VIROLOGY 248, 199–207 (1998)
ARTICLE NO. VY989258

Mapping of a Neurovirulence Determinant within the Envelope Protein of a Polytropic Murine Retrovirus: Induction of Central Nervous System Disease by Low Levels of Virus

David J. Poulsen, Shelly J. Robertson, Cynthia A. Favara, John L. Portis, and Bruce W. Chesebro[1]

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, Montana 59840

Received April 20, 1998; accepted May 27, 1998

Murine leukemia virus (MuLV) clone Fr98 is a recombinant polytropic virus that causes neurological disease characterized by ataxia in susceptible mouse strains. The envelope gene of Fr98 has been previously shown to encode at least two separate neurovirulence determinants. In the present study, the determinant encoded within the *Eco*RI/*Avr*II fragment of the envelope gene was further defined. In these experiments, neurovirulence was associated with a change from a serine to an arginine at position 195 and a glycine to an alanine at position 198 within the envelope protein. Neurovirulent and nonvirulent virus clones, which differed only at these two amino acid residues, showed no difference in the type or location of cells infected. Furthermore, equivalent levels of viral p30 capsid protein were detected in the brains of mice infected with either the neurovirulent or nonvirulent virus clones. These results were consistent with the interpretation that the envelope protein of the neurovirulent virus differed from that of the nonvirulent virus by having a greater toxic effect on central nervous system function.

INTRODUCTION

Retroviruses induce central nervous system (CNS) disease in a variety of hosts, including humans, macaques, ruminants, cats, and rodents. The mechanisms of pathogenesis associated with these viruses remain unclear, and different mechanisms may be used by different viruses. For example, human T-cell leukemia virus, simian immunodeficiency virus, Visna, and caprine arthritis-encephalitis virus all induce neurological diseases associated with a marked CNS infiltration of mononuclear leukocytes, which may contribute to the pathogenic process (Georgsson *et al.,* 1976, 1994; Izumo *et al.,* 1997; Cork *et al.,* 1974). In contrast, human immunodeficiency virus (HIV), feline immunodeficiency virus, and murine leukemia viruses (MuLVs) induce CNS diseases characterized by a more limited inflammatory response, sometimes associated with spongiform degeneration (Georgsson, 1994; Navia *et al.,* 1986; Wiley and Gardner, 1993). Most neurovirulent retroviruses are capable of infecting microglia within the CNS, and a number of studies have suggested that infected macrophages and microglia (Jordan *et al.,*1991) release toxic factors that mediate CNS disease (Lipton, 1991; Gendelman *et al.,* 1994). In addition to microglia, most MuLVs also infect brain capillary endothelial cells. In the case of the ecotropic MuLV TR13, the main pathological feature is hemorrhage associated with a stroke syndrome due to damage of infected brain capillary endothelial cells (Park *et al.,* 1993, 1994). In contrast, other ecotropic MuLVs that can infect endothelia, microglia, and sometimes selected populations of neurons induce tremor and hind limb paralysis associated with severe spongiform degeneration (Gardner, 1988; Masuda *et al.,* 1993; Lynch *et al.,* 1991; Portis *et al.,* 1990; Wong *et al.,* 1989). The polytropic MuLV FMCF98, which was used as the basis for the present study, causes ataxia without paralysis (Buller *et al.,* 1990). FMCF98 does not infect neurons but does infect brain capillary endothelial cells and microglia primarily associated with white matter tracts of the cerebellum and internal capsule, resulting in minimal spongiform lesions compared with the ecotropic MuLVs (Portis *et al.,* 1995).

The envelope gene of FMCF98 was previously shown to be critical for the induction of neurological disease. Insertion of this gene into the nonvirulent ecotropic Friend virus molecular clone FB29 generated the neurovirulent polytropic virus clone Fr98, which induced disease at a more rapid rate than FMCF98 (Portis *et al.,* 1995). Previous studies identified two neurovirulence determinants within the Fr98 envelope gene (Hasenkrug *et al.,* 1996). The expression of either determinant alone was sufficient to induce CNS disease. One determinant was located within an *Sph*I/*Eco*RI fragment near the 5′ end of the envelope gene and appeared to influence viral spread in microglial cells (Robertson *et al.,*1997). The second neurovirulence determinant was located within an *Eco*RI/*Avr*II fragment near the center of the envelope gene. In the present study, two amino acids encoded within this latter region were identified as critical determinants of neurovirulence. Furthermore, quantitative

[1] To whom reprint requests should be addressed. Fax: (406) 363-9286. E-mail: bchesebro@nih.gov.

0042-6822/98

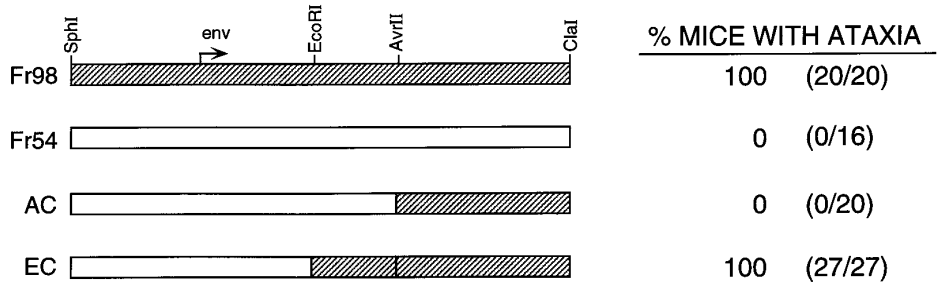

FIG. 1. Influence of the *Eco*RI/*Avr*II fragment on neurovirulence. The 2.45-kb *Sph*I/*Cla*I fragment of parental (Fr98 and Fr54) and chimeric viruses (AC and EC) are shown. The percent of mice that exhibited ataxia at 2–5 weeks after infection with each virus is indicated (right). The number of mice with ataxia over the total in each group is shown in parentheses.

analysis of viral protein levels within the CNS and regional distribution of virus indicated that these envelope amino acid residues did not influence neurovirulence by altering the level or localization of virus infection within the brain.

