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Physical Mapping of the Paralysis-Inducing Determinant of a Wild Mouse Ecotropic Neurotropic Retrovirus

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We have recently shown that a molecularly cloned ecotropic retrovirus, initially isolated from the brain of a paralyzed wild mouse, retained the ability to induce hind limb paralysis when inoculated into susceptible mice (Jolicoeur et al., J. Virol. 45:1159–1163, 1983). To map the viral DNA sequences encoding the determinant of paralysis, we constructed chimeric viral DNA genomes in vitro between parental cloned infectious viral DNA genomes from this neurotropic murine leukemia virus (MuLV) and from nonneurotropic amphotropic 4070-A MuLV. Infectious chimeric MuLVs, recovered after microinjection of NIH 3T3 cells with these recombinant DNAs, were inoculated into newborn SIM.S and SWR/J mice to test the paralysis-inducing potential. We found that the 3.9-kilobase-pair *SalI-ClaI* fragment of the neurotropic MuLV comprising the 3′ end of *pol* and all *env* sequences was sufficient to confer the paralysis-inducing potential to chimeric viruses. Therefore, this region of the neurotropic MuLV genome most likely harbors the primary determinant of paralysis.

Several ecotropic retroviruses isolated from the brains of paralyzed wild mice have been shown to induce a progressive form of hind limb paralysis in a natural population of *mus musculus* or after inoculation into susceptible laboratory mice (for a review, see reference 9). The pathological lesions found in the brains, brainstems, and anterior horns of the spinal cords of paralyzed mice appear as a noninflammatory spongiform degeneration with marked proliferation of astroglial cells (1, 10, 23, 25). These viruses can, however, replicate very efficiently in vitro on mouse fibroblasts without any lytic effect and without affecting the morphology of these cells (2, 9, 17). Among the murine nondefective retroviruses, these neurotropic viruses have unique characteristics. They induce a different disease (paralysis) (10, 22), and their genomes have restriction endonuclease maps which are different from those of ecotropic viruses isolated from inbred mouse strains. However, the map of neurotropic viruses is similar to that of amphotropic viruses, except in the *env* region in which they diverge significantly (5, 17). Amphotropic viruses have been isolated from wild mice and do not induce paralysis in natural wild populations or after inoculation into laboratory mice (9, 14, 27).

The mechanism by which these neurotropic viruses induce paralysis is obscure. Replication of the virus seems to be essential for the appearance of the disease since no paralysis could be induced in *Fv-1*–resistant mice infected with these viruses (2, 23, 25). The *Fv-1* gene is known to block retrovirus replication at an early step of the virus cycle (16, 18, 33). Levels of the major viral protein p30 in the central nervous system (CNS) closely paralleled the severity of the disease, and the suggestion has been made that this protein might be necessary for the disease to occur (23). A role of the *env* gene product gp70 in the etiology of this disease has also been suspected (20, 23). However, no direct evidence for a role of these proteins in the paralytic process has yet been obtained.

In an effort to understand the molecular basis of this retrovirus-induced neurological disease, we recently cloned the genome of Cas-Br-E neurotropic murine leukemia virus (MuLV) in a procaryotic vector (17). The virus recovered from transfection of this cloned viral DNA genome into fibroblast cells had the same biological characteristics as the parental MuLV and has been shown to be able to induce lower limb paralysis after inoculation into newborn mice (17). To localize the viral sequences responsible for the paralysis-inducing potential of this virus, we constructed chimeric viral genomes in vitro with cloned parental viral genomes from the neurotropic Cas-Br-E MuLV (17) and the nonneurotropic amphotropic 4070-A MuLV (5). Infectious chimeric viruses were recovered from cells microinjected with recombinant DNA, and their paralysis-inducing potential was tested in mice. We show here that the portion of the genome comprising the 3′ end of the *pol* sequence and all *env* sequences is sufficient to transfer the paralysis-inducing potential to chimeric viruses.

MATERIALS AND METHODS

Cells and viruses. The origin of NIH 3T3 cells has been given elsewhere (18). The infectious MuLV recovered after microinjection of pBR-NE-8 cloned DNA from Cas-Br-E MuLV has been described previously (17). The cloned viral DNA (p4070-A) from the 4070-A amphotropic MuLV (5) was a generous gift of Allen Oliff (Memorial Sloan-Kettering Cancer Center). Amphotropic 4070-A MuLV was recovered from NIH 3T3 cells microinjected with *Eco*RI-cleaved and religated p4070-A DNA as previously described (17). Parental and chimeric MuLVs were all propagated in NIH 3T3 cells. Titers of viruses were determined on NIH 3T3 cells by the XC assay (30) and by the endpoint dilution and reverse transcriptase assay as previously described (18). Cells were grown in Dulbecco modified Eagle medium supplemented with 5% calf serum (GIBCO Laboratories, Grand Island, N.Y.).

