Unique Sequence and Lesional Tropism of a New Variant of Neuropathogenic Friend Murine Leukemia Virus

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FrC6 murine leukemia virus (MuLV) is a replication-competent, neuropathogenic variant derived from Friend MuLV (F-MuLV) complex. The A8 virus (a molecular clone of the FrC6 virus) induced marked spongiform degeneration in the brain similar to the FrC6 virus, but only mild lesions were found in the spinal cord. In contrast, PVC211 virus, which is also a neuropathogenic F-MuLV variant, caused marked spongiform degeneration in the spinal cord. Virus recovery from the spinal cord of A8 virus-infected rat was the same as that of PVC211-infected rat, indicating that there is no direct correlation between the titer of virus and the intensity of lesions. Furthermore, rats infected with the A8 virus at 3 weeks of age did not undergo spongiform degeneration, although recovery of high titer of virus occurred in the central nervous system (CNS). Studies using chimeric viruses between the A8 virus and nonneuropathogenic F-MuLV clone 57 also indicated that the sequences responsible for virus titers in the CNS and neuropathogenicity are different. The chimeric virus studies proved that the env gene and the LTR and/or 5' leader sequence of A8 are critical for the induction of neuropathogenicity. These sequences in A8 and PVC211 were compared, focusing in on the sites that account for neurovirulence and viral lesional tropism.

INTRODUCTION

Nondefective MuLV can induce several diseases, including leukemia, immune complex autoimmune disease, central nervous system (CNS) disease, thrombocytopenia, hemolytic anemia, and immunosuppressive syndromes (Weiss et al., 1984). A number of MuLVs including Cas-Br-E MuLV isolated from wild mouse (Officer et al., 1973) and its molecular clones (Kay et al., 1991; Lynch et al., 1991), ts mutants of Moloney MuLV (Bilello et al., 1986; McCarter et al., 1977), chimeric Fr-Cas E MuLV which has gp70 of Cas-Br-E on a background of F-MuLV FB29 (Portis et al., 1990), and a mink cell focus-inducing derivative of F-MuLV (Buller et al., 1990), can induce neurodegenerative disease in mice. PVC211 (Kai and Furuta, 1984) and NT40 (Czub et al., 1996), which are variants of F-MuLV, produce neurodegenerative disease in rats. In most models, a noninflammatory spongiform degeneration in the CNS follows an early CNS endothelial cell infection. In some models, oligodendroglia, astrocytes, microglia/macrophages, and/or neurons are also infected, while in others, only endothelial cells are infected (Wiley and Gardner, 1993). These variations may be caused by mouse strain and viral differences in addition to differences in assays. The reconciliation of the various models in which the final neuropathology appears similar, may be the key to understanding their pathogenesis.

The particular distribution of the lesions is important for determining the phenotypically different neuropathogenicity of the virus. Usually, neuropathology begins in the spinal cord and ascends the neuraxis (Wiley and Gardner, 1993). When rats were infected with the F-MuLV variant, PVC211, viral replication in brain capillary endothelial cells was followed by widespread astrogliosis, neuropil vacuolation, and finally, neuronal degeneration in the spinal cord, brain stem, cerebellum, and cortex (Hoffman et al., 1992). In human retrovirus-induced myelopathy, such as that associated with human T-cell leukemia virus type I (HAM), the thoracic portion of the spinal cord is the main target of the disease (Osame et al., 1986; Umehara et al., 1993). It is important to study the determinants responsible for inducing this lesional tropism in the spinal cord. Experimental animal models are useful for investigating these pathomechanisms.

We molecularly cloned a virus, clone A8, from a neuropathogenic FrC6 virus (Watanabe and Takase-Yoden, 1995). Studies on chimeric between the A8 virus and wild-type, nonneuropathogenic F-MuLV clone 57 revealed that the env gene and LTR and/or 5' leader sequence of the A8 virus are critical for induction of spongiform degeneration in CNS. Furthermore, we compared A8 with PVC211, which is closely related but molecularly distinct from A8, in terms of the nucleotide sequences of these regions and the distribution of spongiform lesions.