RESULTS

Envelope sequences influencing neurological disease induced by virus clone EC

Using the coisogenic recombinant virus clones EC and AC, which express chimeric envelope sequences (Fig. 1), it was determined that the *Eco*RI/*Avr*II region of the Fr98 envelope gene strongly influenced neurovirulence (Hasenkrug *et al.,* 1996). Both EC and AC were neuroinvasive, but no signs of clinical disease were observed after intraperitoneal inoculation of neonatal IRW mice with virus clone AC. In contrast, at 3–4 weeks postinfection with the EC virus clone, mice exhibited hyperactivity characterized by repetitive jumping in the corner of the box that could be accentuated by auditory stimulation. At 4–5 weeks postinoculation, EC-infected mice typically developed a progressive ataxia manifested by imbalance and an awkward, uneven gait. Later, in the preterminal stage of disease, mice occasionally exhibited intermittent seizure-like episodes characterized by torticollis and muscle rigidity, which led to immobility and an exhausted appearance. Virus-infected mice that survived past 13 weeks had a gradually increased incidence of leukemia (Buller *et al.,* 1990). Therefore, analysis of CNS disease was terminated at 13 weeks in subsequent experiments.

Because the virus clones EC and AC differed only at 17 amino acid residues between positions 186 and 323 of the envelope protein (Hasenkrug *et al.,* 1996), we were interested in determining which of these sequence differences influenced neurovirulence. Therefore, a series of seven coisogenic recombinant virus clones was created in which specific sequences from the nonvirulent AC virus were substituted into the *Eco*RI/*Avr*II region of the neurovirulent EC virus envelope gene (Fig. 2). All virus constructs were viable and replicated *in vitro* to titers of $\sim 10^5$ FFU/ml. Virus replication *in vivo* was confirmed by measuring plasma viremia titers at 19 days after intraperitoneal inoculation of neonatal IRW mice with $\sim 10^2$ to 10^3 FFU of virus. All recombinant viruses induced plasma viremia titers in the range of 10^4 to 10^5 FFU/ml.

The seven recombinant viruses were observed to differ in their ability to cause CNS dysfunction (Fig. 2). Virus clones EC-25, EC-35, and EC-45 induced ataxia in 14–38% of the mice inoculated. In contrast, ataxia was never observed in mice infected with the virus clones EC-1, EC-12, EC-13, or EC-14. Thus, sequence within the extreme 5′ end of the *Eco*RI/*Avr*II fragment appeared to influence neurovirulence. However, the incidence of disease observed in mice infected with EC-25, EC-35, and EC-45 was lower than that induced by the parental virus clone EC (Fig. 2). This suggested that additional sequences within the 3′ portion of the *Eco*RI/*Avr*II region might be required to induce an incidence of disease equivalent to EC. Alternatively, the difference in disease incidence could have simply been due to the 10-fold range in the virus doses inoculated (Fig. 2).

To resolve this question, newborn IRW mice were inoculated with 10^4 FFU of selected virus clones. Under these conditions, the incidences of disease induced by EC and EC-25 were equivalent (Fig. 2). In addition, a similar pattern of disease kinetics was observed in mice infected with EC and EC-25 (Fig.3). Within 5 weeks postinfection, 100% of the mice infected with EC or EC-25 developed severe ataxia and progressed to a preterminal stage by 4–6 weeks postinfection. In contrast, none of the mice inoculated with 10^4 FFU of AC or EC-1 developed ataxia (Figs. 2 and 3). The envelope gene sequence of virus clone EC-1 differed from the neurovirulent EC virus at only two amino acid codons. Likewise, the neurovirulent virus clone EC-25 differed from the nonvirulent clone AC only at these same two codons (Fig. 2). Both the nonvirulent clones AC and EC-1 encoded serine at position 195 and glycine at position 198 of the envelope protein, whereas the neurovirulent clones EC and EC-25 encoded arginine at position 195 and alanine at 198 (Fig. 2). These results indicated that

