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Retrovirus-induced murine motor neuron disease: Mapping the determinant of spongiform degeneration within the envelope gene

(murine leukemia virus)

YVES PAQUETTE*, ZAHER HANNA*, PIERRE SAVARD*, ROLAND BROUSSEAU†, YVES ROBITAILLE‡, AND PAUL JOLICOEUR*§¶

*Laboratory of Molecular Biology, Clinical Research Institute of Montreal, 110 avenue des Pins ouest, Montréal, PQ, H2W 1R7, Canada; †Biotechnology Research Institute of Montreal, Montreal, PQ, H4P 2R2, Canada; ‡Department of Neuropathology, Montreal Neurological Institute, McGill University, H3A 2B4, Canada; and §Département de Microbiologie et d'Immunologie, Université de Montréal, Montréal, PQ, H3C 3J7, Canada

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ABSTRACT **The Cas-Br-E murine leukemia virus (MuLV) induces a degenerative myeloencephalopathy leading to hind-limb paralysis when inoculated into newborn mice. To map the viral DNA sequences encoding the determinant of neurological degeneration, we constructed chimeric viruses *in vitro* with parental genomes from Cas-Br-E MuLV and from nonparalytogenic MuLVs. We found that a 1.5-kilobase-pair *env* Cas-Br-E fragment was sufficient to confer the full paralysis-inducing potential to chimeric viruses. This region encodes the 19 carboxyl-terminal residues of the leader sequence, all of gp70, and the 45 amino-terminal residues of the transmembrane protein (p15E). Within this *env* region, we identified a 372-base-pair fragment which was necessary for the full paralysis-inducing potential of the virus and which influenced the development of the disease in a strain-dependent manner. This domain encodes the 19 carboxyl-terminal residues of the leader peptide and the first 67 amino-terminal residues of gp70. We propose that Cas-Br-E MuLV induces spongiform degeneration through binding of its gp70 to a specific cellular receptor.**

The Cas-Br-E wild mouse ecotropic murine leukemia virus (MuLV) has been shown to induce a progressive form of hind-limb paralysis after inoculation into susceptible laboratory mice (for review, see refs. 1 and 2). This clinical syndrome is the consequence of a spongiform myeloencephalopathy predominantly involving the brainstem and the anterior horn of the lumbosacral region of the spinal cord (3–6). Using chimeric viruses constructed *in vitro*, we have previously mapped the primary determinant of this spongiform degeneration within a 3.9-kilobase-pair (kbp) *pol–env* fragment of the viral genome (7). Sequencing of this region revealed that the *pol* gene product and p15E diverged very little from homologous proteins of other nonparalytogenic MuLVs, while the Cas-Br-E gp70 sequence was found to diverge significantly from that of other nonparalytogenic MuLVs (8). We also showed that the long terminal repeat (LTR) region of the genome harbors a second determinant of pathogenicity influencing the incidence of the disease and determining in which region of the central nervous system (CNS) this virus induces the spongiform lesions (9).

To determine more precisely which viral gene harbors the primary determinant of the spongiform lesions, we constructed additional chimeric viruses with DNA from parental paralytogenic Cas-Br-E and nonparalytogenic amphotropic, endogenous ecotropic, or Moloney MuLVs. We show here that chimeric viruses harboring only the *env* gene of Cas-Br-E MuLV genome induce spongiform lesions and remain paralytogenic.

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MATERIALS AND METHODS

Mice. SWR/J pregnant females were purchased from The Jackson Laboratory. SIM.S and NFS/N mice were from our breeding colony. The virus suspensions were filtered through a HAWP nitrocellulose filter (pore size, 0.45 μm; Millipore) and injected intraperitoneally (0.15 ml) into newborn (<48 hr) mice.

Cells and Viruses. The Cas-Br-E (pNE-8) molecularly cloned parental MuLV was described previously (10). The origin of NIH 3T3 cells has been given elsewhere (10, 11).

Construction of DNA Recombinants. The cloned viral DNA from Moloney (9), endogenous N-tropic BALB/c (pBR-7) (12), pNE-8 (10), amphotropic 4070-A (13), and chimeric pNEA-1 (7) MuLVs have been described. Recombinant chimeric DNAs were constructed as described (7, 9, 10), using pBR322 or pJRD184 (14) as vectors.