MATERIALS AND METHODS

Viruses and cells

Neuropathogenic FrC6 virus-producing rat glial cells were established by passaging F-MuLV complex (Odaka,
Animals

The ability of viruses to cause neurodegenerative disease was assessed by using newborn Lewis and Fischer rats, as well as 3-week-old Lewis rats, purchased from a commercial breeder. Newborn rats were intraperitoneally inoculated with 0.1 ml and intracerebrally with 0.005 ml of viral supernatant and then observed for signs of neurological disease for 6 weeks to 6 months. Titers of inoculated virus are described in the figure legends.

After exanguination, brains, spinal cords, and thymus were homogenized in cold phosphate-buffered saline (PBS) containing 1 mM MgCl₂ and 1 mM CaCl₂, and infectious virus titers were determined by the XC cell plaque assay (Rowe et al., 1970).

DNA analysis

Basic recombinant DNA procedures such as plasmid preparation, restriction endonuclease digestion, modifying enzyme treatment, and hybridization were performed according to standard methods (Sambrook et al., 1989).

Normal C6 cells were infected with biologically cloned FrC6 virus. At 48 hr postinfection, extrachromosomal DNA was extracted from the cells by differential salt precipitation (Hirt, 1967) with some modifications (Takase-Yoden et al., 1994). The DNA was digested with EcoRI and applied onto a 10–40% sucrose gradient in PBS. After ultracentrifugation at 20°, 18 hr at 25 K rpm using an SW41 rotor (Beckman), each fraction was Southern blotted and fractions containing full-length linear viral DNA were identified. The DNA in these fractions was purified and ligated to the EcoRI-digested λZAP phage vector (Stratagene). An extrachromosomal library was screened using a 57 virus whole-genome probe.

Nucleotide sequences were determined by deoxyribonucleotide chain termination (Sanger et al., 1977) with alkali-denatured plasmid DNA as the template (Hattori et al., 1985) using an A.F.L. DNA sequencer (Pharmacia).

DNA transfection

NIH3T3 or C6 cells (3 x 10⁶) were seeded in 35-mm tissue culture dishes. Plasmid DNAs containing viral genome (4 μg) were digested with EcoRI, multimerized by ligation using T4 DNA ligase for 30 min at 16°, and precipitated with ethanol. DNA was transfected by calcium phosphate coprecipitation (Davis et al., 1986). After 7 days, the biological activities of DNA clones were determined by measuring reverse transcriptase activities. NIH3T3 cells were infected with supernatants of the transfected cells and virus infection was studied in the supernatants of virus-producing cell cultures.

Construction of chimeric virus genome

Chimeric viruses were prepared between the neuropathogenic FrC6 clone A8 and the nonneuropathogenic clone 57 virus (Fig. 5). Clone 57 DNA was originally cloned at the EcoRI site of pBR322 (Oliff et al., 1980) and recloned into pBluescript (Stratagene) for these experiments. Clone A8 DNA was also cloned into pBluescript at the EcoRI site. To construct Rec1, the ClaI-EcoRI fragment containing LTR and the gag gene was replaced by the corresponding fragment of clone 57 DNA. To construct Rec2, the EcoRI–ClaI fragment containing the pol and env genes was replaced with the corresponding fragment of clone 57 DNA. To construct Rec3, the SphI–EcoRI fragment was replaced with the corresponding fragment of clone 57 DNA. To construct Rec5, the EcoRI–SphI fragment of clone 57 was ligated into the SphI–EcoRI fragment of Rec1. To construct Rec6, the EcoRI–SphI fragment of clone A8 was ligated into the SphI–EcoRI fragment of Rec2. To construct Rec7, the HindIII–HindIII fragment was replaced with the corresponding fragment of clone 57 DNA. Structures of chimerae were confirmed by digestion with the restriction enzymes BamHI, DraI, KpnI, NcoI, PstI, and PvuII, the cleavage sites for which are unique either to clone A8 or to clone 57.

Histology

The brains and spinal cords of infected rats were immersed in 4% paraformaldehyde buffered with 0.12 M phosphate (pH 7.3) and fixed. Some infected rats were perfused with 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.3). The tissues were embedded in paraffin for histological staining with hematoxylin and eosin and luxol-fast blue. Paraffin sections were also used for immunohistochemical studies. Epon-embedded materials were prepared from the tissues fixed with 2% glutaralde-
hyde in the phosphate buffer by perfusion followed by postfixation with osmium tetroxide. Ultrathin sections were counterstained with uranyl acetate and lead acetate, and photographed using a JEM-1200EXII (JEOL). Viral antigen was detected with 10 μg/ml purified monoclonal antibody 85-1 (Ikeda et al., 1995) followed by biotinylated sheep anti-mouse IgG (Amersham) and then with avidin–peroxidase complex (Wako). Between each step, the glass slides were washed with PBS. For the peroxidase reaction, 0.1 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (DOTIDE) in 0.1 M Tris buffer (pH 7.6) was used. Normal mouse serum used instead of the monoclonal antibody served as a control.