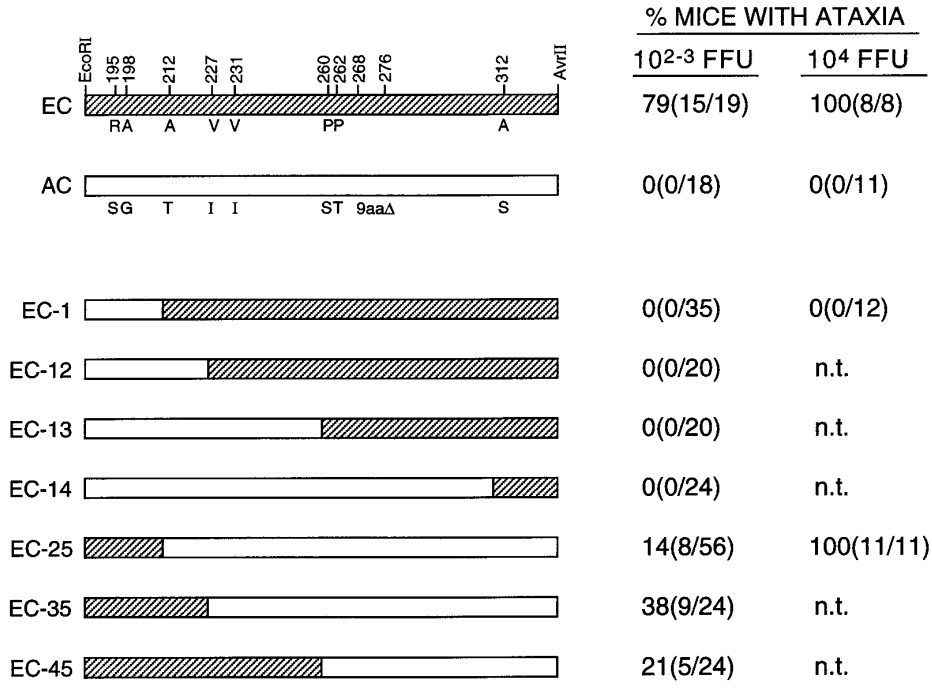

FIG. 2. Influence of specific envelope amino acids on neurovirulence. Map of the 410-bp *Eco*RI/*Avr*II fragment showing the specific amino acid differences encoded by the virulent EC virus, nonvirulent AC virus, and mutant virus clones. The percent of ataxic mice observed after infection of neonatal IRW mice with 10^2 to 10^4 FFU of virus is indicated (right). The number of mice with ataxia over the total in each group is shown in parentheses.

neurovirulence was strongly influenced by this small region of the envelope protein.

Detection of viral p30 capsid protein

In a recent study examining the recombinant polytropic virus clones SE and Fr54, which differ only at 12 amino acid residues encoded within the *Sph*I/*Eco*RI fragment of the envelope gene, a correlation was observed between the level of viral p30 capsid protein detected in the cerebellum of infected mice and the induction of CNS disease (Robertson *et al.,* 1997). Therefore, we were interested in determining whether a similar correlation could explain the differences in neurovirulence observed among the virus clones used in this study.

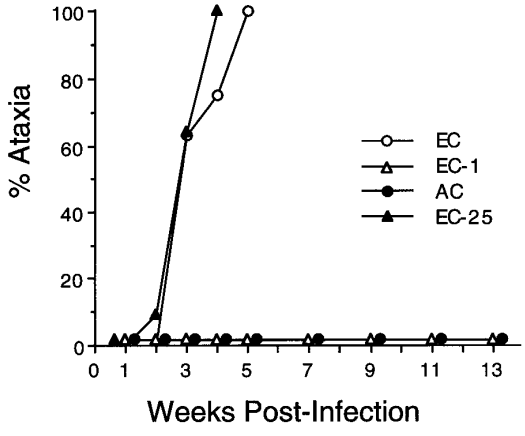

FIG. 3. Kinetics of CNS disease induced after intraperitoneal injection of neonatal IRW mice with 10^4 FFU of recombinant polytropic viruses. The percentage of mice exhibiting ataxia in each experimental group is shown relative to time postinfection.

To examine this possibility, cerebellar homogenates were prepared from mice infected with 10^4 FFU of selected virus clones. The level of virus present in each sample was determined by immunoblot analysis of virus p30 capsid protein (Fig.4). At the time of sacrifice, all the mice infected with the neurovirulent virus clones exhibited ataxia, whereas none of the mice infected with the nonvirulent clones showed signs of disease. No statistically significant differences in the levels of p30 were detected in cerebellar homogenates from mice infected with the neurovirulent clones EC or EC-25 compared with mice infected with the nonvirulent clones EC-1 or Fr54 (Fig. 4). In fact, slightly higher levels of p30 were detected in mice infected with the nonvirulent clone AC compared with those infected with EC or EC-25.

The results of the immunoblot analysis suggested that the induction of CNS disease by the EC virus clone did not correlate with an increase in the amount of viral protein detected. To confirm these results with a more sensitive and quantitative assay, virus p30 levels present in both cerebellar and cerebral homogenates from ataxic EC-infected mice and healthy age-matched EC-1-infected mice were examined by antigen capture ELISA at

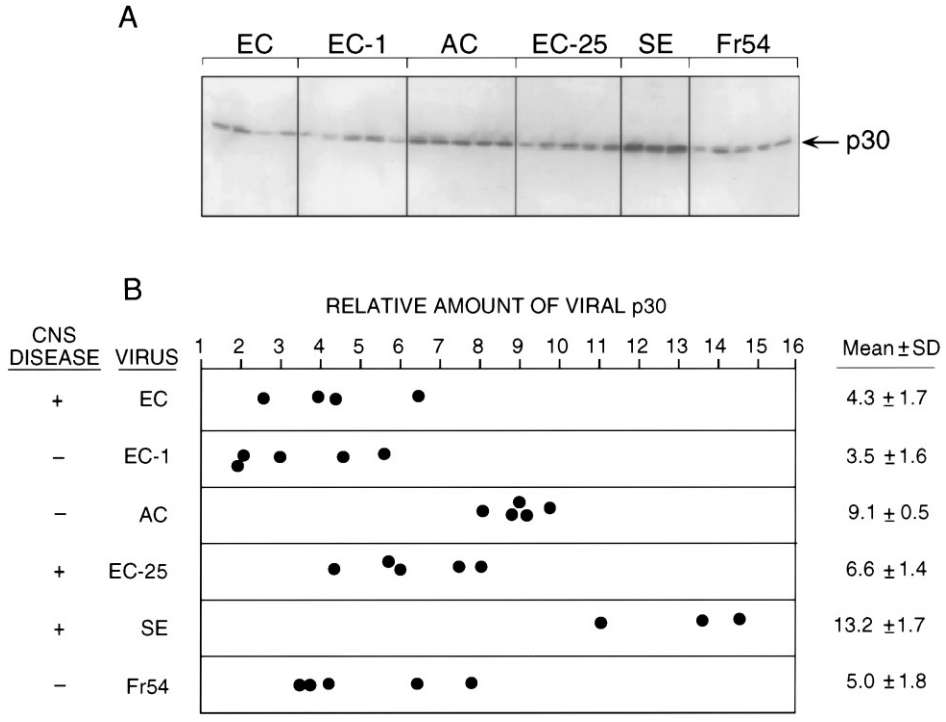

FIG. 4. (A) Western blot analysis of virus p30 antigen levels present in 10% cerebellar homogenates. The virus clones used in each infection are indicated above the gel. Samples from SE-infected mice were included as high virus level controls. Three to five mice per virus clone were sacrificed at 5 weeks postinfection. All mice infected with neurovirulent virus clones exhibited ataxia at the time of sacrifice, whereas all mice infected with nonvirulent virus clones were healthy. (B) Relative amount of viral p30 antigen present in brain samples as measured by quantitative fluorescence analysis for mice infected with each of the viruses indicated. The ability of each virus to induce ataxia is shown (left). The mean values and standard deviations for each group are shown (right).

