A 0.3-kb fragment containing the R-U5-5′ leader sequence is essential for the induction of spongiform neurodegeneration by A8 murine leukemia virus

Sayaka Takase-Yoden*, Rihito Watanabe

Department of Bioinformatics, Faculty of Engineering, Soka University, Tangi-cho 1-236, Hachioji, Tokyo 192-8577, Japan

Received 28 December 2004; returned to author for revision 21 January 2005; accepted 4 March 2005
Available online 11 April 2005

Abstract

Friend murine leukemia virus (Fr-MLV) clone A8 causes spongiform neurodegeneration in the rat brain. The A8-env gene is a primary determinant of neuropathogenicity, and the 1.5-kb Clal–HindIII fragment containing the LTR and 5′ leader from A8 are additionally required for spongiosis. After replacement of the A8 enhancer region of the neuropathogenic chimera with the enhancer region of non-neuropathogenic 57, viral titer in the brain was reduced by two orders of magnitude. However, the A8 enhancer region was not responsible for the induction of spongiosis. The region responsible for neuropathogenesis was located in the 0.3-kb KpnI–AatII fragment of A8 containing the R-U5-5′ leader. The chimeric virus possessing this 0.3-kb fragment of A8 and the A8-env in the 57 background induced a high rate of spongiform neurodegeneration within 7 weeks (9/9 of infected rats). Studies using cultured cells suggest that the 0.3-kb fragment influences the expression of Env protein. Furthermore, these neuropathogenic chimerae, despite low viral replication in the brain, exhibited a stronger expression of Env protein compared with that of non-neuropathogenic viruses.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Spongiform neurodegeneration; Rat; Determinant; R-U5-5′ leader sequence; Env protein; Retrovirus; Murine leukemia virus clone A8

Introduction

A neuropathogenic variant of Friend murine leukemia virus (Fr-MLV), FrC6 virus clone A8, induces spongiform neurodegeneration without inflammatory infiltrates when infected into neonatal rats (Takase-Yoden and Watanabe, 1997; Watanabe and Takase-Yoden, 1995). Studies with chimerae constructed from A8 virus and non-neuropathogenic Fr-MLV clone 57 (Oliff et al., 1980) identified the env gene of A8 as a primary determinant for the induction of spongiform neurodegeneration in the brains of infected rats, and the 1.5-kb Clal–HindIII fragment containing the LTR and 5′ leader sequence of A8 as necessary for neuropathogenicity (Takase-Yoden and Watanabe, 1997). Several MLVs cause spongiform neurodegeneration when infected into neonatal mice and/or rats (Bilello et al., 1986; Czub et al., 1995; Gardner, 1978; Kai and Furuta, 1984; Tanaka et al., 1998; Wong, 1990; Wong et al., 1983). One common feature of these viruses, including A8 virus, is that the primary determinant for the induction of neurodegenerative disease is the env gene. Other viral genes also have an effect on neuropathogenicity. In the case of CasBrE, PVC211, and PVC441, the sequences within the LTR, the 5′ leader region, and the gag-pol also affect neurovirulence (Czub et al., 1992; DesGroseillers et al., 1985; Masuda et al., 1993; Portis et al., 1991, 1994; Tanaka et al., 2000).

Some uninfected neurons may exhibit cytopathogenicity, indicating an indirect mechanism of MLV-induced neuropathogenicity (Portis, 2001; Wiley and Gardner, 1993). However, the patho-mechanism of spongiosis induced by env and other genes is still not understood. It has been reported that microglial infection appears to be necessary for the induction of spongiform neurodegeneration in the brains of mice or rats infected with neuropathogenic viruses (Bazsler and Zachary, 1990, 1991; Czub et al., 1995; Gravel et al., 1995; Gardner, 1978; Kai and Furuta, 1984; Tanaka et al., 1998; Wong, 1990; Wong et al., 1983).
et al., 1993; Lynch et al., 1991). On the other hand, it has been reported that the infection of brain capillary endothelial cells correlates with neuropathogenicity in PVC211-infected rats (Masuda et al., 1996). iNOS expression in the infected cells may play an important role in neurodegeneration (Jinno-Oue et al., 2003). Other cellular effectors involving several lymphokines and cytokines have been found as aberrantly expressed in the brain of infected animals, but it is not clear whether these effectors are required for the development of neurodegenerative lesions (Askovic et al., 2001; Choe et al., 1998; Jolicoeur et al., 2003; Kim et al., 2002; Nagra et al., 1994; Peterson et al., 2001; Zachary et al., 1997). Recently, Dimcheff et al. (2003) reported that endoplasmic reticulum (ER) stress responses induced by the accumulation of uncleaved Env precursor protein correlate with retrovirus-induced spongiform neurodegeneration in FrCas5-infected mice.