Mice. SIM.S (35) ($Fv\text{-}1^{n/n}$) mice were obtained from our breeding colony. Pregnant SWR/J mice were purchased from the Jackson Laboratories (Bar Harbor, Maine). Supernatants from infected NIH 3T3 cells were harvested, titers were determined, and the solution was filtered through a HAWP nitrocellulose filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) before inoculation intraperitoneally (0.15 ml) into newborn (<24-h-old) mice. Inoculated mice

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were observed daily for signs of disease. Diseased mice were sacrificed when they had lower limb paralysis, which was very often accompanied by spasticity and equilibrium disorders, and when they could no longer feed themselves. They were usually sacrificed at a few hours to a few days before anticipated death from the virus.

Construction of DNA recombinants. The infectious viral DNA genomes pBR-NE-8 and p4070-A from Cas-Br-E and amphotropic 4070-A MuLV, respectively, have been described previously (5, 17). For subcloning, 5 to 20 μg of viral DNA was cleaved with restriction enzymes. The digestion conditions were those recommended by the suppliers (Boehringer Mannheim Biochemicals and New England Biolabs). The desired fragments were separated on 1.2% agarose gels and isolated by electroelution (6). These fragments were then ligated with T_4 DNA ligase (New England Biolabs) at 12°C for 16 h in 0.01 ml of a solution containing 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 20 mM dithiothreitol, and 1 mM ATP (ligase buffer) and used to transform (21) *Escherichia coli* JF1161 (32). Colonies were screened by the method of Grunstein and Hogness (12) with ^{32}P-labeled (29) purified homologous viral DNA fragments. Positive clones were isolated and grown in mass culture. Plasmid DNA was extracted and molecularly analyzed with appropriate restriction endonucleases, using agarose gels as described previously (6).

Microinjection procedure. The viral inserts from various recombinant DNAs (2 μg) were excised from pBR322 by digestion with *Cla*I (pNEA-1, pNEA-3) or *Eco*RI (pNEA-2), phenol and chloroform extracted, ethanol precipitated, and ligated with T_4 DNA ligase (50 U) in 0.1 ml of the ligase buffer, as described previously (6). The ligated chimeric viral genomes were phenol extracted and ethanol precipitated. DNA was suspended at a concentration of 200 μg/ml in 1% KCl and microinjected into 500 to 1000 NIH 3T3 cells as described previously (6). Infectious viruses were recovered in the culture supernatant 5 to 10 days later.

Preparation of unintegrated viral DNA. NIH 3T3 cells (8×10^6 cells) were cocultivated with chronically infected cells (4×10^6 cells) in the presence of 2 μg of polybrene per ml. Hirt supernatant DNA was prepared after 48 h of cocultivation as previously described (18). Restriction analysis on unintegrated viral DNA was carried out by the procedure of Southern (31) with ^{32}P-labeled cloned viral DNA probes as described previously (28).

RESULTS

Characterization of parental MuLV used to construct chimeric viruses. Relevant biological characteristics of the parental MuLVs used in this study have been reported previously. Briefly, pBR-NE-8 MuLV, derived from Cas-Br-E MuLV, was originally isolated from the brain of a paralyzed mouse trapped near Lake Casitas (9). This XC^+ N-tropic ecotropic neurotropic molecularly cloned MuLV induced hind limb paralysis when injected into newborn mice after a latent period of 3 to 8 months (17). This MuLV also has the ability to induce nonthymic lymphoma, probably of B-cell or null-cell origin (9, 15, 17). The XC^- N-tropic amphotropic p4070-A MuLV was originally isolated from wild mouse embryos (9). This virus does not induce paralysis after inoculation into newborn mice, but it has been reported to induce a low incidence of lymphoma after a long latent period (9). Newborn mice injected intraperitoneally with p4070-A MuLV had no sign of disease after 7 months (see Fig. 4). Therefore, these two parental MuLVs appeared to have a sufficiently distinct paralysis-inducing potential to identify the sequences responsible for this phenotype by in vitro construction of chimeric molecules.

Construction and characterization of chimeric MuLV genomes. The construction of chimeric viral genomes from parental viral DNA of neurotropic and nonneurotropic (p4070-A) MuLVs was facilitated by the fact that they share several identical restriction endonuclease sites (5, 17). The first recombinant, pNEA-1, was constructed with the 3.9-kilobase-pair (kbp) *pol-env* *Sal*I-*Cla*I fragment from pBR-NE-8, the 4.3-kbp long terminal repeat (LTR)-*gag-pol* *Cla*I-*Sal*I fragment from p4070-A, and *Cla*I-cleaved pBR322 (Fig. 1). Each viral fragment was first purified by an agarose gel electrophoresis-electroelution procedure before ligation and cloning. The second recombinant, pNEA-2, was constructed by ligating three purified fragments: the 3.3-kbp *Pst*I-*Sal*I *gag-pol* fragment from pBR-NE-8, the 5.8-kbp *Pst*I LTR-*env*-pBR322 fragment from p4070-A, and the 3.4-kbp *Sal*I-*Pst* *pol-env*-pBR322 fragment from p4070-A (Fig. 1). These two recombinants (pNEA-1, pNEA-2) harbor only one copy of the LTR sequence derived from the parental p4070-A genome. The third recombinant, pNEA-3, was constructed by inserting the 1.3-kbp *Cla*I-*Pst*I LTR fragment from pBR-NE-8 into the complementary fragment of p4070-A. The 1.2-kbp *Eco*RI-*Cla*I *env* fragment and the 6.3-kbp *Pst*I-*Eco*RI *gag-pol-env* fragment from p4070-A were ligated with the 1.3-kbp *Cla*I-*Pst*I fragment from pBR-NE-8 and with *Cla*I-cleaved pBR322. This recombinant harbors two LTR copies derived from pBR-NE-8 (Fig. 1). Several clones from each construction were picked and studied by restriction endonuclease analysis. One clone of each class of recombinants, which appeared to have the desired structure, was studied further to ascertain the origin of each fragment and to prove the chimeric structure of the viral insert of each recombinant. The *env* gene of the two parental viral genomes could be distinguished by the presence of an *Eco*RI site (at 6.9 kbp) on the amphotropic MuLV genome (Fig. 2, lane c), its absence on the neurotropic MuLV genome (Fig. 2, lane b), and the presence of additional *Xba*I sites on the neurotropic MuLV genome, giving rise to two additional fragments (1.0 and 0.9 kbp) (Fig. 2, lane p). pNEA-2 and pNEA-3 DNAs had an additional *Eco*RI site in their viral insert (Fig. 2, lanes d and e) but no additional *Xba*I sites (Fig. 2, lanes s and t), indicating that their *env* gene was derived from the genome of the p4070-A MuLV. pNEA-1 DNA had additional *Xba*I sites in its genome (Fig. 2, lane r), indicating that its *env* gene was derived from the genome of pBR-NE-8 MuLV.

