were all clustered within two regions of pBR-NE-8 MuLV DNA, one at the 5′ end and one in the middle of gp70-coding sequences (data not shown). With this set of probes, we could subsequently derive longer probes specific to pBR-NE-8 MuLV DNA and of a length suitable for standard hybridization conditions.

The probes NE-8A and NE-8B were derived from the *env* region and corresponded to the 595-bp *Xba*I-*Bam*HI and 1,050-bp *Taq*I-*Bam*HI fragment, respectively (Fig. 1). They both hybridized exclusively to homologous pBR-NE-8 DNA at high stringency and not to any other MuLV DNA genome tested, namely amphotropic, Moloney, BALB/c endogenous ecotropic and nonecotropic MuLVs, or radiation leukemia viruses (Fig. 1B and C). Probe NE-8B did not hybridize to any fragment of normal mouse DNA (NIH Swiss, C57BL/Ka, SIM.R, C3H/HeJ, SWR/J, C58/J, A/HeJ, DBA 2/J, or AKR/J) digested with *Eco*RI, although it could easily detect the virus-specific fragments in DNA of a tumor induced in an NIH Swiss mouse by pBR-NE-8 MuLV (Fig. 2B). These results indicated that these *env* sequences, which were unique to pBR-NE-8 MuLV, were not derived from an endogenous retroviral sequence present in the strains tested. Not surprisingly, sequencing of this region revealed a poor homology with corresponding sequences from other MuLVs (see below).

Isolation and characterization of U3 LTR-specific probe from Cas-Br-E MuLV. The U3 LTR region of each MuLV is unique and has been shown to determine the tissue (10) and disease (3, 4, 9, 11) specificity of these viruses. Because of the presence of numerous endogenous LTR copies in the mouse genome, specific U3 LTR probes are required to detect newly acquired LTR-containing fragments in mouse DNA after infection. Using this LTR region, we derived a probe specific to the Cas-Br-E MuLV U3 LTR. The probe corresponds to the 280-bp *Stu*I-*Sac*I fragment (Fig. 1). At high stringency, it hybridized to homologous pBR-NE-8 MuLV DNA, as expected, but also to amphotropic 4070-A and Moloney MuLV DNAs (Fig. 1D). Comparison of pBR-NE-8 (see Fig. 7), amphotropic 4070-A (R. Friedrich, personal communication), and Moloney (45) LTR nucleotide sequences revealed indeed a high degree of homology among these sequences. The amphotropic 4070-A and pBR-NE-8 sequences differed by only seven point mutations. The U3 LTR probe did not hybridize to the other viral genomes tested (BALB/c endogenous ecotropic and nonecotropic MuLVs and radiation leukemia viruses (Fig. 1D) or to any *Eco*RI fragment from NIH Swiss, C57BL/Ka, SIM.R, C3H/HeJ, SWR/J, C58/J, A/HeJ, DBA2/J, or AKR/J mouse DNAs but detected the virus-specific fragments in DNA of a tumor induced in an NIH Swiss mouse by pBR-NE-8 MuLV (Fig. 2A). Again, these results indicated that the U3 LTR region of Cas-Br-E MuLV was not derived from a specific class of endogenous viral sequences present in inbred strains of laboratory mouse and suggested that Cas-Br-E and amphotropic 4070-A MuLVs might have a common origin.

Studies of Cas-Br-E MuLV-induced tumor DNAs with *env*- and U3 LTR-specific probes. The Cas-Br-E MuLV and its molecularly cloned derivative pBR-NE-8 MuLV induce hind-limb paralysis and leukemia in mice (25). Mink cell focus-forming (MCF) MuLVs have been isolated from leukemic spleens of Cas-Br-E MuLV-inoculated mice but not from the brains of these mice (22). Little is known about the molecular structure of these MCF viruses. Previous studies showed that several such recombinants are generated after inoculation of different strains of MuLVs into mice (13, 20). The MCF MuLVs have acquired a new *env* gene, and some have retained the LTR from the inoculated MuLV (13). Utilization of various ecotropic MuLV-specific probes has revealed the general structure of these MCF proviruses in the AKR system (20). With the availability of *env* and LTR pBR-NE-8 MuLV-specific probes which do not hybridize to endogenous viral fragments, we could study the general structure of the proviruses in pBR-NE-8 MuLV-induced leukemic DNAs.

Some of these tumor DNAs were digested with restriction endonucleases and hybridized with the three specific probes. *env* probe NE-8A detected few well-defined *Eco*RI fragments in each tumor, suggesting that they were clonal or oligoclonal, and the hybridization pattern was unique for each tumor (Fig. 3B). Most of the *Eco*RI fragments hybridizing with probe NE-8A were larger than 9 kbp. Most of the fragments detected with the longer *env* NE-8B probe were the same as those detected with probe NE-8A (data not shown). Because *Eco*RI does not cleave the genome of the inoculated pBR-NE-8 MuLV, this result suggested that probe NE-8A was detecting mainly the integrated full-length genome of the inoculated pBR-NE-8 MuLV. The fragments detected with probe NE-8A also hybridized with U3 LTR probe, but in each tumor DNA, this U3 LTR probe detected several additional *Eco*RI fragments ranging from 2 to 20 kbp (Fig. 3A), indicating the presence of several additional newly acquired integrated viral genomes in each tumor. Taken together, these results are reminiscent of the data obtained with ecotropic MuLV-specific probes in tumor DNAs known to contain MCF MuLV proviruses derived from other ecotropic MuLVs (20).