Synthetic Oligonucleotides. Oligonucleotides (Fig. 1) were synthesized on an Applied Biosystems model 380A nucleic acid synthesizer, using the solid-phase phosphoramidite chemistry (15). They were purified on a polyacrylamide/urea gel, annealed, and cloned in pUC18. The double-stranded inserts were sequenced by the dideoxynucleotide method (17).

Transfection Procedure. The permuted viral inserts were cleaved with appropriate restriction endonucleases and religated to obtain nonpermuted forms of the viral genome. The DNAs (5–10 μg) were transfected by the calcium phosphate procedure (18) on NIH/3T3 cells. Viruses recovered from the supernatant of these transfected cells were titered by XC assay (19) or by end-point dilution.

Preparation of Unintegrated Viral DNA. NIH 3T3 cells were cocultivated with chronically infected cells in the presence of Polybrene at 2 μg/ml. Hirt supernatant DNA was prepared after 48 hr of cocultivation as described (7, 11). Restriction analysis of unintegrated viral DNA was performed by hydridization of Southern blots with ^{32}P-labeled probes, as described (7, 11).

RESULTS

Construction of Chimeric MuLV Genomes. To determine which region of the Cas-Br-E viral genome carries its paralysis-inducing determinant, we constructed chimeric viral genomes *in vitro* with cloned viral DNA from parental paralytogenic Cas-Br-E (pNE-8) MuLV and nonparalytogenic amphotropic 4070-A, endogenous BALB/c N-tropic (N-Cl-35, pBR-7), or Moloney MuLVs (Fig. 2). The recombinant pNE-XC harbored the 3′ end of *pol* and the *env* from pNE-8

Abbreviations: MuLV, murine leukemia virus; LTR, long terminal repeat; CNS, central nervous system.

¶To whom reprint requests should be addressed at: Clinical Research Institute of Montreal, 110 Pine Avenue West, Montréal, PQ, H2W 1R7, Canada.

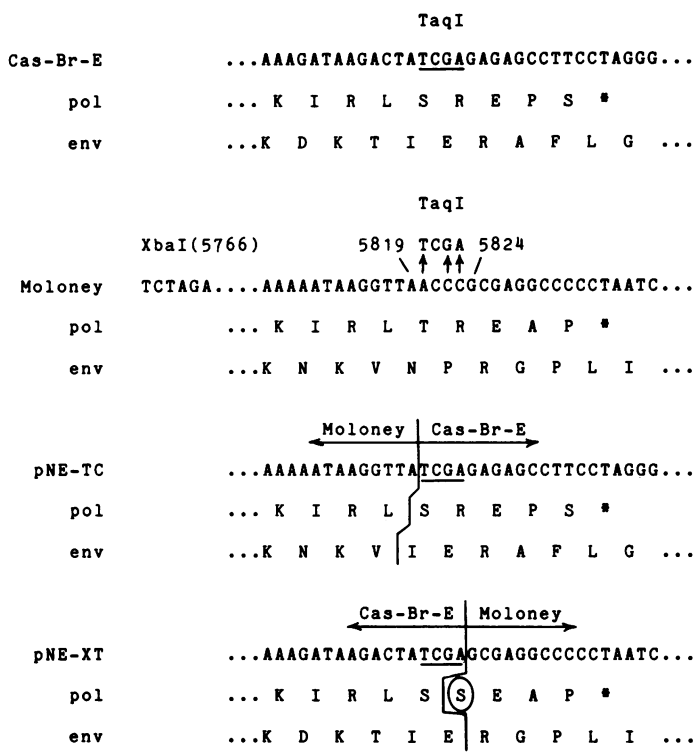

FIG. 1. Nucleic acid and amino acid sequence of the chimeric junction region of the pNE-TC and pNE-XT recombinants. To construct recombinant pNE-TC, we used a 55-base-pair (bp) chimeric *Xba* I–*Taq* I oligonucleotide having the Moloney MuLV sequence from nucleotide 5766 (*Xba* I site) to nucleotide 5819, followed by a novel *Taq* I recognition site (nucleotides 5820–5823). The nucleotide changes introduce no amino acid modification in the pol or env proteins. To construct recombinant pNE-XT, we used a 105-bp chimeric *Taq* I–*Bst*EII oligonucleotide having the Moloney MuLV sequence from nucleotide 5824 to nucleotide 5923, in front of which the *Taq* I recognition site (nucleotides 5820–5823) was introduced. This modification introduces a mutation in the pol protein, an arginine residue being replaced by a serine residue (circled). Each oligonucleotide was flanked by *Hin*dIII and *Eco*RI sites for inserting into pUC18. Arrows indicate nucleotides modified in the Moloney MuLV sequence to introduce the *Taq* I site. Positions are numbered according to Shinnick *et al.* (16).