5 weeks postinfection. Homogenates from ataxic SE-infected mice, shown previously to have high levels of viral p30 in the cerebellum (Robertson *et al.,* 1997), were used as additional controls. No statistically significant differences were observed between the levels of virus p30 present in either the cerebellum or cerebrum of mice infected with EC or EC-1 (Fig. 5). In contrast, compared with EC and EC-1, SE-infected mice had a threefold increase in viral p30 in cerebellar homogenates and a slight but statistically significant increase in the level of p30 in cerebral homogenates. These results confirmed the results of immunoblot analysis and indicated that EC and EC-1 replicated to equivalent levels within the cerebellum and cerebrum yet differed dramatically in neurovirulence.

Immunohistochemical analysis of viral envelope

One possible explanation for the difference in neurovirulence observed between EC and EC-1 was that changes in the envelope sequence might have allowed these two viruses to infect different cell types or subpopulations of cells within different regions of the brain. To examine this possibility, immunohistochemical analysis was performed on brain sections from ataxic, EC-infected or age-matched, healthy EC-1-infected mice

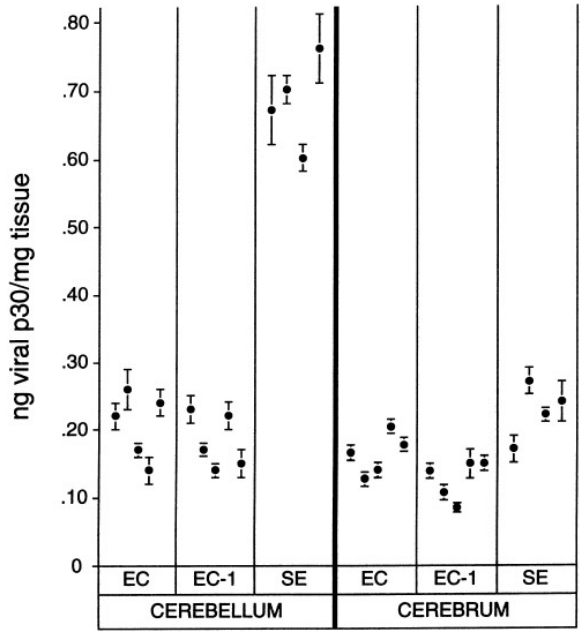

FIG. 5. Detection of virus p30 by antigen capture enzyme-linked immunosorbent assay. The concentrations of virus p30 present in 10% cerebral and cerebellar homogenates from 5-week-old, ataxic EC- or SE-infected mice or healthy age-matched EC-1-infected mice are shown. Each point represents a single mouse, and bars represent the standard deviation of replicate determinations.