Our results can best be interpreted by the presence of a mixture of pBR-NE-8 and MCF proviruses (derived from pBR-NE-8 MuLV) in these tumor DNAs. Probe NE-8A, which maps within the recombination region of MCF, would detect almost exclusively ecotropic parental pBR-NE-8 pro-

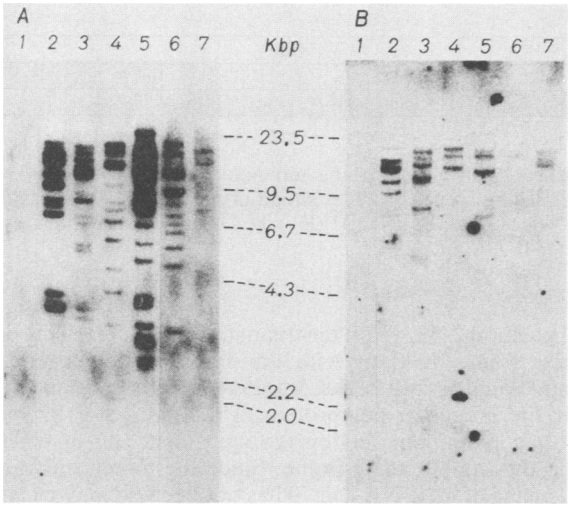

FIG. 3. *Eco*RI analysis of proviruses in Cas-Br-E MuLV-induced tumors. DNAs (15 μg) from leukemic organs were extracted, digested with *Eco*RI, and fractionated by electrophoresis on a 1% agarose gel. DNA fragments were transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled U3 LTR (A) or NE-8A (B) Cas-Br-E MuLV probes. Lanes: 1, control NIH Swiss liver DNA; 2, tumor N006 DNA; 3, tumor N007 DNA; 4, tumor N012 DNA; 5, tumor 1284-11 DNA; 6, tumor N011 DNA; 7, tumor N010 DNA.

viruses on fragments longer than 9 kbp (because this viral genome is not cleaved by *Eco*RI) and would not detect MCF proviruses (because of its lack of homology to the MCF sequences). Most MCF proviruses studied to date seem to harbor an *Eco*RI site at position 6.9 on the viral genome. If MCF proviruses are present in these DNAs, they most likely harbor an *Eco*RI site within their genome. *Eco*RI cleavage within each of these putative MCF MuLV genomes would generate two LTR-containing fragments hybridizing with U3 LTR probe. This could explain the numerous bands, some of smaller molecular weight, detected with this probe and undetectable with the *env* probes NE-8A or NE-8B, the *env* sequences having probably undergone recombination. The other interpretation of our finding of small (<9 kbp) *Eco*RI fragments hybridizing with U3 LTR probe would be to postulate the presence of several defective pBR-NE-8 MuLV proviruses that had retained their LTR regions. However, the presence of deleted proviruses is rare in MuLV system, and in the systems in which all proviruses of a single tumor have been cloned, such deleted proviruses have not been observed frequently (our unpublished observations).

To distinguish between the two possibilities, we digested the same tumor DNAs with *Eco*RI-*Sac*I and hybridized them with the U3 LTR probe. If MCF proviruses of the same type as those previously described were present in tumor DNAs, this probe should hybridize to a 1.7-kbp 3′ end *Eco*RI-*Sac*I fragment (position 6.9 to 8.6) (Fig. 4). The results of this experiment are presented in Fig. 4A. As expected, in each tumor, the U3 LTR probe hybridized to an amplified 4.7-kbp *Sac*I fragment generated from ecotropic pBR-NE-8 MuLV proviruses, confirming the presence of ecotropic MuLV proviruses in each tumor. In three of four tumor DNAs shown, a relatively intense comigrating 1.7-kbp *Eco*RI-*Sac*I fragment could also be detected, suggesting the presence of recombinant MuLVs of the MCF type in these tumors. The absence of a similar fragment in other tumors indicated the absence of proviruses of the same class in these tumors and suggested that MCF MuLV proviruses in these tumors, if present, have a different restriction endonuclease cleavage map or different fragment lengths, or both. The numerous other hybridizing fragments presumably represent the 5′-end cell-virus junction fragments. Hybridization of the same filter with the *env* probe NE-8B revealed the same amplified 4.7-kbp *Sac*I fragment generated from ecotropic pBR-NE-8 MuLV proviruses in all four tumors shown (Fig. 4B) and in eight additional tumors tested (data not shown). In some tumor DNAs, additional fragments of different lengths were also detected. These results are compatible with the presence of ecotropic and MCF-type recombinant proviruses in each tumor.