MuLV. To construct it, we exchanged most of the Cas-Br-E *pol* gene from a previously characterized paralytogenic pNEA-1 MuLV genome (7). Recombinant pNE-TC was constructed to harbor only the *env* gene from Cas-Br-E MuLV spanning the leader region of gp70 to the amino-terminal third of p15E. The pNE-SC recombinant was identical to pNE-TC MuLV, except in the amino-terminal portion of gp70, which was substituted for Moloney sequences. The

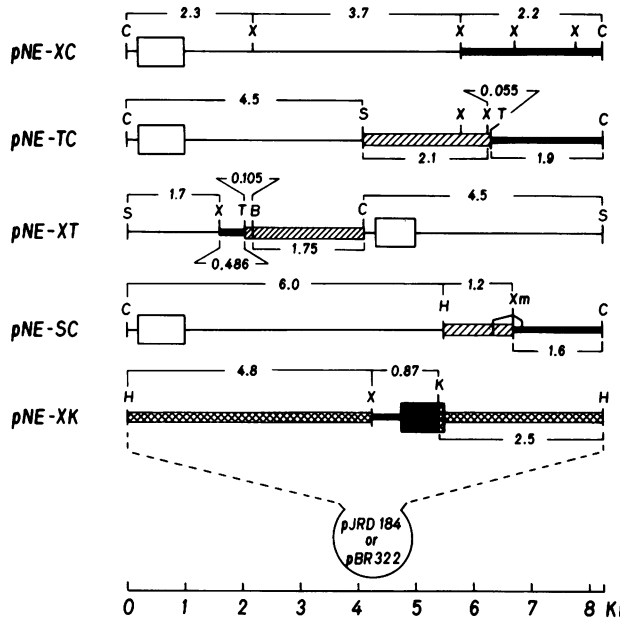

FIG. 2. Construction of chimeric MuLVs. The recombinant clones were constructed by ligating the fragments from various parental DNA genomes at the indicated restriction sites into pBR322 or pJRD184 vectors. The 0.055-kbp fragment of pNE-TC and the 0.105-kbp fragment of pNE-XT are synthetic oligonucleotides shown in Fig. 1. Symbols: Thick line, pNE-8 (Cas-Br-E) MuLV; thin line, 4070A (amphotropic) MuLV; hatched bar, Moloney MuLV; cross-hatched bar, pBR-7 (BALB/c endogeneous N-tropic, N-Cl-35) MuLV; boxes, LTRs; B, *Bst*EII; C, *Cla* I; H, *Hin*dIII; K, *Kpn* I; S, *Sal* I; T, *Taq* I; X, *Xba* I; Xm, *Xma* I.

pNE-XT and pNE-XK recombinants harbored only a short region of Cas-Br-E MuLV, respectively from the integrase and the p15E-LTR regions. Each chosen recombinant was characterized to determine its structure and to confirm the origin of each fragment, using a combination of restriction endonuclease analysis and hybridization with specific probes (data not shown).