Nucleotide sequence accession number

The DDBJ, EMBL, and GenBank Accession No. of the sequences in this paper is D88386.

RESULTS

Molecular cloning of an infectious DNA of FrC6 MuLV

Extrachromosomal DNA was extracted 48 hr after infection of the glial cell line C6 with FrC6 virus, once the presence of a single EcoRI endonuclease restriction site in the proviral DNA was confirmed by hybridization (data not shown). The DNA was digested with EcoRI and ligated to the EcoRI-digested λZAP phage vector. The extrachromosomal DNA library was screened using a 57 virus whole-genome probe. Ten positive clones were randomly selected and after two or three rounds of plaque purification, the Bluescript plasmid containing insert was excised from positive λZAP phage. Six of these clones were of the correct size according to restriction mapping.

The viral inserts were excised from the plasmid by EcoRI digestion, multimerized by ligation, and transfected into C6 cells. After 7 days, the biological activity of each clone was determined by measuring the reverse transcriptase activity. Two of three clones were biologically active and one of these, designated A8, was applied to subsequent experiments.

Replication and pathogenicity of FrC6 MuLV clone A8 in rats

To investigate the level of replication and pathogenicity in vivo, we injected the A8 virus into newborn or 3-week-old Lewis rats. Brains and thymus were homogenized after exanguination and infectious virus titers were determined. When inoculated into newborn rats, A8 or 57 was recovered from the brain at similar titers at 1 week postinfection (Fig. 1A). At 8 weeks postinfection, the virus recovery of A8 in the brain and the thymus was 1000- and 300-fold higher than that of 57, respectively. When inoculated into 3-week-old rats, A8 was recovered from brain and thymus, but 57 did not show detectable proliferation (Fig. 1B).

The A8 virus caused neurological disease as did the biologically cloned FrC6 virus when injected into newborn Lewis rats. Pathological changes in the CNS of rats infected with A8 consisted of spongiform degeneration without cell infiltration (Figs. 2B, 2C, 2D, and 2F). The lesions caused by A8 were most prominent in the brain stem and the thalamus, where over 100 vacuoles formed clusters (Figs. 2C and 2D). These clusters often occupied more than 30% of the brain stem (+ + + + + + lesion in the Table 1). In the brain cortex, the vacuolar changes were moderate. Small clusters usually consisted of 10 to 20 scattered vacuoles (Fig. 2B). The lesions in the spinal
FIG. 2. Spongiosis induced by infection with the A8 and PVC211 virus. Paraffin sections were prepared from the tissues of rats infected with the A8 (B, C, D, F) or PVC211 virus (A, E). (A) No lesion (−), in the brain cortex of a Fischer rat infected with PVC211. (B) Clusters of vacuoles, forming mild spongiform lesions in brain cortex (arrowhead). Each cluster contains 20–100 vacuoles. This lesion, which can be observed in one field by using the light microscopic subjective lens at ×10 magnification (field (×10)), represents the ++ described in the legend to Table 1. (C) Sagittal section of pons and cerebellum (right side). More than 100 vacuoles composing a distinct spongiform extend within one field (×10). This lesion represents ++++. In the cerebellum, a +++ lesion is evident (arrow). (D) Higher magnification of the +++ lesion in C. (E) Fields (×10) with spongiform lesions composed of over 100 vacuoles occupy over 30% of the total area of the spinal cord. Representative of ++++ lesion. (F) Less than 20 vacuoles (arrowhead) are located in the section of the spinal cord of the rat infected with the A8 virus. Representative of + lesion. This animal exhibited a +++ lesion in thalamus. Abbreviations: PVC211, tissue from a rat infected with PVC211 virus; A8, tissue from a rat infected with A8 virus. Bars indicate 100 μm (D, E, F) and 250 μm (A, B, C).
### TABLE 1

Topographical Distribution of Spongiform Lesions in the CNS of the Rats Infected with A8 and PVC211 Viruses