The *gag-pol* region of the two parental viral genomes could be distinguished by the presence of an additional *Sac*I site (at 2.9 kbp) on the amphotropic p4070-A MuLV genome, thus generating two fragments of 2.1 and 0.95 kbp (Fig. 2, lane g) instead of a 3.1-kbp fragment, which was generated by *Sac*I digestion of the parental pBR-NE-8 MuLV genome (Fig. 2, lane f). *Sac*I digestion of pNEA-1 (Fig. 2, lane h) and pNEA-3 (Fig. 2, lane j) DNAs generated the 2.1- and 0.95-kbp fragments, indicating that the *gag-pol* region of these recombinants was derived from the p4070-A genome. Digestion of pNEA-2 DNA with *Sac*I did not generate these two fragments (Fig. 2, lane i), thus indicating that the *gag-pol* region of this recombinant was derived from the pBR-NE-8 genome.

The LTR region of the two parental viral genomes could be distinguished by the presence of two LTR copies on pBR-NE-8 DNA and only one LTR copy in the p4070-A DNA. Therefore, a restriction endonuclease which cleaves within the LTR, such as *Sac*I, would give rise to a complete permuted LTR fragment of 0.5 kbp after cleaving pBR-NE-8

DNA (Fig. 2, lane f) but would not generate such a fragment on p4070-A DNA (Fig. 2, lane g). Moreover, *Pvu*I and *Cla*I which cleave the two parental genomes close to the LTR would generate a larger LTR-containing fragment (1.3 kbp) with pBR-NE-8 DNA (Fig. 2, lane k) than they would with p4070-A DNA (0.85 kbp) (Fig. 2, lane l). *Sac*I digesion of pNEA-1 and pNEA-2 DNAs did not generate a 0.5-kbp fragment (Fig. 2, lanes h and i), and *Pvu*I-*Cla*I digestion of the same DNAs generated a 0.85-kbp fragment (Fig. 2, lanes m and n), thus indicating that the LTR region of these two recombinants was derived from p4070-A DNA. However, digestion of pNEA-3 DNA with *Sac*I generated a 0.5-kbp fragment (Fig. 2, lane j), and its digestion with *Pvu*I-*Cla*I generated a 1.3-kbp fragment (Fig. 2, lane o), as expected for a region derived from pBR-NE-8 DNA. Therefore, these results indicate that each chosen recombinant has the expected structure.

Recovery and characterization of infectious chimeric MuLVs. To recover infectious chimeric viruses, the viral inserts excised from pNEA-1 and pNEA-3 DNAs with *Cla*I and from pNEA-2 DNA with *Eco*RI were ligated with T_4 DNA ligase to obtain nonpermuted forms of viral DNA and microinjected into NIH 3T3 cells. Infectious viruses were recovered from the culture supernatant 5 to 10 days later, and titers were determined on NIH 3T3 cells by an XC assay for XC^+ chimeric MuLVs or by endpoint dilution for XC^- chimeric MuLVs.

These recovered viruses were further characterized by analysis of their unintegrated viral DNA obtained by Hirt extraction of acutely infected cells. These unintegrated viral DNAs and appropriate control DNAs were cleaved with various restriction endonucleases and analyzed by the agarose gel-Southern transfer procedure. The parental amphotropic MuLV DNA was cleaved once with *Eco*RI at 6.9 kbp