Nucleotide sequence of pBR-NE-8 DNA region harboring the determinant of paralysis. By constructing chimeric viruses whose genomes were derived from parental neurotropic paralytogenic Cas-Br-E MuLV (pBR-NE-8 MuLV) and from nonneurotropic, nonparalytogenic amphotropic 4070A MuLV, we previously found that the determinant of paralysis resided within the 3.9-kbp *Sal*I-*Cla*I *pol-env* fragment of pBR-NE-8 MuLV (7). More recently, using the same approach, we found that the determinant resided within a shorter 2.3-kbp *Xba*I-*Cla*I fragment (P. Savard, E. Rassart, and P. Jolicoeur, unpublished results). This fragment encodes the end of *pol* and all of *env* (see Fig. 7). To determine the molecular basis of this biological characteristic and to construct finer chimeric MuLV viruses, we sequenced this viral DNA region. The sequence was determined by the Maxam and Gilbert procedure (33), and the sequencing

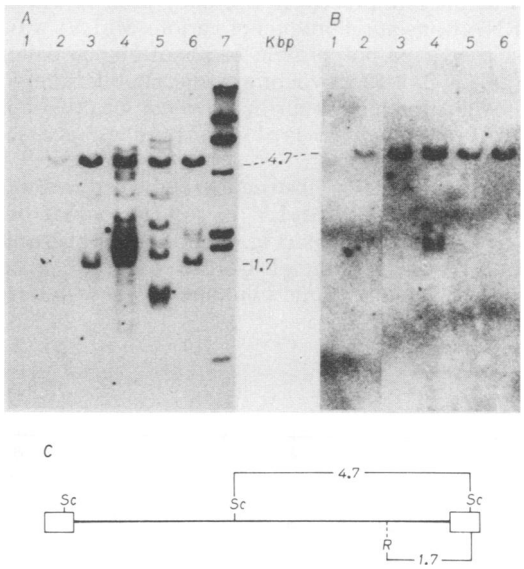

FIG. 4. *Eco*I-*Sac*I analysis of proviruses in Cas-Br-E MuLV-induced tumors. DNAs from leukemic organs were digested with *Eco*RI and *Sac*I and fractionated by electrophoresis on a 1% agarose gel. DNA fragments were transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled U3 LTR (A) or NE-8B (B) Cas-Br-E MuLV probes. Lanes: 1, control NIH Swiss liver DNA; 2, Cas-Br-E MuLV-infected NIH 3T3 cell DNA; 3, tumor N012 DNA; 4, tumor 1284-11 DNA; 5, tumor N011 DNA; 6, tumor N009 DNA. *Hin*dIII-digested λ DNA was used as marker (lane 7). (C) Partial restriction map of Cas-Br-E MuLV genome, showing the size of the expected ecotropic MuLV-specific fragments and the position of the *Eco*RI site in putative MCF-type recombinants of Cas-BR-E MuLV. Open boxes, LTR. Numbers are lengths in kilobase pairs. Restriction sites: R, *Eco*RI; Sc, *Sac*I.

strategy is illustrated in Fig. 5. For the nucleotide sequence and the deduced amino acid sequence of the pBR-NE-8 *pol-env* region, see Fig. 7. The positions of the stop codons in the three possible reading frames are shown in Fig. 6.

The first long open reading frame encountered represents the carboxyterminus of the *pol* gene, which terminates at position 847 (Fig. 7). A comparison of the deduced pBR-NE-8 *pol* amino acid sequence with that of the corresponding sequence of the nonparalytogenic Moloney MuLV (45) revealed the complete identity of the 216 amino acids following the *Hin*dIII site (residues 28 to 243 in Fig. 8; nucleotides 82 to 730 in Fig. 7), indicating that this *pol* region does not harbor the determinant of paralysis. However, more variation was observed within the last 41 amino acids of the *pol* gene and 14 differences were found between the pBR-NE-8 and Moloney MuLV sequences. This *pol* region was previously reported to be polymorphic among different strains of MuLVs such as AKV and Moloney MuLVs (19, 45).

The second long open reading frame present in the sequenced region corresponds to the *env* gene (Fig. 6). Previous studies with other retroviruses showed that the *env* gene of murine retroviruses is transcribed as a spliced mRNA (15, 43). Shinnick et al. (45) noticed a putative splice acceptor site (CACTTACAG) in Moloney MuLV, 276 bp upstream from the *env* precursor amino terminus. However, the splice acceptor site could be CTCTCCAAG (2), a sequence 11 bp upstream from the splice acceptor site described by Shinnick et al. (45). Since both of these sequences are present in pBR-NE-8 DNA, at the same location (positions 504 and 515; Fig. 7) as in Moloney MuLV, we postulate a similar splicing mechanism for the neurotropic pBR-NE-8 *env* mRNA as the one present in Moloney MuLV. The spliced *env* mRNA translation product of various MuLVs was found to be a precursor polyprotein of about 80,000 daltons (Pr gp80) (14, 26, 34). Removal of a leader peptide from Pr gp80 generates gp80, which is further cleaved into two polypeptides, gp70 and Prp15E. Prp15E is further processed into p12E (47, 48).