Recovery and Characterization of Infectious Chimeric MuLVs. To recover infectious chimeric viruses, the recombinant viral inserts were transfected into NIH 3T3 cells. The recovered viruses were further characterized by restriction endonuclease analysis of their unintegrated viral DNA obtained by Hirt extraction of acutely infected NIH 3T3 cells, using specific probes. Clones pNE-SC, pNE-XT, and pNE-TC, analyzed with the *pol* probe, showed fragments typical of the 4070A MuLV *gag–pol* region, namely a 3.7-kbp *Xba* I fragment (not shown) and a 1.6-kbp *Hin*dIII–Sac I fragment (Fig. 3*B*, lanes 4–6). With the Cas-Br-E MuLV (pNE-8)-specific *env* probe, clones pNE-SC and pNE-TC showed a 1065-bp *Xba* I fragment typical of the pNE-8 envelope, plus a novel 500-bp *Xba* I fragment spanning the Moloney–pNE-8 MuLV DNA junction (Fig. 3*D*, lanes 4 and 5). With the *int* probe, a 441-bp *Xba* I fragment typical of Moloney MuLV was detected (Fig. 3*C*, lanes 4 and 5). In contrast, the *Xba* I digest of clone pNE-XT did not give any signal with the *env* probe (Fig. 3*D*, lane 6), as expected, since this genome does not contain this region of the pNE-8 envelope. However, with the *int* probe, a 3.0-kbp *Xba* I fragment could be detected, spanning the *pol–env*–LTR region (Fig. 3*C*, lane 6). In the *Xba* I digest of the pNE-XC chimeric viral DNA, two fragments (1065 and 940 bp) typical of the *env* region of the pNE-8 parental DNA were detected (Fig. 3*A*, lane 3). With the same DNA, *Hin*dIII generated 8.5- and 5.4-kbp fragments (respectively from supercoiled and linear molecules), longer than those of the pNE-8 control (7.5 and 4.2 kbp) (Fig. 3*A*, lane 1), indicating that the 5′ region of this chimeric virus was not derived from pNE-8 but came from the parental 4070A MuLV.

To confirm the presence of the NE-8 LTR in the pNE-XK chimeric MuLV, the viral DNA was digested with *Sac* I or *Sac* I–*Cla* I and hybridized with the NE-8-specific U3 probe. The pNE-XK DNA hybridized well with this probe, as