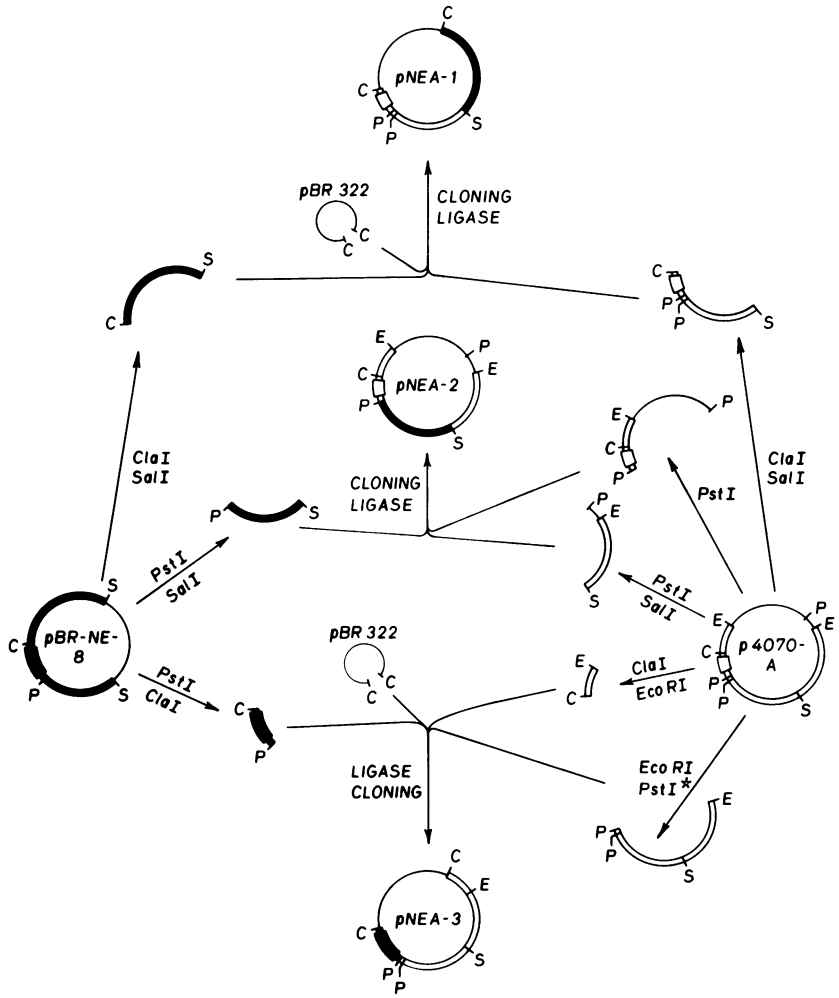

FIG. 1. Construction of pNEA-1, pNEA-2, and pNEA-3 viral DNA recombinants. The parental pBR-NE-8 and p4070-A DNAs were cleaved with *Cla*I and *Sal*I, and the purified 3.9-kbp fragment from pBR-NE-8 and the 4.3-kbp fragment from p4070-A were ligated with *Cla*I-cleaved pBR322 (1 μg) before transfection onto *E. coli* and cloning to generate pNEA-1. The recombinant pNEA-2 was constructed by ligating the 3.3-kbp *Pst*I-*Sal*I fragment of pBR-NE-8 and the 3.4-kbp *Pst*I-*Sal*I and the 5.8-kbp *Pst*I fragments of p4070-A. These ligated fragments were transfected onto *E. coli* to generate pNEA-2 recombinant DNA. The 1.3-kbp *Cla*I-*Pst*I fragment of pBR-NE-8, the 1.2-kbp *Cla*I-*Eco*RI fragment of p4070-A, and the 6.3-kbp *Eco*RI-*Pst*I fragment of p4070-A were ligated together in the presence of *Cla*I-cleaved pBR322 to generate pNEA-3 recombinant DNA. Solid areas indicate viral DNA of neurotropic pBR-NE-8 or fragments derived from it; open areas indicate viral DNA of amphotropic p4070-A or fragments derived from it; lines indicate pBR322; and boxes represent the LTRs. Abbreviations: C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sal*I; asterisk, partial digestion. The restriction endonuclease sites on pBR322 are not all illustrated.