As expected, the overall structure of the deduced *env* gene product of pBR-NE-8 MuLV is similar to that of other MuLV *env* gene products (Fig. 7 and 9). The first putative initiator ATG codon of the pBR-NE-8 *env* gene is at position 789, and the reading frame continues without interruption

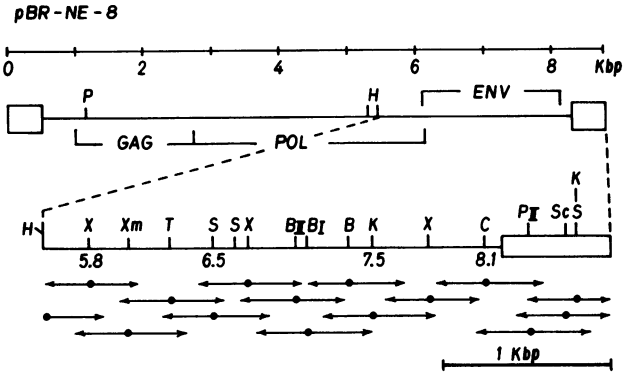

FIG. 5. Strategy for sequencing the 3′ end region of Cas-Br-E MuLV. The LTRs are represented as open boxes. The arrows indicate DNA regions sequenced from the restriction site. In most regions, both strands were sequenced. Restriction sites: B, *Bam*HI; BI, *Bgl*I; BII, *Bgl*II; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; PII, *Pvu*II; S, *Sma*I; Sc, *Sac*I; T, *Taq*I; X, *Xba*I; Xm, *Xma*III.

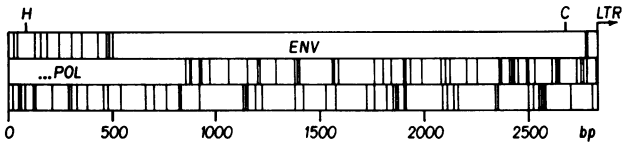

FIG. 6. Distribution of the termination codons present in the three reading frames of the + strand of the 3′ end of the Cas-Br-E MuLV genome. The *Hin*dIII (H) and *Cla*I (C) restriction sites are indicated.

until position 2773, 44 bp upstream from the LTR. This open reading frame overlaps with the end of the *pol* gene, as already found for other MuLVs (19, 45), and represents most likely the precursor polyprotein of the *env* gene (Pr gp80). No other methionine codon is found upstream of position 789 in the same reading frame. Since the direct amino acid sequencing of pBR-NE-8 gp70 has not been done, we cannot determine the amino terminus of gp70 from our DNA sequence. Moloney (35), Rauscher (44), and Friend (6, 31) MuLV gp70 proteins have been found to start with an alanine residue (Fig. 9), but AKV (30) MuLV gp70 protein was found to harbor a valine residue at the same position. The amino acid homology between pBR-NE-8 and other MuLVs in this region is too poor (Fig. 9) to allow speculation on the first residue of gp70 from Cas-Br-E MuLV.

The carboxy terminus of pBR-NE-8 gp70 is probably located just before the amino terminus of p15E, which is a glutamic acid residue in all MuLVs sequenced to date (nucleotide 2185; Fig. 7) (35). The Prp15E in Moloney MuLV is processed into p12E by cleavage of the last 17 amino acids at the carboxy terminus (30, 45) from the leucine residue at position 686 (Fig. 9). Since pBR-NE-8 MuLV Prp15E has the same length as that of Moloney MuLV and is identical to it in this cleavage region (Fig. 9), we postulate that a 17-amino-acid peptide is cleaved off pBR-NE-8 MuLV Prp15E for maturation into p12E, as in Moloney MuLV (45).

Comparison of the *env* amino acid sequence of pBR-NE-8 MuLV with that of other MuLVs. The neurotropic Cas-Br-E MuLV, or its molecularly cloned derivative pBR-NE-8 MuLV, has the unique property of inducing paralysis. Since this determinant resides within the sequenced *pol-env* region (7), a direct comparison of pBR-NE-8 *env* sequences with the corresponding sequences of other nonparalytogenic MuLVs could be of interest. Such a comparison of the amino acid *env* sequences of ecotropic pBR-NE-8, Moloney, AKV, and Friend MuLVs and of MCF-247 MuLV is shown in Fig. 9.

The region of pBR-NE-8 MuLV Pr gp80 corresponding to the leader sequences (positions 1 to 32; Fig. 9) is very different from that of other MuLVs, although it has conserved a highly hydrophobic content that is typical of membrane precursor protein (12). It is more related to the MCF-247 than to other MuLV leader sequences. However, the amino acid sequence of this peptide of each MuLV is highly polymorphic, and the significance of these variations remains to be elucidated.