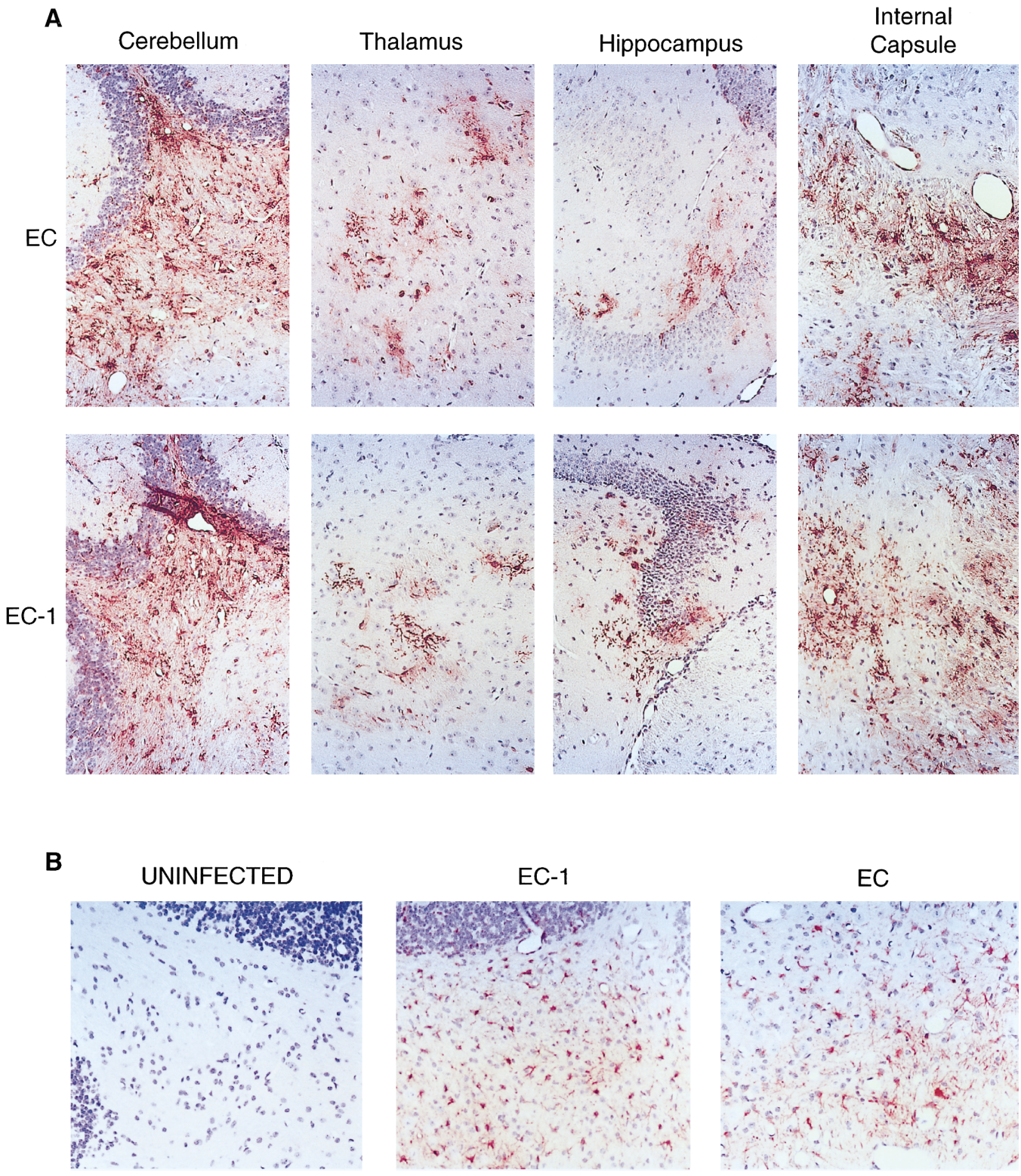

FIG. 6. Immunohistochemical staining of horizontal brain sections from ataxic EC-infected or healthy age-matched EC-1-infected mice. (A) Infected cells were detected using goat anti-gp70 antibody. (B) Activated astrocytes expressing GFAP were detected with rabbit anti-GFAP antibody, as detailed in Materials and Methods.

(Fig. 6A). No difference was observed in the distribution or apparent type of cells infected by either EC or EC-1 Virus envelope expression was detected primarily in highly arborized cells, reminiscent of microglia that have been previously shown to be infected by both Fr98 and Fr54 (Robertson *et al.,* 1997). Vascular endothelial cells

were also infected to a lesser extent by both viruses. EC-and EC-1-infected cells were mostly associated with the white matter tracts of the internal capsule and cerebellum but also, to a lesser extent, within the lateral thalamic nucleus and the CA4 region of the hippocampus. Thus, both viruses appeared to infect the same cell types within the same regions of the brain.

Detection of activated astrocytes

Astrocyte activation is one of the earliest responses after CNS injury (Norenberg, 1994). To examine whether astrocyte activation differed in EC-versus EC-1-infected mice, brain sections adjacent to those examined for virus envelope expression were stained for the astrocyte activation marker GFAP. Immunohistochemical analysis indicated that GFAP-positive astrocytes colocalized to regions of the brain infected by EC and EC-1. Furthermore, as shown in Figure 6B, infection with either EC or EC-1 resulted in similar levels of astrocytosis and GFAP expression. These results were similar to those observed previously for the avirulent virus clone Fr54 and the neurovirulent clone Fr98 (Robertson *et al.,* 1997) and suggest that astrocytosis is not a pathological correlate of clinical CNS disease in this case but rather a response to viral infection.

DISCUSSION

In the present experiments, expression of specific amino acid residues at positions 195 and 198 of the polytropic MuLV envelope protein was found to strongly influence neurovirulence. To gain insight into how these residues might affect disease induction, we examined their relationship with previously defined domains within the polytropic MuLV envelope protein. Residues 195 and 198 did not lie within the defined VRA, VRB, or VRC receptor binding domains, nor were they associated with the proline-rich region (Battini *et al.,* 1995). Thus, these residues may not play a direct role in receptor interactions. We also compared the predicted EC and EC-1 envelope protein secondary structures with the known secondary structure of the recently published crystallographic analysis of the N-terminal 236-amino-acid residues from the ecotropic MuLV envelope protein (Fass *et al.,* 1997; Rost *et al.,* 1993, 1994). Indeed, the predicted secondary structures of the EC and EC-1 envelopes showed considerable similarity with the common immunoglobulin-like core stalk of the ecotropic envelope protein composed of an antiparallel β sandwich (Dimitrov, 1997; Fass *et al.,* 1997). Based on this comparison, the amino acid residues at positions 195 and 198 in the EC and EC-1 envelope proteins were predicted to reside within a short α-helical loop located on the opposite side of the monomeric molecule from the receptor binding elements. Given the predicted location of residues 195 and 198, they could interact with the stalk of an adjacent molecule in the mature envelope complex, which exists as a trimer. In this way, residues at these positions might influence the processing and stability of the mature trimeric molecule. Indeed, a similar mechanism has been proposed to explain the effect of a valine-to-isoleucine change at position 25 within the envelope protein of the neurovirulent ecotropic MuLV *ts* 1 (Kamps *et al.,* 1991; Yu and Wong, 1992).

The neurovirulent virus clone EC and the nonvirulent clone EC-1 both infected similar populations of microglia and replicated to equivalent levels within the cerebellum and cerebrum (Figs. 5 and 6A). Furthermore, the levels of viral protein detected in the CNS of mice infected with EC or EC-1 were comparable with the lower levels of virus protein detected in the brains of mice infected with the nonvirulent clone Fr54. Under these conditions of relatively low viral protein and equal levels of infection, two alternatives may explain the difference in neurovirulence between EC and EC-1. First, nonvirulent virus clones such as EC-1 may stimulate a protective response that the EC envelope is unable to induce. For example, it has been suggested that astrocytes may play a role in limiting MuLV-induced pathology within the CNS (Lynch *et al.,* 1995). Thus, the envelope sequence from EC-1 may induce a more robust protective response from astrocytes. However, we did not observe any major differences in the levels of astrocyte activation as assessed by GFAP expression after infection with either EC or EC-1. The second possible explanation for the difference in neurovirulence is that the EC envelope protein might be more neurotoxic than the EC-1 envelope protein. The EC envelope protein itself might be directly toxic to neurons or, alternatively, the EC envelope protein might indirectly mediate neurovirulence by altering the neurotoxins produced by infected microglia (Fig. 6A) or nearby uninfected, activated astrocytes (Fig. 6B).