<table>
<thead>
<tr>
<th>Inoculated(^a) with</th>
<th>Weeks postinfection</th>
<th>Spongiform lesions(^b)</th>
<th>Virus recovery(^c) (XC-PFU/g)</th>
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<td>Thalamus</td>
<td>Cerebellum</td>
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<td>4.6</td>
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<td>+</td>
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<td></td>
<td>8.4</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

\(^a\) New born Lewis rats were inoculated with the A8 (2.8–3.8 × 10^5 XC-PFU/rat except pi 10 weeks: 1.7 × 10^6 XC-PFU/rat) or PVC211 virus (2.4 × 10^4 XC-PFU/rat), intraperitoneally and intracerebrally.

\(^b\) ‐, No lesion; ++, less than 20 vacuoles in the total area; +++, at ×10 magnification (field (×10)), 20 to 100 vacuoles counted in the field; ++++, clusters consisting of over 100 vacuoles spread within one field (×10); +++++, clusters consisting of over 100 vacuoles scattered over 2 fields (×10); ++++++, clusters of vacuoles occupy over 30% of the total area.

\(^c\) Virus recovery was determined by XC plaque assay as described under Materials and Methods.
TABLE 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Rat*</th>
<th>Spongiform lesionb (spinal cord/thalamus)</th>
<th>Viral recoveryc (spinal cord/brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8</td>
<td>Lewis</td>
<td>0.36 ± 0.03 (14)</td>
<td>1.94 ± 0.24 (7)</td>
</tr>
<tr>
<td></td>
<td>Fischer</td>
<td>0.25 ± 0.25 (4)**</td>
<td>NT</td>
</tr>
<tr>
<td>PVC211</td>
<td>Lewis</td>
<td>1.25 ± 0.26 (12)*</td>
<td>2.30 ± 0.49 (4)*</td>
</tr>
<tr>
<td></td>
<td>Fischer</td>
<td>1.72 ± 0.43 (11)</td>
<td>2.04 ± 0.82 (4)</td>
</tr>
</tbody>
</table>

a New born Lewis and Fischer rats were intraperitoneally and intracerebrally inoculated with the A8 virus, as described in Table 1; Fischer rat, 1.7 × 10⁴ XC-PFU/rat or PVC211 virus (2.4 × 10⁴ XC-PFU/rat).
b,c The symbol (+) indicating the intensity of neuropathologic lesions and virus titers recovered from the tissues of infected animals shown in Table 1 except for Fischer rats, is substituted by numbers for statistical comparison. The amount of virus recovered from spinal cords and brains of PVC211-virus-infected Fischer rat was 5.5 × 10⁵-2.5 × 10⁶ and 2.7 × 10³-2.3 × 10⁵ XC/PUF/g, respectively. In each animal, numbers of (+) and virus titers in the spinal cord were divided by the numbers of (+) and the virus titer in the brain, respectively.
d Difference is not significant compared to PVC211-infected Fischer rat.
e Difference is not significant compared to A8-infected Lewis rat or PVC211-infected Fischer rat.
* P < 0.01 vs PVC211-infected Lewis rat. Difference is not significant compared to A8-infected Fischer rat.** P < 0.02 vs PVC211-infected Fischer rat.

which contains the gag gene, except for 0.2 kb of the 5’ terminus of the gag coding region, and the pol gene, except for 0.8 kb of the 3’ terminus of the pol coding region, does not include the unique sequence that causes neurodegenerative disease. Rec2, which contains the E. coli – Clal fragment of the 57 virus and Rec6, which contains the 2.6-kb Sphl–Clal fragment of 57 on a background of A8 caused very slight, if any, neurodegenerative disease. In the 75-bp HinIII–Sphl fragment containing the 3’ terminus of the pol coding region, no amino acid substitutions were found (data not shown). Therefore, the Sphl–Clal fragment of the A8 virus containing the 0.7-kb 3’ terminus of the pol gene and the env gene except for the 0.1-kb 3’ terminal p15E region, is essential for neurodegenerative disease. Rec3 failed to induce spongiform degeneration within 6 – 9 weeks postinfection, although the animals remained infected with Rec5 for 17 – 20 weeks postinfection. Therefore, the additional determinant for neurological disease resides within the 1.5-kb Clal–HinIII fragment of the A8 virus containing LTR, 5’ leader sequence, and the 0.26-kb 5’ terminal gag region.