The amino acid sequence of pBR-NE-8 MuLV gp70 has conserved all the cysteine residues and might have a similar conformation to those of other MuLV gp70 proteins. However, its sequence is unique and shows several differences from other gp70 MuLV sequences. These amino acid differences are distributed along the gp70 molecule. They appear to be clustered in small domains of the molecule, such as at positions 58 to 60, 97 to 102, 114 to 119, 146 to 154, 174 to

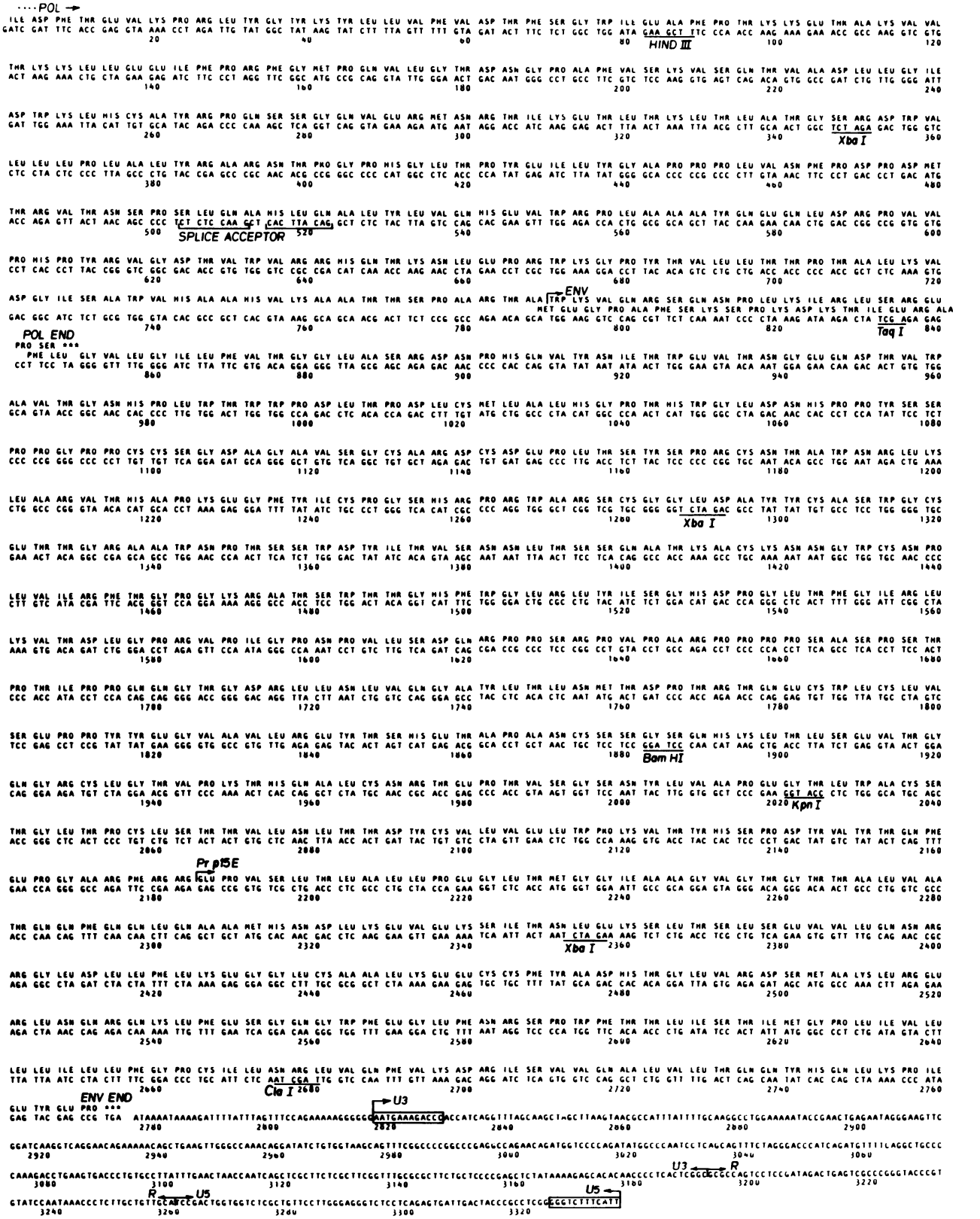

FIG. 7. Nucleotide sequence of the 3′ end of the Cas-Br-E MuLV genome. The deduced amino acid sequence of the *pol* and *env* gene products is shown above the nucleotide sequence. Some restriction sites and the potential *env* mRNA splice acceptor sequences are indicated. Symbols: ***, termination codon of *pol* and *env* gene products; arrows, beginning of *env*, of the Prp15E protein, and of the different LTR regions; boxes, inverted repeats.

177, 244 to 249, 360 to 369, 391 to 398, and 436 to 442, and in one larger domain (positions 299 to 345) (Fig. 9). The high proline content seen between positions 285 and 341 is conserved, although the pBR-NE-8 sequence is significantly different from the other sequences.

The gp70 proteins of retroviruses are known to be glycosylated (42). Glycosylation occurs on asparagine residues in sequences Asn-X-Thr and Asn-X-Ser (32). Figure 9 shows seven potential glycosylation sites in the pBR-NE-8 MuLV gp70 molecule. Four of these sites are common with those of ecotropic and MCF MuLVs. One site is present only in gp70 from ecotropic MuLVs (position 207), and one site is unique to pBR-NE-8 MuLV gp70 (position 194). Another site is absent only in pBR-NE-8 MuLV gp70 (position 396). Therefore, two different putative glycosylation sites (positions 194 and 396) distinguish the gp70 of pBR-NE-8 MuLV from those of other MuLVs.