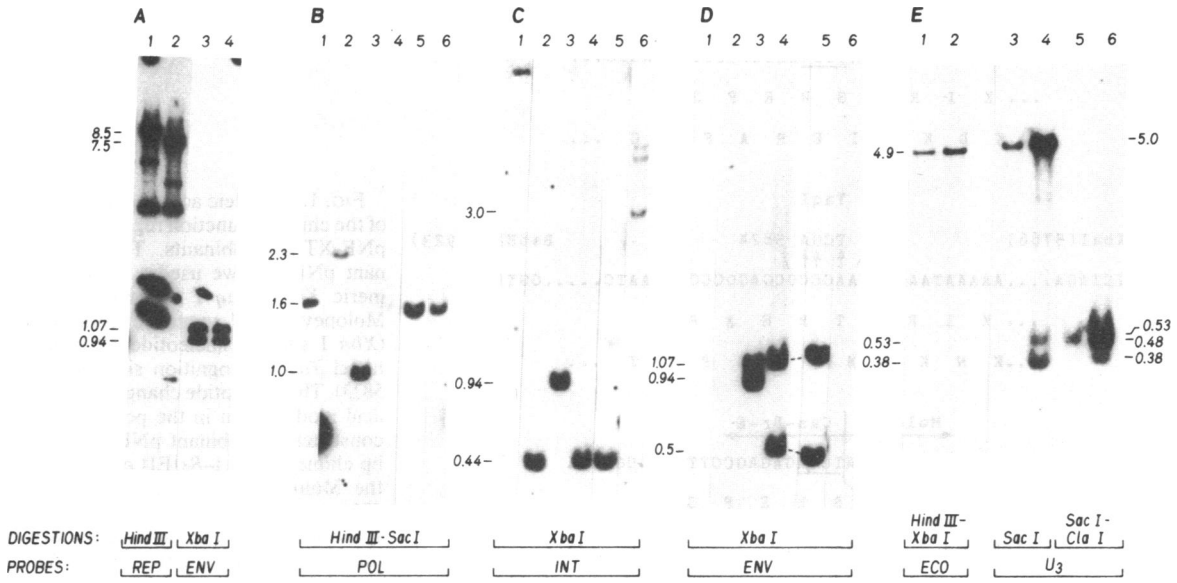

FIG. 3. Hybridization analysis of unintegrated viral DNA. The Hirt supernatant DNA from cells infected with parental or chimeric viruses was digested with appropriate restriction enzymes, electrophoresed on 1% agarose gels, and transferred to nitrocellulose or nylon membranes. The probes used for hybridizations (see Fig. 4) are the following: the representative retroviral probe (REP) is derived from the whole genome of BALB/c B-tropic endogenous ecotropic MuLV; the POL probe comes from the 1.0-kbp *Hin*dIII fragment of pNE-8; the INT probe is made out of the 441-bp *Xba* I fragment of Moloney MuLV which maps at the 3′ end of the *pol* gene; the ecotropic MuLV-specific (ECO) (20) and the pNE-8 ENV and U3-specific probes (8) have been described. The probe inserts were excised with appropriate restriction endonucleases, purified on agarose gels, and ^{32}P-labeled by random hexanucleotide primer extension (21). (*A*) The viral DNA from pNE-XC MuLV digested with *Hin*dIII (lane 1) or *Xba* I (lane 3), compared to the pNE-8 DNA cut with *Hin*dIII (lane 2) or *Xba* I (lane 4). Lanes 1 and 2 were hybridized with the REP probe and lanes 3 and 4 with the pNE-8-specific ENV probe. The 8.5- and 7.5-kbp fragments are derived from circular molecules. The blotches around 1.5–2 kbp are artefacts. (*B*, *C*, and *D*) DNA from pNE-TC (lane 4), pNE-SC (lane 5), and pNE-XT (lane 6) MuLVs compared to the DNA from parental Moloney (lane 2) and pNE-8 (lane 3) MuLV and from cloned 4070A (lane 1) MuLV. (*B*) DNAs cut with *Hin*dIII–*Sac* I and hybridized with the POL probe. (*C*) DNAs cut with *Xba* I and hybridized with the INT probe. The double bands at 4.5 and 5 kbp represent LTR fusion fragments from supercoiled DNA with one and two LTRs, respectively. (*D*) Same filters as in panel *C*. The filter was washed and hybridized with the pNE-8-specific ENV probe. (*E*) DNA from clone pNE-XK cut with *Xba* I–*Hin*dIII (lane 1), *Sac* I (lane 3), or *Sac* I–*Cla* I (lane 5), compared to the DNA from parental pNE-8 cut with *Sac* I (lane 4) and *Sac* I–*Cla* I (lane 6) and to the DNA from parental pBR7 cut with *Xba* I–*Hin*dIII (lane 2). The filter was hybridized with the ECO probe (lanes 1 and 2) or with the U3NE-8 probe (lanes 3–6). The 0.53-kbp fragments in lanes 4 and 6 are permuted LTRs derived from circular molecules.

expected for a molecule containing the LTR from pNE-8; as in the NE-8 control, it shows a band at 5.0 kbp coming from the 3′ part of the viral DNA molecule, plus a small, 338-bp, fragment corresponding to the 5′ LTR (Fig. 3*E*, lane 3). The pNE-8 lane also shows a 521-bp fragment originating from circular molecules carrying two LTRs in tandem. When cut with *Sac* I plus *Cla* I, the 5.0-kbp *Sac* I fragment generated a 480-bp fragment from both DNAs, confirming the presence of a *Cla* I site in the chimeric molecule (Fig. 3*E*, lanes 5 and 6). To prove that the coding region of pNE-XK was derived from the pBR7 parent, this viral DNA was cleaved with *Xba* I–*Hin*dIII and hybridized with a gp70 ecotropic MuLV-specific (*eco*) probe. In both cases, the pNE-XK DNA showed fragments identical to those of the pBR7 control (Fig. 3*E*, lanes 1 and 2). This analysis is illustrated in Fig. 4.