The A8 virus, and Rec2, Rec6, and Rec7, which have the 1.5-kb Clal–HinIII fragment of A8, replicated with high titers in the rat brain, while the 57 virus and Rec5, which has the Clal–HinIII fragment of 57, proliferated at low efficiency (Fig. 5). In the thymus of infected rats, viral recovery of A8, Rec2, Rec6, and Rec7 was higher than that of 57 and Rec5. Accordingly, a determinant for high level viral replication in the rat resides within the Clal–HinIII fragment of the A8 virus.

Viral replication of A8, 57, and chimeric viruses in glial cell lines

To determine whether or not the A8 virus retained the same ability as the FrC6 virus to replicate in cultured glial cell lines, we inoculated A8 and 57 viruses into the glial cell line F10 and measured viral production. As shown in Fig. 5, A8 replicated in glial cells. In contrast, 57 was not very infective in F10 cells, but in NIH3T3 cells, both 57 and A8 proliferated with a titer of 2 – 6 × 10⁵ XC-PFU/ml (data not shown).

To determine which sequences of the A8 virus are responsible for replication in F10 cells, we inoculated chimeric A8 and 57 viruses into cultured-glial cells. All viruses proliferated well in NIH3T3 cells with a titer of 10⁵ – 10⁶ XC-PFU/ml (data not shown). As shown in Fig. 5, A8 replicated in glial cells. In contrast, 57 was not very infective in F10 cells, but in NIH3T3 cells, both 57 and A8 proliferated with a titer of 2 – 6 × 10⁵ XC-PFU/ml (data not shown).

FIG. 3. Immunohistochemistry to detect A8 viral antigen in the CNS. (A) Using the monoclonal antibody, Mab 85-1, A8 viral envelope protein was detected mainly in the walls of blood vessels (arrow) in the thalamus of Lewis rat infected with the A8 virus. A small cluster of vacuoles is seen adjacent to the antigen-positive blood vessel (arrowhead). (B) In uninfected rat CNS, viral antigen was not detected. Bars indicate 100 μm.
5. Rec2, Rec6, and Rec7, which contain a Clal–HindIII fragment of the A8 virus, proliferated in glial cells almost to the same level as the complete virus. In contrast, Rec1, Rec3, and Rec5, which contain the Clal–HindIII fragment of the 57 virus, replicated at low efficiency in F10 cells. Therefore, the LTR, 5′ leader region and 0.26-kb 5′ termi-

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FIG. 4. Electron micrograph of the thalamus from a rat infected with the A8 virus. (A) Most of the vacuoles are surrounded by a membranous structure and contain no cell structures that indicate the origin of vacuolations. Enlarged axons (arrow) are situated near the vacuolations. Bar indicates 1 μm. (B) Occasional degenerative postsynaptic dendrites are evident. Note the structure of the synaptic junction (arrow). Bar indicates 100 nm.
FIG. 5. Structure, pathogenicity, and ability of replication in rat tissue and cultured-glial cells of A8, 57, and derived recombinant viruses. The viral genomes are represented in their nonpermutated form, flanked at their ends by the LTR sequence. In recombinant viral genomes, solid regions are sequences derived from the A8 virus and open regions are sequences derived from the 57 virus. Each viral DNA was transfected to NIH3T3 or C6 cells. The viral preparations used for virus-infection studies were obtained from NIH3T3 cells infected with the supernatants of the transfected cells. Viruses were inoculated intraperitoneally and intracerebrally into newborn Lewis rats (A8: 6 rats, 2.8 ± 10^5; 8 rats, 3.8 ± 10^5; or 1 rat, 1.7 ± 10^6 XC-PFU/rat. Rec7: 2.4 ± 10^6 XC-PFU/rat. Rec6: 4 rats, 2.1 ± 10^6 or 11 rats, 2.0 ± 10^6 XC-PFU/rat. Rec2: 3.8 ± 10^6 XC-PFU/rat. Rec5: 8 rats, 4.6 ± 10^5 or 2 rats, 1.1 ± 10^5 XC-PFU/rat. 57: 3 rats, 1.8 ± 10^4 or 15 rats, 9.3 ± 10^4 XC-PFU/rat). Six to 9 weeks later, neurodegeneration in the CNS and virus recovery were determined. Means and SEM are indicated. The numerals enclosed in parentheses represent numbers of rats. Asterisk (*) indicates only 6 vacuoles in the rat pons infected with 4.6 ± 10^3 XC-PFU/rat. Rat cultured-glial cells (F10) were also inoculated with virus at a m.o.i. of 0.1 or 1. After 4 days of culture, virus production was determined by XC plaque assay. Means ( ± 3) are indicated.