The Prp15E pBR-NE-8 MuLV amino acid sequence is very similar to the corresponding sequence from other MuLVs (Fig. 9), suggesting that this protein is not likely to harbor the determinant of paralysis.

DISCUSSION

The Cas-Br-E MuLV, or its molecularly cloned derivative pBR-NE-8 MuLV, is a bipotential pathogenic retrovirus, having the ability to induce hind-limb paralysis and leukemia in inoculated mice (7). By restriction endonuclease analysis and T_1 oligonucleotide fingerprinting, its genome was previously shown to be distinct from the genome of other murine retroviruses, most notably in the *env* region (5, 7, 29). We have exploited these nucleic acid differences to derive probes specific to the Cas-Br-E MuLV genome. At high stringency, the *env*-specific probes did not hybridize to any of the MuLV genomes tested, while the U3 LTR-specific probe hybridized also, but to a lower extent, to the amphotropic (4070-A) and Moloney MuLV genomes. None of them hybridized to *Eco*RI fragments of mouse genomic DNA. These probes will be useful tools in the study of the virus cycle and in the detection of Cas-Br-E MuLV nucleic acids in brains of paralyzed mice or in infiltrated tissues of leukemic mice, since they will not detect endogenous retroviral sequences. We have started to exploit their uniqueness to detect proviruses in pBR-NE-8 MuLV-induced tumors. We found that all these tumors appeared clonal (or oligoclonal), like most tumors induced by MuLVs. Moreover, we could document the presence of typical MCF-type MuLV proviruses in leukemic tissues and possibly the presence of other recombinant MuLV proviruses whose structure seemed different from those of MCF MuLVs. Further studies on the structure of these proviruses require their molecular cloning in a procaryotic vector. The U3 LTR-specific probe will be quite adequate for this task.

To better understand the molecular nature of the paralysis-inducing potential of this virus, we sequenced the 3′ end region of the genome (from the *Hin*dIII site to the LTR), which is known to include the determinant of paralysis. We have indeed recently found that a chimeric virus harboring the *Xba*I-*Cla*I fragment (nucleotides 347 to 2675 in Fig. 7) of pBR-NE-8 MuLV and all other regions of the nonparalytogenic amphotropic 4070-A MuLV could induce paralysis (Savard, et al., unpublished experiments). The result with this chimeric virus, in addition to the information obtained by the present sequencing work and the possibility of deducing the amino acid sequence, has allowed us to map the determinant of paralysis within a shorter region of the

```
                          20                  40
pBR-NE-8  IDFTEVKPRLYGYKYLLVFVDTFSGWIEAFPTKKETAKVVTKKLLEEIFP
MOL-ECO   .....I..G.........I..............................
AKV-ECO   .....G.................V......R...R..S.........

      60                  80                 100
RFGMPQVLGTDNGPAFVSKVSQTVADLLGIDWKLHCAYRPQSSGQVERMNRTIKETLTKL
..........................................................
.........S......T.Q...S....................................

      120                 140                160
TLATGSRDWVLLLPLALYRARNTPGPHGLIPYEILYGAPPPLVNFPDPDMTRVTNSPSLQ
..........................................................
...A.T.....................................H....SEL.......

      180                 200                220
AHLQALYLVQHEVWKPLAAAYQEQLDRPVVPHPYRVGDTVWVRRHQTKNLEPRWKGPYTV
..........................................................
......QT..R.I.K...E..RD...Q..I...F.I..S...................

      240                 260                280
LLTTPTALKVDGISAWVHAAHVKAA---TTSPARTAWKVQRSQNPLKIRLSREPS*
............A..I........DPGGGPSS.LT.R............T..AP*
................I.............P.IKPS.R............T.GAP*
```

FIG. 8. Alignment of the amino acid sequence of the carboxy terminus of the *pol* gene of Cas-Br-E MuLV with comparable sequences of ecotropic MuLVs. The amino acid sequence deduced from the nucleotide sequence of Cas-Br-E MuLV *pol* gene was compared with the homologous sequences of Moloney (45) (MOL-ECO) and AKR (19) (AKV-ECO) MuLVs. The standard one-letter abbreviations of amino acids were used. Symbols: dots, amino acid identities with the top sequence from pBR-NE-8 (Cas-Br-E) MuLV; dashes, an amino acid gap; *, termination codon; numbers, relative position of amino acids counting the gaps introduced to align the sequences. The *Hin*dIII (H), *Xba*I (X), and *Taq*I (T) sites correspond, respectively, to positions 27, 116, and 280.