Paralysis-Inducing Potential of Chimeric MuLVs. To identify the viral sequences responsible for the induction of neurological disease, the chimeric MuLVs were inoculated intraperitoneally into newborn (<48 hr) susceptible SWR/J, SIM.S, or NFS/N mice. Mice were checked frequently for manifestations of the disease (spasticity, tremulousness, hind-limb paralysis, splay reflex), and those showing signs of paralysis were sacrificed a few days before their anticipated death. As shown in Fig. 5, mice inoculated with pNE-XK or pNE-XT chimeric MuLVs showed no sign of paralysis or other neurological disease more than 200 days after injection, indicating that the integrase, the amino-terminal half of the *env* leader peptide, and the 3′ end of *env*, encoding most of p15E, do not harbor the determinant of paralysis. However, these viruses replicated well *in vivo*, since 8 out of 18 SWR/J and 7 out of 23 SIM.S mice inoculated with pNE-XK MuLV and 3 out of 7 pNE-XT MuLV-inoculated NFS/N or SWR/J mice had lymphomas at the time they were sacrificed. Mice inoculated with pNE-XC or pNE-TC chimeric MuLVs showed a high incidence of paralysis (80–100%), indicating that the *env* gene (and not the *pol* gene) harbors the determinant of paralysis. The longer latency seen with pNE-TC MuLV might have been caused by the lower virus titer at injection. SWR/J mice injected with the pNE-SC virus also developed neurologic disease; however, except for 4 of these mice which showed the typical signs of hind-limb paralysis, the majority of them developed a milder form of paralysis which did not progress as rapidly as with the other chimeric viruses or as with the parental Cas-Br-E MuLV. Most (19/21) of these pNE-SC MuLV-inoculated SWR/J also had leukemia. Interestingly, SIM.S mice inoculated with pNE-SC MuLV did not show clinical signs of neurological disease, even though a significant percentage of them (10/22) eventually developed leukemias. These results indicated that the amino-terminal region of Cas-Br-E gp70 is not essential for the appearance of the neurologic disease in SWR/J mice. However, since the substitution of this region led to a less aggressive form of hind-limb paralysis in SWR/J mice and to the absence of neurological disease in SIM.S mice, this 372-bp fragment (*Taq* I–*Xma* I) is likely to harbor part of the determinant.

Pathology. Examination of brain and spinal cord specimens from four or five mice inoculated with each paralytogenic chimeric MuLV showed spongiform lesions indistinguishable in their appearance and distribution from those induced by the parental Cas-Br-E MuLV (data not shown).

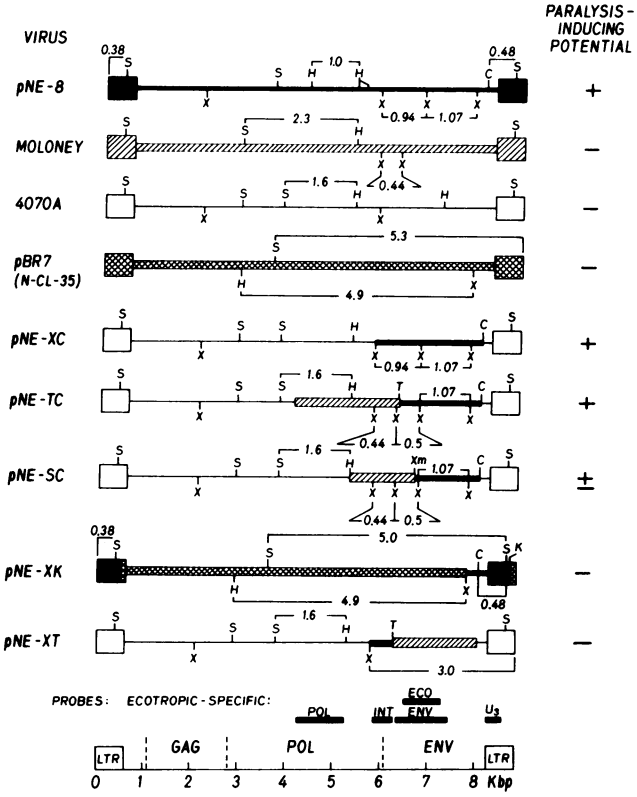

FIG. 4. Linear forms of the parental and chimeric viral DNA genomes and their paralysis-inducing potential; these genomes are represented as linear double-stranded molecules with two LTRs (boxes). The probes used for hybridizations are indicated at their correct position on the viral genomes. Numbers linking restriction sites on the viral genomes indicate the length of fragments (in kbp) detected by these probes. S, *Sac* I; see Fig. 2 legend for other key.

DISCUSSION

Paralysis-Inducing Determinant of Cas-Br-E MuLV. Our results show that the *env* gene of Cas-Br-E MuLV is the region which is necessary and sufficient to induce spongiform degeneration. We could exclude all *pol* sequences since pNE-TC (containing only the last two amino acid residues of Cas-Br-E int protein) and pNE-SC (containing only Cas-Br-E *env*, but no *pol*, sequences) MuLVs were still capable of inducing spongiform degeneration. Within the Cas-Br-E MuLV *env* region we could also identify two regions which appear nonessential for inducing neurological disease. The carboxyl terminus of the transmembrane *env*-encoded protein (p15E) seems indeed unlikely to harbor the determinant of paralysis, since the chimeric pNE-XK MuLV (which contains 139 amino acid residues of the carboxyl terminus of Cas-Br-E p15E) was totally incapable of inducing paralysis. In addition, our previous sequencing data (8) showed that the 12 amino acid residues upstream of these 139 amino acid residues were identical in Cas-Br-E and other nonparalytogenic MuLVs. Therefore, the 3′ end *env* border of the paralysis-inducing determinants of Cas-Br-E MuLV does not extend beyond residue 45 of p15E. Since pNE-TC chimeric MuLV was fully paralytogenic, the *Taq* I site borders the paralysis-inducing determinant at the 5′ end of *env*.