DISCUSSION

The A8 virus, which was molecularly cloned from a variant of F-MuLV, FrC6, induced neuropathogenic disease in rats and proliferated in cultured rat glial cells (F10) with a high titer. PVC211, which is also a neuropathogenic virus derived from F-MuLV (Kai and Furuta, 1984) induces similar spongiform degeneration in the CNS. Hoffman et al. (1992) reported that these lesions were most severe in the spinal cord, brain stem, and cerebellum. In the present study, spongiform degeneration was also apparent in the spinal cord of the PVC211-infected rat. In the spinal cord of A8 virus-infected rats, spongiform lesions were weakly apparent (Fig. 2F, Tables 1 and 2). Both A8 and PVC211 viruses replicated in the spinal cord at titers of 4.8 ± 10^4–6.0 ± 10^5 XC-PFU/g, which were double that in the brain of each rat (Tables 1 and 2). The ratios of the virus titers in spinal cord and brain of PVC211 virus-infected rats were comparable with those reported by Kai and Furuta (1984). These results indicate that in spinal cord, there is no direct correlation between the titer of virus and the intensity of spongiform lesions.

We constructed a series of chimeric between the A8 virus and wild-type F-MuLV clone 57, which did not cause disease and replicated at low efficiency in rat brain and
TABLE 3
Comparison of Amino Acids in the env Gene Products of A8, 57, and PVC211 Viruses*

<table>
<thead>
<tr>
<th>Position</th>
<th>A8</th>
<th>S7</th>
<th>PVC211</th>
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<tr>
<td>7</td>
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<tr>
<td>392</td>
<td>Gin</td>
<td>Arg</td>
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<tr>
<td>413</td>
<td>Thr</td>
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<tr>
<td>414</td>
<td>Gly</td>
<td>Asp</td>
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<td>424</td>
<td>Ala</td>
<td>Thr</td>
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<td>427</td>
<td>Met</td>
<td>Thr</td>
<td>Met</td>
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<tr>
<td>676</td>
<td>Gin</td>
<td>End</td>
<td>Gin</td>
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</tbody>
</table>

* Amino acid sequences of the env product of A8, 57, and PVC211 virus were deduced from the nucleotide sequences and compared. Shadowed boxes indicate amino acids that differ between A8 and PVC211 viruses. Unique amino acids in neuropathogenic viruses are underlined. Open boxes indicate the amino acids responsible for cell tropism (Masuda et al., 1996).

Amino acid sequences were deduced from the nucleotide sequences and compared. Shadowed boxes indicate amino acids that differ between A8 and PVC211 viruses. Unique amino acids in neuropathogenic viruses are underlined. Open boxes indicate the amino acids responsible for cell tropism (Masuda et al., 1996).

The amino-terminal methionine of the envelope protein precursor is numbered 1. Positions 1 to 34, leader peptide; 35 to 479, gp70; 480 to 676, p15E.

Structural elements are described by Linder et al. (1994).

A10 cells (Figs. 1 and 5). We then examined the biological activities of these viruses, to define the gene(s) responsible for the neuropathogenicity and/or virus titers. Rec7, which has both the SpH1–Cla1 fragment containing the env gene and the Cla1–HindIII fragment containing the LTR and the 5′ leader sequence of the A8 virus, induced neuropathogenicity, whereas Rec2, Rec5, and Rec6, which have either the SpH1–Cla1 fragment or the Cla1–HindIII fragment of the A8 virus, did not (Fig. 5). These results indicate that the determinants of spongiform degeneration in the CNS of infected rats reside within these two fragments of the A8 virus. Rec2 and Rec6 proliferated in rats (Fig. 5). Furthermore, these viruses were recovered from the brain as was Rec7, with titers of 6.4 ± 6.2 × 10^6 and 1.6 ± 0.9 × 10^5 XC-PFU/g, respectively, whereas both viruses did not, or scarcely induced spongiform degeneration in infected rats (Fig. 5). Therefore, the Cla1–HindIII fragment of the A8 virus is sufficient for high titer of the virus in the brain but is not sufficient to induce neuropathogenicity. Here again, the discrepancy of viral proliferation and neuropathogenicity was indicated.