Results from *in vitro* studies have suggested a number of ways in which retrovirus envelope proteins might influence neurovirulence. For example, it has been reported that the HIV gp120 envelope protein can predispose cultured cerebellar neurons to *N*-methyl-D-aspartate-mediated neurotoxicity by causing an increase in the intracellular Ca^{2+} concentration (Lannuzel *et al.,* 1995; Lipton, 1994). HIV gp120 has also been shown to both inhibit the uptake and induce the release of glutamate by astrocytes (Dreyer and Lipton, 1995; Vesce *et al.,* 1997). It is possible the EC envelope protein influences neurovirulence through similar mechanisms.

Although differences in the levels of viral p30 protein were detected in the cerebellums of mice infected with SE and EC virus clones, no obvious differences were observed in the clinical disease produced by these two viruses. Mice infected with either the EC or SE virus clones exhibited similar neuromotor deficits, as well as the same incidence and rate of disease progression (Hasenkrug *et al.,* 1996). Furthermore, the disease-induc-

ing effects of the EC and SE neurovirulence determinants appeared to be additive in that expression of both determinants by the virus clone Fr98 resulted in an acceleration of CNS disease induction (Hasenkrug *et al.,* 1996). This complementation might be expected if the increased level of virus infection induced by the SE determinant further amplified the virulent effects of the EC determinant, resulting in a more rapid kinetics of disease. However, more precise knowledge of the mechanisms of both of these determinants will be required to explain their cooperative effects *in vivo*.

MATERIALS AND METHODS

Construction of recombinant viruses

All recombinant clones were derived from the BC3 plasmid that contained the *Sph*I/*Cla*I fragment encoding the *env* gene of EC inserted into the cloning vector pSP72 (Promega). The construction of virus clones EC, AC, Fr54, and FR98 has been previously described (Hasenkrug *et al.,* 1996). Specific amino acid coding changes were introduced into EC by overlapping polymerase chain reaction mutagenesis. Sequence changes were confirmed by double-stranded sequence analysis. *Sph*I/*Cla*I fragments containing the altered *env* sequences were subcloned into the nonneurovirulent virus clone FB29 (Hasenkrug *et al.,* 1996), in which the *Sph*I/*Cla*I fragment had been replaced with a short polylinker composed of multiple restriction enzyme digestion sites. Virus stocks were prepared by transfecting *mus dunni* fibroblasts with the plasmid DNA of recombinant clones and harvesting supernatants from confluently infected cultures. Virus titers were determined by a focal infectivity assay (Sitbon *et al.,* 1985) using the envelope specific monoclonal antibody 720 (Robertson *et al.,* 1991). For viremia titration, 19-day-old mice were anesthetized by methoxyflurane inhalation and bled by retroorbital puncture. Blood was collected in heparinized capillary tubes, and plasma was separated by centrifugation, stored on ice, and diluted for immediate titration by the focal infectivity assay (Sitbon *et al.,* 1985).

Animals and clinical disease

Inbred Rocky Mountain White (IRW) mice were bred and housed at the Rocky Mountain Laboratories animal facility. Within 24 h after birth, mice were injected intraperitoneally with various doses of viruses as detailed within each experiment. Mice were observed daily for clinical signs of CNS disease as previously described (Portis *et al.,* 1995). All animal experiments were done in accordance with the standards established by the Rocky Mountain Laboratory Animal Care and Use Committee and the National Institutes of Health.

Immunoblotting

Western blot analysis was performed as previously described (Robertson *et al.,* 1997). Viral p30 capsid protein was detected using the rat monoclonal antibody R187 (Chesebro *et al.,* 1983) followed by an alkaline phosphatase-conjugated goat anti-rat IgG (Southern Biotechnology Associates) and developed with the Vistra ECF (enhanced chemifluorescence) system (Amersham Life Science). The fluorescence intensities of specific bands were quantified using the Storm 860 image analysis system and ImageQuant software (Molecular Dynamics).

Capture enzyme-linked immunosorbent assay

Viral p30 capsid protein levels present in 10% (w/v) brain homogenates were quantified as previously described (Wehrly and Chesebro, 1997). The anti-p30 rat monoclonal antibody R187 was used as the capture antibody (Chesebro *et al.,* 1983). Bound p30 was then detected with rabbit anti-p30 antiserum (Lynch and Portis, 1993) and a secondary donkey anti-rabbit antibody conjugated to horseradish peroxidase (HRP; Amersham Life Science). The total protein concentration present in each brain homogenate (either whole cerebellum or one half of the cerebrum) was determined by measuring the A_{205nm} in a solution of 0.01% Brij 35 (Sigma Chemical) dissolved in distilled water. Equivalent amounts of total protein were assayed for each brain homogenate. The p30 levels present in four serial dilutions from each brain homogenate were compared in triplicate against a dilution series standard. The standard used was the neurovirulent ecotropic MuLV KP (Portis *et al.,* 1994) that had been purified by sucrose density gradient centrifugation. The purified KP standard was determined to have a total protein concentration of 580 μg/ml. To determine the percentage of p30 present in the virus standard, viral proteins were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and stained with SYPRO-red (Molecular Probes). The relative fluorescence intensities of each protein band within the standard was quantified as before using the Storm 860 image analysis system and ImageQuant software (Molecular Dynamics). Under these conditions, p30 was determined to represent $\sim$40% of the total proteins present in the standard.