genome by excluding much of the *pol* gene. Indeed, we found a complete identity of the amino acids (residues 28 to 243 in Fig. 8 and nucleotides 82 to 730 in Fig. 7) encoded by the Cas-Br-E and Moloney MuLV *pol* genes. Since Moloney MuLV is nonparalytogenic, the determinant of paralysis must reside within the 1,945-bp fragment delineated by residue 243 (nucleotide 730) at the end of the *pol* gene and by the *Cla*I site (nucleotide 2675) at the end of Prp15E. It therefore appears that the *env* gene is an excellent candidate for harboring the determinant of paralysis, but the numerous amino acid substitutions seen in the protein encoded by this region as compared with the corresponding sequences from other nonparalytogenic MuLVs preclude the mapping of the determinant of paralysis more precisely. Within the *env* gene, the encoded Prp15E from Cas-Br-E MuLV is unlikely to carry the determinant of paralysis, since its sequence diverged very little from that of Prp15E of other nonparalytogenic MuLVs. However, several domains within Cas-Br-E gp70 have diverged significantly from sequences of other nonparalytogenic MuLVs, and any one of them could harbor the determinant of paralysis. The neurotropism of the Cas-Br-E MuLV could be caused by its ability to recognize new cellular receptor(s) and consequently infect new types of cells, notably brain cells. Alternatively, other domains of Cas-Br-E gp70, not responsible for receptor recognition, could be involved in paralysis.

Recently, Yuen et al. (49), studying a temperature-sensitive mutant of Moloney MuLV which induces hind-limb paralysis, also mapped the determinant of paralysis within a 1.6-kbp *Hin*dIII-*Bam*HI fragment spanning the end of *pol* and the beginning of gp70. If the molecular nature of the defect leading to paralysis by each virus is identical, it would indicate that the sequences responsible for paralysis map within the 820-bp fragment delineated by residue 243 (nucleotide 730) at the end of the *pol* gene (Fig. 7), as we found, and by the *Bam*HI site which would be present at a site equivalent to nucleotide 1550 in our sequence (Fig. 7), as found by Yuen et al. However, it is conceivable that the