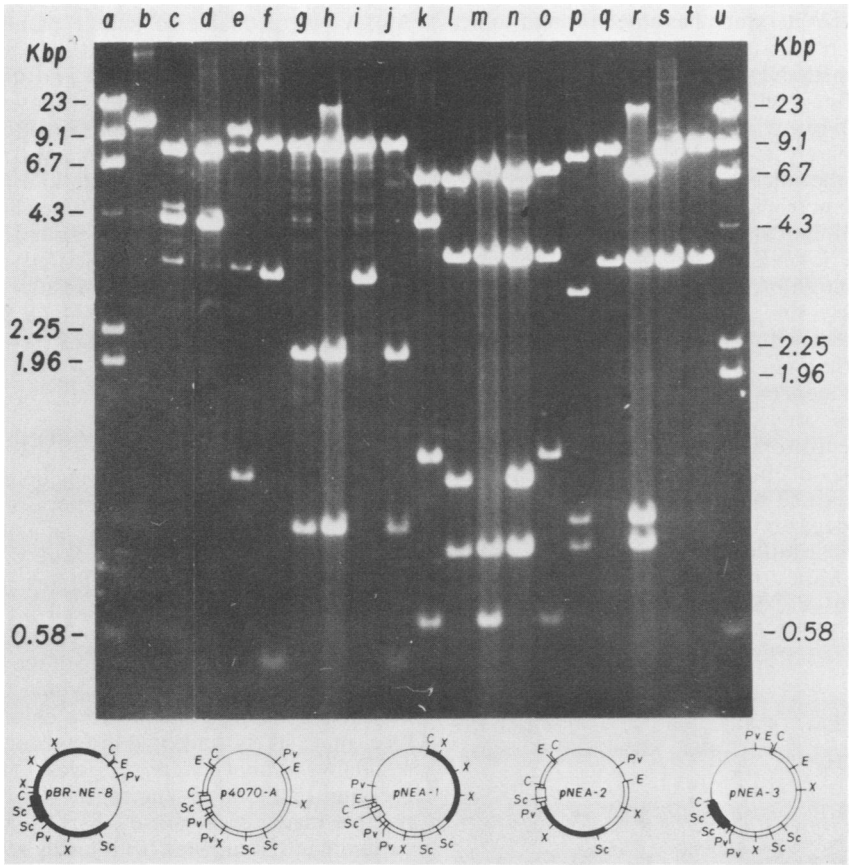

FIG. 2. Molecular characterization of pNEA-1, pNEA-2, and pNEA-3 recombinant DNAs. Parental pBR-NE-8 and p4070-A and recombinant pNEA-1, pNEA-2, and pNEA-3 DNAs (2 μg each) were digested with restriction endonucleases, and DNA fragments were separated on 1.4% agarose gel as described previously (6). The gel was stained with ethidium bromide, and DNA was revealed by UV illumination. pBR-NE-8 (lanes b, f, k, and p), p4070-A (lanes c, g, l, and q), pNEA-1 (lanes h, m, and r), pNEA-2 (lanes d, i, n, and s), and pNEA-3 (lanes e, j, o, and t) DNAs were digested with *Eco*RI (lanes b through e), *Sac*I (lanes f through j), *Pvu*I-*Cla*I (lanes k through o), and *Xba*I (lanes p through t). Markers consisted of *Hin*dIII-digested λ DNA (lanes a and u). At the bottom of the figure, solid areas indicate viral DNA of pBR-NE-8 or fragments derived from it, open areas indicate viral DNA of p4070-A or fragments derived from it, and lines indicate pBR322. The boxes represent the LTRs. Abbreviations: C, *Cla*I; E, *Eco*RI; Pv, *Pvu*I; Sc, *Sac*I; X, *Xba*I.

in the *env* region, as previously reported (5), to generate two fragments of 6.9 and 1.9 kbp (Fig. 3, lane a). Digestion of Hirt DNA from cells infected with pNEA-2 and pNEA-3 MuLVs also generated 6.9- and 1.9-kbp virus-specific fragments (Fig. 3, lanes c and d), indicating that the *env* region of these viruses was derived from the amphotropic parent. Viral DNA from cells infected with pNEA-1 MuLV was not digested by *Eco*RI (Fig. 3, lane b), documenting the absence of an *Eco*RI site on this genome and therefore indicating a different origin for its *env* gene, which was very likely from pBR-NE-8. This was confirmed by digestion with *Kpn*I. An additional *Kpn*I site is present in the *env* region of the neurotropic MuLV (at 7.5 kbp) (5, 17). Fragments of 4.1 and 1.4 kbp and two comigrating 1.3-kbp fragments were generated with DNA from pNEA-1 MuLV-infected cells (Fig. 3, lane h), indicating the presence of an extra *Kpn*I site in the *env* region as in the parental pBR-NE-8 genome (Fig. 3, lane g). Fragments of 5.4, 1.4, and 1.3 kbp were generated with DNA from the pNEA-3 MuLV (Fig. 3, lane f) and from the p4070-A MuLV-infected cells (Fig. 3, lane e), indicating the absence of this *Kpn*I site in the *env* region. *Sac*I was shown to cleave within the LTRs and in the *pol* region (at 3.8 kbp) of the parental neurotropic MuLV DNA to generate 5.1- and 3.1-kbp fragments (Fig. 3, lane j). An additional *Sac*I site was present in the amphotropic MuLV DNA (at 2.9 kbp), generating 5.1-, 2.1-, and 0.95-kbp fragments (Fig. 3, lane i). Digestion of viral DNA from pNEA-2 MuLV-infected cells with *Sac*I generated the 5.1- and 3.1-kbp fragments (Fig. 3, lane l), indicating the absence of the additional site in the *gag* region and thus suggesting that the *gag* region was derived from the neurotropic parent. However, *Sac*I digestion of viral DNA from pNEA-1 and pNEA-3 MuLV-infected cells generated the 5.1-, 2.1-, and 0.95-kbp fragments (Fig. 3, lanes k and m), indicating that the *gag* region was derived from the parental amphotropic MuLV. Together, these results indicate that the genome of the viruses recovered after microinjection had the expected structure of the chimeric DNA molecules that were microinjected and was not grossly rearranged during their passage through NIH 3T3 cells.