Immunohistochemistry

Brains were collected from 4-week-old ataxic EC-infected mice or healthy EC-1-infected mice. Animals were perfused with 4% formaldehyde in phosphate-buffered saline (PBS), and brains were postfixed for an additional 4 h in 4% formaldehyde in PBS at 4°C. Paraffin-embedded brains were cut into 8-μm-thick sections and prepared for staining for virus envelope protein or GFAP. After the removal of paraffin, sections to be stained for

virus envelope protein were incubated at 100°C in citrate buffer (pH 6.0) for 20 min to reactivate antigens masked by fixation (McQuaid *et al.,* 1995; Usuda *et al.,* 1996; Yang *et al.,* 1995). Sections were blocked in PBS with 5% bovine serum albumin overnight at 4°C and then washed in PBS. Virus envelope was detected by incubating each section for 1 h at 37°C with a goat anti-gp70 antibody (kindly provided by Roland Friedrich, Institute of Medical Virology, Geissen, Germany) diluted 1:2000 in PBS containing 5% fetal calf serum. Sections were washed and reacted for 1 h at 37°C with a secondary rabbit anti-goat antibody conjugated to HRP (ICN Biochemicals Inc.) that had been diluted 1:250 in PBS with 5% bovine serum albumin. Sections were washed and reacted with 3-amino-9-ethylcarbazole (AEC) as a substrate for HRP (Robertson *et al.,* 1997) for 10 min and counterstained with Harris hematoxylin.

GFAP staining was performed on paraffin-embedded sections with primary rabbit anti-GFAP antibody diluted 1:2000 in PBS with 5% fetal calf serum (Dakopatts) followed by a secondary goat anti-rabbit conjugated to HRP (BioRad) diluted 1:250. GFAP-positive cells were visualized by reacting sections with AEC and counterstained with Harris hematoxylin.

REFERENCES

Battini, J. L., Danos, O., and Heard, J. M. (1995). Receptor-binding domain of murine leukemia virus envelope glycoproteins. *J. Virol.* 69, 713–719.

Buller, R. S., Wehrly, K., Portis, J. L., and Chesebro, B. (1990). Host genes conferring resistance to a central nervous system disease induced by a polytropic recombinant Friend murine retrovirus. *J. Virol.* 64, 493–498.

Chesebro, B., Britt, W., Evans, L., Wehrly, K., Nishio, J., and Cloyd, M. (1983). Characterization of monoclonal antibodies reactive with murine leukemia viruses: Use in analysis of strains of Friend MCF and Friend ecotropic murine leukemia virus. *Virology* 127, 134–148.

Cork, L. C., Hadlow, W. J., Gorham, J. R., Piper, R. C., and Crawford, T. B. (1974). Pathology of viral leukoencephalomyelitis of goats. *Acta Neuropathol.* (Berl) 29, 281–292.

Dimitrov, D. S. (1997). How do viruses enter cells? The HIV coreceptors teach us a lesson of complexity. *Cell* 91, 721–730.

Dreyer, E. B., and Lipton, S. A. (1995). The coat protein gp120 of HIV-1 inhibits astrocyte uptake of excitatory amino acids via macrophage arachidonic acid. *Eur. J. Neurosci.* 7, 2502–2507.

Fass, D., Davey, R. A., Hamson, C. A., Kim, P. S., Cunningham, J. M., and Berger, J. M. (1997). Structure of a murine leukemia virus receptor-binding glycoprotein at 2.0 angstrom resolution. *Science* 277, 1662–1666.

Gardner, M. B. (1988). Neurotropic retroviruses of wild mice and macaques. *Ann. Neurol.* 23(Suppl.), S201–S206.

Gendelman, H. E., Lipton, S. A., Tardieu, M., Bukrinsky, M. I., and Nottet, H. S. (1994). The neuropathogenesis of HIV-1 infection. *J. Leukoc. Biol.* 56, 389–398.

Georgsson, G., Nathanson, N., Palsson, P. A., and Petursson, G. (1976). The pathology of Visna and maedi in sheep. *Front. Biol.* 44, 61–96.

Georgsson, G. (1994) Neuropathologic aspects of lentiviral infections. *Ann. NY Acad. Sci.* 724, 50–67.

Hasenkrug, K. J., Robertson, S. J., Portis, J., McAtee, F., Nishio, J., and Chesebro, B. (1996). Two separate envelope regions influence induction of brain disease by a polytropic murine retrovirus (FMCF98). *J. Virol.* 70, 4825–4828.

Izumo, S., Umehara, F., Kashio, N., Kubota, R., Sato, E., and Osame, M. (1997). Neuropathology of HTLV-1-associated myelopathy (HAM/TSP). *Leukemia* 11(Suppl. 3), 82–84.

Jordan, C., Watkins, B., Kufta, C., and Dubois-Dalaq, M. (1991). Infection of brain microglial cells by human immunodeficiency virus type 1 is CD4 dependent. *J. Virol.* 65, 736–742.

Kamps, C., Lin, Y., and Wong, P. (1991). Oligomerization and transport of the envelope protein of Moloney murine leukemia virus-TB and of *ts* 1, a neurovirulent temperature-sensitive mutant of MoMuLV-TB. *Virology* 184, 687–694.

Lannuzel, A., Lledo, P. M., Lamghitnia, H. O., Vincent, J. D., and Tardieu, M. (1995). HIV-1 envelope proteins gp120 and gp160 potentiate NMDA-induced $[Ca^{2+}]_i$ increase, alter $[Ca^{2+}]_i$ homeostasis and induce neurotoxicity in human embryonic neurons. *Eur. J. Neurosci.* 7, 2285–2293.

Lipton, S. A. (1991). HIV-related neurotoxicity. *Brain Pathol.* 1, 193–199.