However, a high level of viral proliferation seems to be necessary, if not sufficient, to induce spongiform degeneration in the CNS. Rec5, having the SpH1–Cla1 fragment of the A8 virus on a background of the 57 virus, did not induce spongiform degeneration in the CNS. Since the level of recovery of Rec5 from the rat brain was lower, the amount of env protein might not be sufficient to induce spongiform degeneration. In a transgenic model carrying the env gene and LTR of the Cas-Br-E MuLV, a spongiform change and astroglisis consistent with the natural disease appeared after an extended period in the absence of viral replication (Kay et al., 1993). However, in this transgenic mouse, the lesions were mild and the level of env expression, that was promoted by LTR, was low. Recent work revealed that expression of the env protein of the Cas-Br-E MuLV is not sufficient to induce spongiform neurodegeneration in vivo (Lynch et al., 1996). These findings indicate that the viral genes other than env also contribute to the neuropathogenicity.

The U3 region of the LTR in other viruses is an important determinant of target cell and disease specificity (Wiley and Gardner, 1993). Paquette et al. (1990) reported that the substitution of the U3 LTR affects the distribution of lesions. After replacing the LTR of Cas-Br-E MuLV with that of ts-Mo BA1 MuLV, the spongiform degeneration in the deep cerebellar nuclei and the spinal cord appeared to be more severe. The construction of the nuclear-factor binding sites in the U3 region of A8-LTR (manuscript in preparation) differed from that of 57-LTR (Manley et al., 1989) or PVC211 virus (Masuda et al., 1992). Differences in the U3 region between A8 and PVC211 may be responsible for the distribution of lesions in the CNS.

A 5′ leader sequence is located between the primer-binding site and the beginning of gag, and contains a donor site for the generation of spliced subgenomic mRNA and the packaging signal for the incorporation of genome RNA into virions. This region also contains the internal ribosomal entry mechanism that promotes the translation of gag polyprotein precursors (Berlioz and Darlix, 1995). The 5′ leader sequence of the A8 virus contains 24 nucleotide mutations relative to the 57 virus (manuscript in preparation). Masuda et al. (1993) and Czub et al. (1992) reported that the 5′ leader sequence is related to a shorter onset of neurological signs. Therefore, it is possible that these differences in nucleotides are important for neuropathogenicity.

When the A8 virus was inoculated into 3-week-old rats, typical neurological changes were not evident in the brain, although viral recovery from the brain at 8 weeks postinfection was comparable (8.4 ± 3.2 × 10^4 XC-PFU/
g) to that from the brain when inoculated into newborn rats (2.1 ± 0.9 × 10³ XC-PFU/g) (Figs. 1A and 1B). Not only did this finding reconfirm the discrepancy between virus titers and pathogenicity, it also indicates that the A8 virus induces pathogenic effects upon developing brain tissue. Brooks et al. (1981) speculated that age-related changes in the cell surface of astrocytes may influence the course of neurotropic retrovirus infection in these cells. However, Lynch et al. (1995) indicated that the developmental resistance of the CNS to infection of FlCas lies at the postnatal change of the blood-brain barrier, resulting in age-dependent resistance to disease expression, since this resistance can be bypassed by directly introducing virus-infected cells into the brain.

The viruses that proliferated at low efficiency in F10 cells, such as the 57 virus, Rec1, Rec3, and Rec5, did not cause neuropathogenicity in infected rats (Fig. 5). Therefore, effective viral proliferation in F10 cells is a necessary condition for neuropathogenicity. The viruses that replicated efficiently in F10 cells, such as the A8 virus, Rec2, Rec6, and Rec7, also recovered with high titers from rat brains. These findings imply that viral replication in F10 cells is a useful indicator with which to screen the neuropathogenicity in vivo of viral variants.