Paralysis-inducing potential of chimeric MuLVs. To determine the paralysis-inducing potential of each chimeric MuLV and thus locate the viral sequences responsible for inducing paralysis, the pNEA-1, pNEA-2, and pNEA-3 MuLVs recovered after microinjection of recombinant DNAs were inoculated intraperitoneally into newborn SIM.S and SWR/J mice. Both mouse strains have previously been shown to be susceptible to the parental neurotropic

MuLV (17, 23–25), and SWR/J mice have been found to be the most susceptible to paralysis among the inbred strains tested (23–25). Parental pBR-NE-8 MuLV induced paralysis in both mouse strains after a relatively short latency (Fig. 4 and 5). As reported previously (23–25), SWR/J mice were very susceptible to the paralysis induced by this virus, with 100% (nine of nine) of the mice being paralyzed after 157 days. The parental amphotropic p4070-A MuLV did not induce paralysis in the 18 inoculated mice even after a long latent period. The chimeric pNEA-1 MuLV induced paralysis in both strains of mice (6 of 9 SIM.S mice and 15 of 16 SWR/J mice) with nearly the same incidence and latent periods as that obtained with the parental neurotropic MuLV, indicating that the *pol-env* region between the *Sal*I and *Cla*I sites harbor sequences sufficient to induce paralysis. The form of paralysis was clinically similar to the one seen with the parental neurotropic pBR-NE-8 MuLV. Moreover, histological analysis of the brains of mice inoculated with pNEA-1 MuLV revealed pathological damage (noninflammatory spongiform degeneration) which was identical in the type, severity, and localization to the damage induced by the parental pBR-NE-8 MuLV (data not shown). Chimeric pNEA-2 and pNEA-3 MuLVs, each harboring, respectively, the *gag-pol* and the LTR regions from the neurotropic MuLV, were unable to induce paralysis in the inoculated mice (0 of 10 SIM.S mice and 0 of 11 SWR/J mice for pNEA-2 MuLV; 0 of 6 SIM.S mice and 0 of 7 SWR/J mice for pNEA-3 MuLV). This result indicates that these regions of the genome did not contribute significantly to the paralysis-inducing potential of neurotropic MuLV. However, pNEA-2 and pNEA-3 MuLVs were able to establish infection in the inoculated mice, since eight of eight mice tested 8 to 10 months postinoculation were viremic and had a relatively high titer (1×10^4 to 5×10^4 PFU/ml) of infectious viruses in their serum. Therefore, these results indicate that the *Sal*I-*Cla*I *pol-env* fragment of pBR-NE-8 MuLV is sufficient and necessary to transfer the paralysis-inducing potential to chimeric MuLVs.

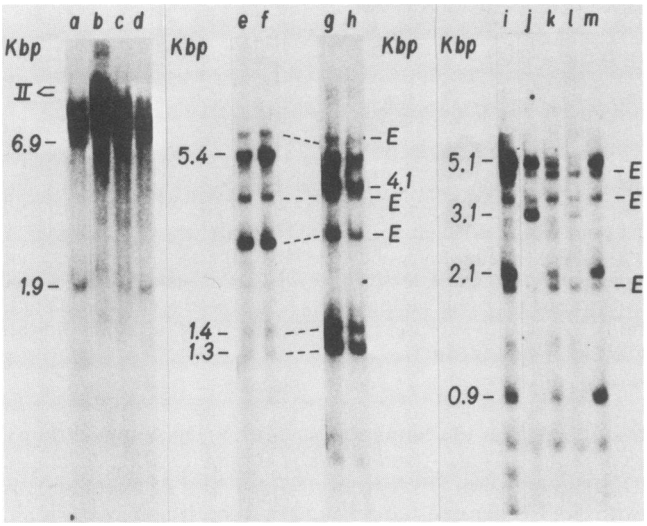

FIG. 3. Agarose gel electrophoresis of unintegrated viral DNA from NIH 3T3 cells infected with pNEA-1, pNEA-2, and pNEA-3 MuLVs. The recombinant viral DNAs pNEA-1, pNEA-2, and pNEA-3 were microinjected into NIH 3T3 cells, and infectious viruses were spread throughout the culture within a few days after microinjection. These chronically infected NIH 3T3 cells (4×10^6) were cocultivated with NIH 3T3 cells (8×10^6) in the presence of 2 μg of polybrene per ml for 48 h. After this incubation, Hirt supernatant DNA was extracted. Hirt DNAs were digested with restriction endonucleases and DNA fragments, separated by 1% agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled 8.2 kbp cloned-viral DNA. Virus-specific DNA was detected by autoradiography. Hirt supernatant DNA from cells infected with p4070-A (lanes a, e, and i), pBR-NE-8 (lanes g and j), pNEA-1 (lanes b, h, and k), pNEA-2 (lanes c and l), and pNEA-3 (lanes d, f, and m) MuLVs were digested with *Eco*RI (lanes a through d), *Kpn*I (lanes e through h), and *Sac*I (lanes i through m). Several endogenous viral sequences (E) also present in uninfected NIH 3T3 cells hybridized to this probe. Because of the presence of supercoiled viral DNA in the preparation, the 6.9-kbp fragment (lanes a through d) is not well visualized here, although it was easily detected on the original film.