Lynch, W., Czub, S., McAtee, F., Hayes, S., and Portis, J. (1991). Murine retrovirus-induced spongiform encephalopathy: productive infection of microglia and cerebellar neurons in accelerated CNS disease. *Neuron* 7, 365–379.

Lynch, W. P., Robertson, S. J., and Portis, J. L. (1995). Induction of focal spongiform neurodegeneration in developmentally resistant mice by implantation of murine retrovirus-infected microglia. *J. Virol.* 69, 1408–1419.

Masuda, M., Hoffman, P. M., and Ruscetti, S. K. (1993). Viral determinants that control the neuropathogenicity of PVC-211 murine leukemia virus *in vivo* determine brain capillary endothelial cell tropism of the virus *in vitro*. *J. Virol.* 67, 4580–4587.

McQuaid, S., McConnell, R., McMahon, J., and Herron, B. (1995). Microwave antigen retrieval for immunocytochemistry on formalin-fixed, paraffin-embedded post-mortem CNS tissue. *J. Pathol.* 176, 207–216.

Munk, C., Lohler, J., Prassolov, V., Just, U., Stockschlader, M., and Stocking, C. (1997). Amphotropic murine leukemia viruses induce spongiform encephalomyelopathy. *Proc. Natl. Acad. Sci. USA* 94, 5837–5842.

Navia, B. A., Cho, E. S., Petito, C. K., and Price, R. W. (1986). The AIDS dementia complex: II. Neuropathology. *Ann. Neurol.* 19, 525–535.

Norenberg, M. D. (1994). Astrocyte response to CNS injury. *J. Neuropathol. Exp. Neurol.* 53, 213–220.

Park, B. H., Lavi, E., Blank, K. J., and Gaulton, G. N. (1993). Intracerebral hemorrhages and syncytium formation induced by endothelial cell infection with a murine leukemia virus. *J. Virol.* 67, 6015–6024.

Park, B. H., Lavi, E., Stieber, A., and Gaulton, G. N. (1994). Pathogenesis of cerebral infarction and hemorrhage induced by a murine leukemia virus. *Lab. Invest.* 70, 78–85.

Portis, J. L., Czub, S., Garon, C. F., and McAtee, F. J. (1990). Neurodegenerative disease induced by the wild mouse ecotropic retrovirus is markedly accelerated by long terminal repeat and gag-pol sequences from a nondefective Friend murine leukemia virus. *J. Virol.* 64, 1648–1656.

Portis, J. L., Czub, S., Robertson, S., McAtee, F., and Chesebro, B. (1995). Characterization of a neurologic disease induced by a polytropic murine retrovirus: evidence for differential targeting of ecotropic and polytropic viruses in the brain. *J. Virol.* 69, 8070–8075.

Portis, J. L., Spangrude, G. J., and McAtee, F. J. (1994). Identification of a sequence in the unique 5′ open reading frame of the gene encoding glycosylated gag which influences the incubation period of neurodegenerative disease induced by a murine retrovirus. *J. Virol.* 68, 3879–3887.

Robertson, M. N., Miyazawa, M., Mori, S., Caughey, B., Evans, L. H., Hayes, S. F., and Chesebro, B. (1991). Production of monoclonal antibodies reactive with a denatured form of the Friend murine leukemia virus gp70 envelope protein: use in a focal infectivity assay, immunohistochemical studies, electron microscopy and western blotting. *J. Virol. Methods* 34, 255–271.

Robertson, S. J., Hasenkrug, K. J., Chesebro, B., and Portis, J. L. (1997). Neurological disease induced by polytropic murine retroviruses: neurovirulence determined by efficiency of spread to microglial cells. *J. Virol.* 71, 5287–5294.

Rost, B., and Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* 232, 584–599.

Rost, B., and Sander, C. (1994). Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* 19, 55–72.

Sitbon, M., Nishio, J., Wehrly, K., Lodmell, D., and Chesebro, B. (1985). Use of a focal immunofluorescence assay on live cells for quantitation of retroviruses: distinction of host range classes in virus mixtures and biological cloning of dual-tropic murine leukemia viruses. *Virology* 141, 110–118.

Usuda, N., Arai, H., Sasaki, H., Hanai, T., Nagata, T., Muramatsu, T., Kincaid, R. L., and Higuchi, S. (1996). Differential subcellular localization of neural isoforms of the catalytic subunit of calmodulin-dependent protein phosphatase (calcineurin) in central nervous system neurons: immunohistochemistry on formalin-fixed paraffin sections employing antigen retrieval by microwave irradiation. *J. Histochem. Cytochem.* 44, 13–18.

Vesce, S., Bezzi, P., Rossi, D., Meldolesi, J., and Volterra, A. (1997). HIV-1 gp120 glycoprotein affects the astrocyte control of extracellular glutamate by both inhibiting the uptake and stimulating the release of the amino acid. *FEBS Lett.* 411, 107–109.

Wehrly, K., and Chesebro, B. (1997). p24 antigen capture assay for quantification of human immunodeficiency virus using readily available inexpensive reagents. *Methods Companion Methods Enzymol.* 12, 288–293.

Wiley, C. A., and Gardner, M. (1993). The pathogenesis of murine retroviral infection of the central nervous system. *Brain Pathol.* 3, 123–128.

Wong, P. K., Prasad, G., Hansen, J., and Yuen, P. H. (1989). ts1, a mutant of Moloney murine leukemia virus-TB, causes both immunodeficiency and neurologic disorders in BALB/c mice. *Virology* 170, 450–459.

Yang, Q., Hamberger, A., Hyden, H., Wang, S., Stigbrand, T., and Haglid, K. G. (1995). S-100 beta has a neuronal localization in the rat hind brain revealed by an antigen retrieval method. *Brain Res.* 696, 49–61.

Yu, Y., and Wong, P. K. (1992). Studies on compartmentation and turnover of murine retrovirus envelope proteins. *Virology* 188, 477–485.