Interestingly, we could also identify a region within *env* which seems essential for the induction of the complete neurological disease. Comparison of pathogenicity between pNE-TC and pNE-SC chimeric MuLVs indeed showed that pNE-SC MuLV was significantly less pathogenic. The genomes of the two viruses differ by only the 19 carboxyl-terminal residues of the env leader peptide and by the 67 amino-terminal residues of gp70 encoded between the *Taq* I and the *Xma* I sites, indicating that this env domain (86 amino acid residues) harbors at least part of the paralysis-inducing determinant of the virus. However, since pNE-SC MuLV induced paralysis in some SWR/J mice, these 86 amino-terminal residues of env do not seem to be absolutely essential, suggesting that other sequences within env are critical for the induction of this neurological degeneration. Our combined data indicate that these other sequences lie within a 413-amino-acid env domain, between amino acid residue 68 of gp70 and residue 45 of p15E. Since the 45 p15E amino acid residues were previously found to be highly conserved between Cas-Br-E and other nonparalytogenic MuLVs, gp70 remains the best candidate to harbor the determinant of paralysis of Cas-Br-E MuLV. The Cas-Br-E gp70 sequences are highly divergent from those of other nonparalytogenic MuLVs. In contrast to pNE-TC MuLV,

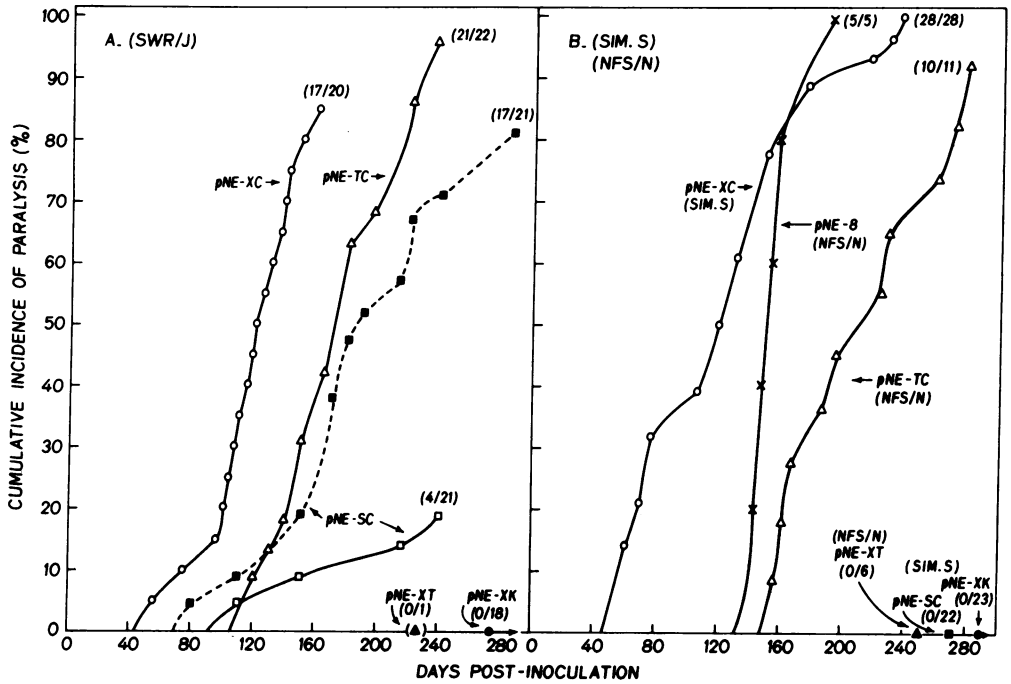

FIG. 5. Cumulative incidence of paralysis induced by inoculation of chimeric MuLVs into SWR/J (*A*) or SIM.S or NFS/N (*B*) mice. Animals were killed when they showed signs of advanced disease. Numbers in parentheses indicate the number of paralyzed mice/total number of mice inoculated. Horizontal arrows on the abscissa indicate that the mice were killed later than 300 days. Titers (plaque-forming units/ml) of viruses were pNE-XC (○), 1×10^5; pNE-TC (△), 1.5×10^4; pNE-SC (□, ■), 2.6×10^6; pNE-XT (▲), 2.0×10^4; pNE-XK (●), 1×10^5; and pNE-8 (×), 5×10^5. Mice inoculated with pNE-SC MuLV showed either typical and severe hind-limb paralysis (□——□) or only mild hind-limb paralysis (■ - - - ■).

the pNE-SC MuLV was pathogenic for SWR/J mice, but it did not induce clinical neurological disease when inoculated into SIM.S mice, although it replicated in these mice, almost half of them (10/22) eventually developing leukemias. The amino-terminal 86 residues of env seem responsible for this host range of the virus, and they appear to be essential for induction of paralysis in SIM.S mice but not in SWR/J mice. This result suggests that this env domain interacts with a cellular factor(s) that is (are) critical for the outcome of the disease and that is (are) genetically determined.