DISCUSSION

To study the paralysis-inducing determinant of neurotropic MuLV, we constructed chimeric viral DNA genomes in vitro between parental genomes of a neurotropic MuLV and a nonneurotropic amphotropic MuLV. Viruses derived from these chimeric molecules were tested for their paralysis-inducing potential in mice. We have shown here that a 3.9-kbp fragment of the neurotropic genome which comprises the 3′ end of the *pol* gene and all of the *env* gene (between the *Sal*I and *Cla*I sites) is necessary and sufficient to confer the paralysis-inducing potential to a chimeric virus and, therefore, most likely harbor the determinant of paralysis. The results we obtained were very clear-cut, representing a total presence or a total absence of the paralytic determinant with no intermediate response. This suggests that the whole determinant is encoded within this region. The *pol* gene is known to code for reverse transcriptase in all retroviruses (34) and for an endonuclease in the avian myeloblastosis viruses (11). This protein might play a role in the paralysis-inducing potential of these viruses. Indeed, nonneurotropic ecotropic or amphotropic MuLVs isolated from wild mice have a genome with restriction endonuclease sites different from those of the neurotropic ecotropic pBR-NE-8 MuLV in this region (5).

The gp70 protein encoded by the *env* gene is also an excellent candidate protein to harbor the paralysis-inducing determinant. Of all the regions between the *Sal*I and *Cla*I sites involved in paralysis, this region was shown by restriction enzyme (5, 17) and T_1-resistant oligonucleotide (20) analyses to be the most divergent between neurotropic MuLV and other nonneurotropic ecotropic or amphotropic MuLVs from wild mice. Also, tryptic peptide analysis of gp70 from wild mouse ecotropic neurotropic MuLV and nonneurotropic amphotropic MuLV revealed significant differences between these gp70 proteins, with 30 of 39 peptides being nonidentical (3, 8). Moreover, recombinant mink cell focus-inducing-type MuLVs, which were isolated from the spleens of paralyzed mice inoculated with neurotropic MuLV and which harbor gp70 molecules different from the inoculated parental neurotropic viruses, were unable to induce paralysis when inoculated into newborn mice (24). Finally, in studies of temperature-sensitive mutants of Moloney MuLV that are able to induce lower limb paralysis, defectiveness in the processing of the precursor *env* gene product gPr80^{env} has been shown (37).

It has been reported previously that p30 levels in the brain closely paralleled the severity of the disease (23). In our study with chimeric MuLVs, this and other *gag* proteins seem to be excluded as being essential in the induction of paralysis. Indeed, pNEA-1 viruses, in which the genome

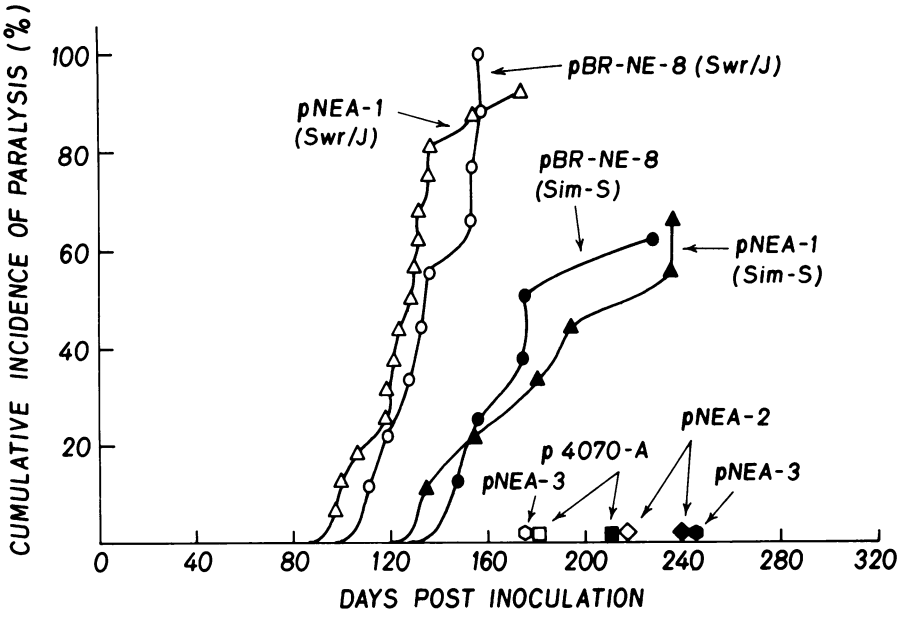

FIG. 4. Cumulative incidence of paralysis induced by intraperitoneal inoculation of parental and chimeric MuLVs into SWR/J or SIM.S mice. Groups of 8,9,9,10, and 6 SIM.S mice and groups of 9,9,16,11, and 7 SWR/J mice were inoculated with, respectively, pBR-NE-8 (4×10^5 PFU/ml), p4070A (1×10^6 PFU/ml), pNEA-1 (1.5×10^6 PFU/ml), pNEA-2 (1×10^6 PFU/ml), or pNEA-3 (1×10^6 PFU/ml) MuLVs. Open symbols indicate SWR/J mice; closed symbols indicate SIM.S mice.

encodes a p30 protein derived from the nonneurotropic amphotropic MuLV induced paralysis, and pNEA-2 MuLVs, in which the genome encodes p30 protein derived from the neurotropic MuLV, could not induce paralysis. The high levels of p30 protein in damaged CNS tissues most likely reflect the high replication efficiency of the neurotropic viruses in the brain. However, we cannot exclude the possibility that some *gag* proteins, sharing identical domains in neurotropic and amphotropic MuLVs, would be involved in the paralytic process together with the primary determinant of paralysis that we identified in the *pol-env* region.