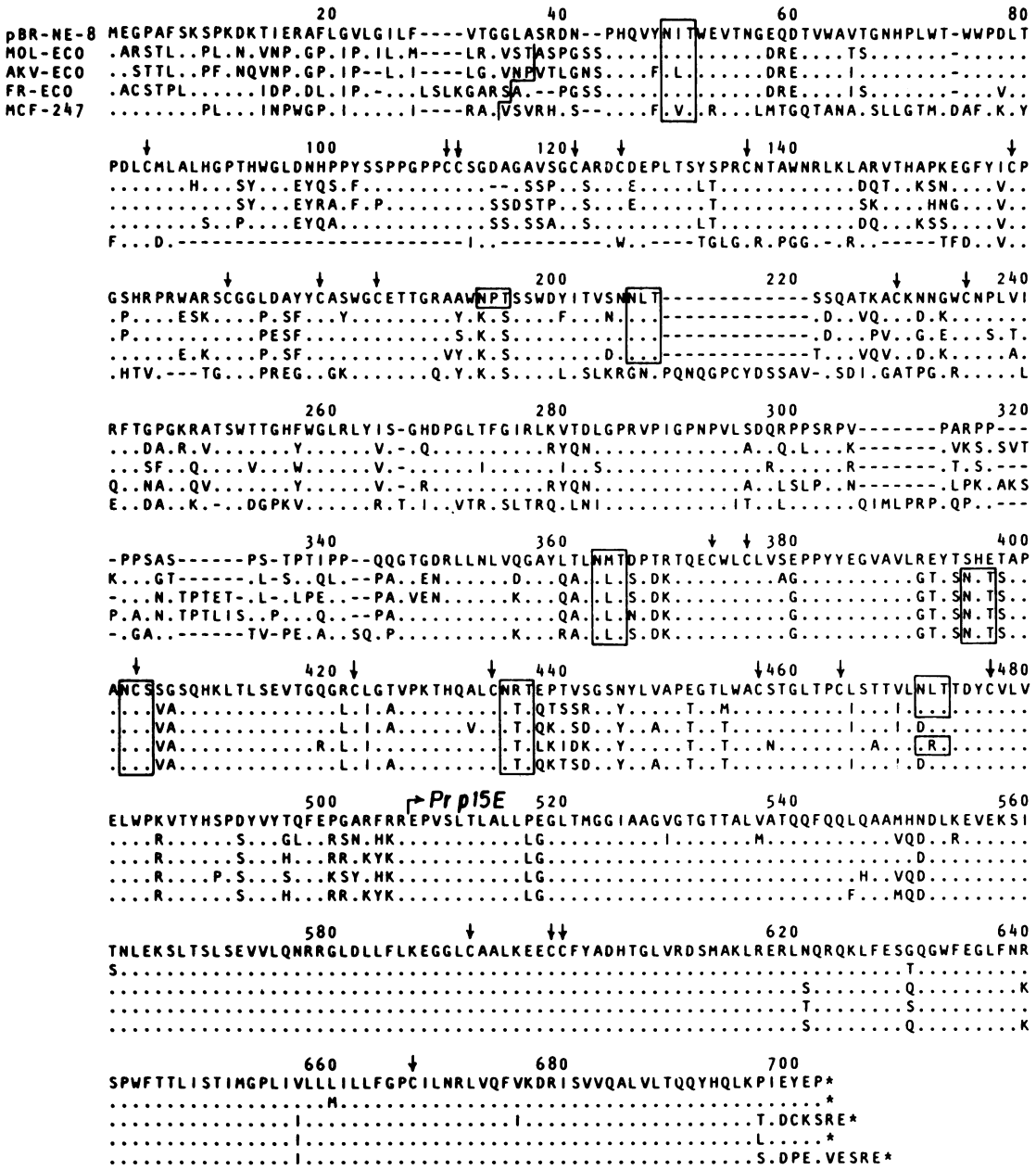

FIG. 9. Alignment of the amino acid sequence of the *env* gene of Cas-Br-E MuLV with comparable sequences of ecotropic and MCF MuLVs. The amino acid sequence deduced from the nucleotide sequence of Cas-Br-E MuLV *env* gene was compared with the homologous sequences of Moloney (45) (MOL-ECO), AKR (19, 30) (AKV-ECO), Friend (28) (FR-ECO), and MCF-247 (23, 27) MuLVs. The standard one-letter abbreviations of amino acids were used. Symbols: dots, amino acid identities with the top sequence of pBR-NE-8 (Cas-Br-E) MuLV; dashes, one amino acid gap per dash; *, termination codon; open rectangles, potential glycosylation sites; arrows, cysteine residues; numbers, relative position of amino acids counting the gaps introduced to align the sequences; vertical lines at positions 34 to 37, amino terminus of gp70 for each MuLV strain.

determinant of paralysis maps at different locations within these two viruses, since the temperature-sensitive Moloney MuLV also showed an important defect of maturation of the *env* polyprotein Pr gp80 (49), which was not seen with Cas-Br-E MuLV (22). The construction of chimeric MuLV genomes harboring smaller regions from the paralytogenic parental genome should yield to the more precise mapping of this determinant.

Origin of Cas-Br-E MuLV. The Cas-Br-E MuLV and other similar paralytogenic MuLVs were isolated from the brains of paralyzed wild mice trapped in Lake Casitas, Calif. (16, 17). Amphotropic nonparalytogenic MuLVs were also isolated from the same animals (16–18, 36). Our present data shed some light on the molecular origin of this neurotropic MuLV. The availability of *env* probes, which encompass a large portion of the region known to harbor the determinant of paralysis and which do not hybridize to any of the other viral genomes tested, has allowed us to test whether these sequences are derived from endogenous mouse sequences. Our data are very clear regarding this point, and we could

not detect any fragment hybridizing with these Cas-Br-E MuLV *env* probes in several mouse strain DNAs tested, suggesting that these *env* sequences are not part of the normal genome of *M. musculus*. Therefore, these neurotropic *env* sequences are unlikely to have been captured by recombination with endogenous mouse sequences, unless such sequences are present in the genome of some wild mice. These paralytogenic viruses have probably emerged by successive additions of point mutations, small insertions, and deletions within the *pol-env* region of the molecule. Interestingly, the U3 LTR regions of Cas-Br-E (Fig. 7) and amphotropic (R. Friedrich, personal communication) MuLVs are very homologous, suggesting a common origin for these viruses. The initial isolation of these two classes of MuLVs in the same mouse already suggested this common origin. It is conceivable that the Cas-Br-E leukemogenic and paralytogenic MuLV emerged from the nonparalytogenic amphotropic MuLV.

Sequencing of the 3′ end *pol* region from Cas-Br-E MuLV revealed complete identity of 216 residues of the deduced amino acid sequences (residues 28 to 243 in Fig. 8) with that of Moloney MuLV. This conservation is remarkable, since the same sequences from endogenous AKR (19) and Moloney (45) MuLVs do not share this homology and are more divergent. This observation tends to support the hypothesis of a common origin for Cas-Br-E and Moloney MuLVs. The conservation of few restriction sites on both genomes (*Pst*I at position 1.2, *Hin*dIII at 5.3, *Xba*I at 5.8, *Sma*I at 6.5, *Kpn*I at 7.5, *Cla*I at 8.1, and within the LTRs, *Pvu*II and *Sac*I) that were never present in the several endogenous viral genomes isolated from different mouse strains also favors a common origin for these two viruses. Then, both the leukemogenic Moloney and paralytogenic Cas-Br-E MuLVs could have emerged from nonparalytogenic amphotropic MuLVs. The amphotropic MuLV appears to be present in the population of wild mice as an exogenous retrovirus, since its genome was previously found to be absent from the normal mouse genome (1). Our data tend to confirm these findings. Indeed, the pBR-NE-8 MuLV U3 LTR probe (*Stu*I-*Sac*I fragment), whose sequence is identical, except for five nonclustered point mutations, to the corresponding U3 LTR sequences from amphotropic MuLV (Friedrich, personal communication), could not detect homologous endogenous viral sequences at high stringency (Fig. 2). It appears that amphotropic MuLV, the putative progenitor of Cas-Br-E MuLV, is not endogenous to *M. musculus*. Its origin remains unknown. It could have originated from another species and established itself in some wild mice. Alternatively, an endogenous provirus could have sustained multiple genetic alterations and evolved to the point of appearing as an exogenous genome.

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