The determinant of paralysis of another neurovirulent retrovirus, ts-1 Moloney MuLV, has also recently been mapped within env, and only four amino acid residues were found to distinguish this paralytogenic strain of Moloney from the wild-type nonparalytogenic Moloney MuLV (22). In one out of four of these amino acid positions, the Cas-Br-E sequence was identical to that of wild-type Moloney MuLV, in two sites the Cas-Br-E residue was distinct from that of ts-1 and of wild-type Moloney MuLV, and in the last site (position 7250) it was similar to that of ts-1 Moloney but also to that of other nonparalytogenic MuLVs (8). This comparison indicates that the modifications which led to the emergence of a paralytogenic *env* gene in ts-1 Moloney and Cas-Br-E MuLVs are distinct.

A Model for Retrovirus-Induced CNS Spongiform Degeneration. The *env* region of some retroviruses has been implicated in pathogenesis. The ability of spleen-focus-forming virus (SFFV) to induce erythroleukemia has clearly been shown to be caused by its *env* gene product (gp55) (23). Secondary determinants of leukemogenicity of different strains of MuLVs have also been found in the *env* region of these viruses (24–26), and the gp120 *env* protein of the human immunodeficiency virus (HIV) seems responsible for some of the cytopathic effects of the virus (27), although its role in pathogenicity is not yet clear. The mechanism by which the *env* gene product (most likely gp70) of Cas-Br-E MuLV induces spongiform degeneration within the CNS remains unclear. Since the only well-known function of retroviral *env* gene product is to recognize a cellular receptor to initiate infection, we propose that the Cas-Br-E MuLV produces this spongiform degeneration through binding of its gp70 to a specific cellular receptor. (*i*) Through this receptor, the Cas-Br-E gp70 could facilitate penetration into specific CNS cells. However, the specificity of this virus for anterior horn cells does not appear to be determined by its *env* gene. We have indeed previously shown that the Cas-Br-E LTR determines this topographical specificity (7). (*ii*) Bound to this receptor, the gp70 (virion-associated or free) could compete with a physiological trophic factor, thus preventing its action. (*iii*) Alternatively, by binding to a receptor, the gp70 molecules could interfere with its normal function. For example, they could overstimulate it or they could initiate opening of ion channels or induce inappropriate synthesis of membranes or synthesis of abnormal membranes, leading to a change in plasma membrane permeability (28) and consequently to spongiform degeneration. This model assumes that Cas-Br-E gp70 has evolved to the point of recognizing a normal physiologically important CNS receptor. The *env* gp120 of HIV (29–31) and several viral proteins (32) have been shown to recognize cellular receptors. The proposed model is consistent with one major feature of the pathological process, the absence of inflammation in the spongiform degenerative lesions (3, 5, 6). If correct, this model may help to identify this receptor and its physiological ligand in the CNS. We hope that the availability of such a mouse model of retrovirus-induced CNS degenerative diseases will help us to understand the human neurological diseases induced by human retroviruses such as human T-cell leukemia virus type I (33) and HIV (34).

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