Our data have also allowed us to exclude a 1.3-kbp region harboring the LTR as the primary determinant of paralysis on the neurotropic viral genome. This result was rather unexpected since in studies with leukemogenic viruses it has been revealed that this region confers the disease specificity to a virus (4) and that the LTR direct tandem repeats (enhancer sequences) confer tissue specificity to these viruses (7). It was therefore not unreasonable to expect that the LTR of the neurotropic MuLV might confer some disease specificity to this virus, but this does not appear to be the case. However, the choice of the amphotropic MuLV genome as one of the parents might have been very fortunate, and the neurotropic MuLV LTR could be substituted for the amphotropic MuLV LTR in this function. Indeed, both the neurotropic ecotropic MuLV and the amphotropic MuLV appear to replicate in B lymphocytes, since both have been reported to induce B-cell lymphomas (9, 15). It has been reported that neurotropic retroviruses could not induce paralysis when inoculated into splenectomized mice (9), indicating that their replication in spleen cells (possibly in B lymphocytes) might be essential for the progressive development of paralysis. The appropriate LTR sequences might be critical in targeting these viruses to their specific lymphoid cells, as reported with other retroviruses (4, 7), and the amphotropic and neurotropic MuLVs might share this specific determinant on their LTR sequences. Construction of chimeric viral genomes with different LTRs should allow clarification of the role of this replication step in lymphoid cells in the development of the disease.

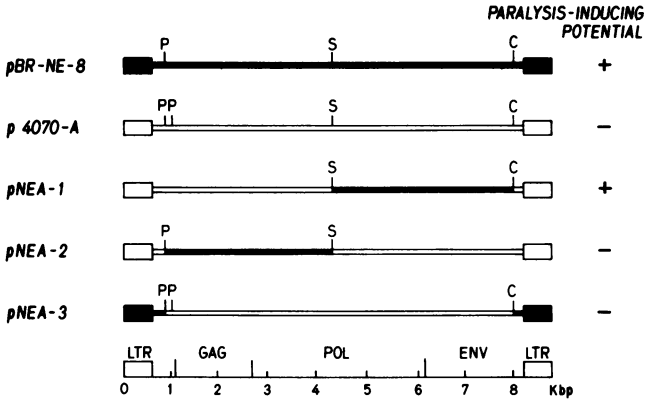

FIG. 5. Nonpermuted form of parental and chimeric viral DNA genomes with their paralysis-inducing potential. The viral genomes are represented as linear double-stranded molecules flanked at their ends by LTR sequences (boxes). The positions of *gag*, *pol*, and *env* are shown at the bottom. The solid areas indicate pBR-NE-8 (neurotropic MuLV) or fragments derived from it. The open areas indicate p4070-A (amphotropic MuLV) or fragments derived from it. The paralysis-inducing potential is summarized from Fig. 4. Abbreviations: C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sal*I.

The mechanism by which ecotropic retroviruses induce paralysis remains obscure. The viral protein involved in paralysis, most likely the gp70 *env* protein, could recognize specific receptors in specific cells of the nervous system and facilitate the penetration of the viral genome into these cells, thus initiating virus replication, which is lytic for these cells. The same *env* protein or another peptide encoded by the *pol* gene could also have a lytic effect when accumulating at high levels in some cells of the nervous system. The retrovirus-transforming *mos* and *abl* proteins have been reported to be

lethal to cells at high copy numbers (26, 38). It has been suspected that the CNS injuries are due to primary direct viral effects and that the virus reaches the CNS through the blood stream and infects endothelial, glial, and neuronal cells (9, 23). A direct neurotoxicity effect on the neuronal membranes by the viral *env* gene product once the viruses reach a high titer has also been proposed (9). This model would also be compatible with our results. Some proteins encoded by the *Sal*I-*Cla*I fragment that we identified as conferring the paralysis-inducing potential could allow the unintegrated viral DNA to accumulate at high levels in the nondividing neurons, thus leading to cell death. Such a phenomenon seems to explain cell lysis in vitro by the spleen necrosis virus (19), the cytopathic avian leukosis virus RAV-2 (36), and the visna virus (13).

Finally, the paralysis-inducing sequences we identified could promote recombination with endogenous *env* sequences to generate MCF-type MuLVs. Such MCF MuLVs have indeed been isolated in the spleens of paralyzed mice (15, 24), and they could have some role in some step of the paralytic process. However, these MCF MuLVs were not detected in brain tissues (15, 24) and they did not induce paralysis upon inoculation into susceptible newborn mice (24). They were also detected late after inoculation of the ecotropic neurotropic virus, which was long after the appearance of CNS damage (24).

The data shown here represent the first attempt to identify and map the viral sequences involved in paralysis induced by retroviruses. These have been mapped within a 3.9-kbp fragment. The construction of finer recombinants within this *pol-env* region should allow more precise determination of the specific protein and the domain of this protein that are involved in paralysis.

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