Our studies indicate that not only a high level of viral production in the brain, but also expression of the env protein of the A8 virus is necessary to cause neuropathological changes. The SpI–Clal fragment of the A8 virus contains the 3′ terminal pol and env gene except for the 3′ terminus of the p15E coding region. Although in this fragment, nucleotide mutations in the carboxy terminus of the pol product and in the signal peptide of the env gene product, compared with the corresponding region of 57 virus, caused changes to two and one amino acid (Table 3), respectively, these changes were relatively conservative and minor compared with the variability of these regions among ectropic MuLVs. The p15E coding region containing the SpI–Clal fragment did not contain any mutations that led to amino acid changes compared with the 57 virus. Therefore, it is most likely that one of the determinants of spongiform degeneration resides within the gp70 coding region of the env gene of the A8 virus. It has been postulated that four amino acids (Arg-114, Lys-163, Ala-311, and Gly-379) of PVC211 clone 3d are unique in neuropathogenic virus compared with ecotropic MuLVs (Masuda et al., 1993) such as F-MuLV clone K-1 (Obata et al., 1984), F-MuLV clone FB29 (Perryman et al., 1991), M-MuLV (Shinnick et al., 1981), and Akv MuLV (Herr, 1984). In the present study, the same analysis showed that the A8 virus encodes three unique amino acids (Arg-114, Ala-311, and Gly-379) in the gp70 protein (Table 3, underlined). One of these amino acids (Gly-379) is shared by neuropathogenic Cas-Br-E, while all three are shared by PVC211 clone 3d. These results indicate that Arg-114, Ala-311, and Gly-379 are important for neuropathogenicity.

Structural element I and II is localized on the surface of the gp70 molecule and thought to play an essential role in receptor binding (Linder et al., 1994; Masuda et al., 1996). Asn-113, Arg-114, and Ser-118 of A8-env are located within structural element I (Table 3). Compared with 57-env, Asn-113 and Arg-114 differ, but Ser-118 is common. Asn-113 and Arg-114 of A8-env are the same as those of PVC211-env, suggesting that the two are crucial for neuropathogenicity mediated by receptor-binding. Although A8 and PVC211 caused similar spongiform degeneration in rat CNS, differences in the distribution of the lesions in the CNS were observed (Table 1). The env gene of these viruses is essential for neurovirulence (Fig. 5). Relative to the gp70 protein, three amino acid differences were found (Table 3). Of these amino acids, Ser-118 and Glu-163 of A8-env are localized in structural elements I and II of the gp70 protein, respectively. These differences in amino acids may cause the different interaction with molecule(s) responsible for brain function and induce various intensities of spongiform lesions. In HIV infection, it was reported that the gp120 glycoprotein or its fragment competes with vasoactive intestinal polypeptide (VIP) on neuronal cells, and neuronal injury arises through increases in the intracellular Ca²⁺ concentration mediated by the NMDA receptor (Lipton, 1992).

The PVC211 virus effectively infects rat brain capillary endothelial cells (Hoffman et al., 1992), and this tropism correlates with the neuropathogenicity of PVC211 (Masuda et al., 1993). Our neuropathogenic isolate also had affinity for the vascular wall (Fig. 3). Masuda et al. (1996) reported that Gly-150, which is surrounded by preferential proteolytic sites (Linder et al., 1994), and Lys-163, which is localized in structural element II, are responsible for this endothelial cell tropism mediated by gp70-receptor interaction (Table 3, open box). Sequencing indicated that compared with PVC211-env, Gly-150 of A8-env is common, but Glu-163 of A8-env is not (Table 3). Glu-163 of A8-env is the same as that of nonendothelial cell tropic 57 virus env (Masuda et al., 1993). These results indicate that Gly-150, is at least in the env region, important for endothelial cell tropism and neuropathogenicity.

The A8 virus was isolated from the same source as PVC211 (Kai and Furuta, 1984), i.e., FLV complex passed through mice (Odaka, 1973), but by a different method (Watanabe and Takase-Yoden, 1995). Although A8 and PVC211 both proliferated in the CNS and caused similar neurodegenerative diseases, these viruses resulted in different neuropathological manifestations in terms of the distribution of lesions. In this paper, we proposed that the differences in the env gene and/or LTR between A8 and PVC211 were responsible for the different lesions distribution in the CNS. Particularly, the env gene was essential for induction of neurodegeneration in both A8 and PVC211. The product of A8-env gene differed by 3 amino acids (Val-35, Ser-118, Glu-163) compared with PVC211. Analysis and comparison of these
viruses should make it easier to focus in on the site for mutational analysis to determine the viral lesional tropism.

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