In this study, we identified the minimal genetic determinant within the LTR and 5′ leader of A8 that controls neuropathogenicity. Studies using chimeric A8 and non-neuropathogenic MLV clone 57 revealed that the 0.3-kb KpnI–AarII fragment containing 0.04-kb of R, U5, and the 5′ half of the 5′ leader of A8 is essential for the induction of spongiform neurodegeneration. Furthermore, we compared the amount of viral DNA, viral transcripts, and viral protein in the cultured cells infected with chimeric viruses, and discussed the role of the 0.3-kb KpnI–AarII fragment of A8 in viral replication.

Materials and methods

Viruses and cells

Neuropathogenic FrC6 virus clone A8 was obtained as described previously (Takase-Yoden and Watanabe, 1997, Watanabe and Takase-Yoden, 1995). The infectious DNA of clone 57 of Fr-MLV (Oliff et al., 1980) and A8 was transfected into NIH3T3 cells, and a virus-producing cell culture was established. The supernatants of these cells were used to infect NIH3T3 cells, and virus-producing cultures were used in the experiments. Virus titers were determined by XC cell-plaque assay on C182 cells in the presence of 10 μg/ml of Polybrene (Rowe et al., 1970). C182 cells were grown on minimum essential medium supplemented with 10% calf serum. XC cells, NIH3T3 cells, and rat glial F10 cells (Sun et al., 1993; Wekerle et al., 1987) were grown on Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

Construction of chimeric viruses

Chimeric viruses combining A8 and 57 were prepared as follows: R7a was constructed by replacing the AarII–HindIII fragment containing the 3′ half of the 5′ leader sequence, gag and pol genes of A8 with the corresponding fragment of 57. The construction of R7c involved replacing the BlpI–HphI fragment of A8-LTR of R7a with the BlpI–BsiEI fragment of 57-LTR. Ligation of the HphI and BsiEI sites was performed using an XhoI linker. The construction of R7g involved replacing the KpnI–HindIII fragment containing the 3′ half of the R region and U5 of LTR, the 5′ leader sequence, gag and pol genes of A8 with the corresponding fragment of 57. R7e was constructed by replacing the HindIII–HindIII fragment containing gag and pol genes and the CloI–KpnI fragment containing the 3′ terminus of the env gene, the intergenic region, the U3, and the 5′ half of the R region in the LTR of A8 with the corresponding fragments of 57. R7f was constructed by replacing the AarII–HindIII fragment and the CloI–KpnI fragment of A8 with the corresponding fragments of 57. R7b was constructed by replacing the HindIII–HindIII fragment containing gag and pol genes and the CloI–AarII fragment containing the 3′ terminus of the env gene, the intergenic region, LTR, and the 5′ half of the 5′ leader sequence of A8 with the corresponding fragments of 57. R5a was constructed by replacing the BlpI–BsiEI fragment of 57-LTR of Rec5 with the BlpI–HphI fragment of A8-LTR. The XhoI-linker was used to ligate the HphI and BsiEI sites. Rec5 was constructed by replacing the SphI–ClaI fragment containing the env gene of 57 with the corresponding fragment of A8. The structures of chimeric viruses were confirmed by digestion with restriction enzymes and sequence analysis. Basic recombinant DNA procedures were performed according to standard methods (Sambrook et al., 1989).

Animal experiments

The ability of viruses to cause disease was assessed using newborn Lewis rats purchased from a commercial breeder. Newborn rats were inoculated intraperitoneally with 0.1 ml and intracerebrally with 0.005 ml of viral supernatant. The titers of the inoculated virus were A8: 9 rats, 2.8 × 10² XC-PFU/rat; 4 rats, 3.8 × 10⁵ XC-PFU/rat; 1 rat, 1.7 × 10⁶ XC-PFU/rat. R7a: 6.4 × 10⁵ XC-PFU/rat. R7c: 8.4 × 10⁴ XC-PFU/rat. R7g: 2.8 × 10⁴ XC-PFU/rat. R7e: 1.5 × 10⁵ XC-PFU/rat. R7f: 2.8 × 10⁴ XC-PFU/rat. R7b: 4.4 × 10⁵ XC-PFU/rat. R7: 8.4 × 10⁴ XC-PFU/rat. R7: 8.4 × 10⁴ XC-PFU/rat. R5a: 1.5 × 10⁴ XC-PFU/rat. Rec5: 1.0 × 10⁴ XC-PFU/rat. 57: 3 rats, 1.8 × 10⁴ XC-PFU/rat; 15 rats, 9.3 × 10⁴ XC-PFU/rat. Neurodegeneration in the CNS was determined at 5 w-pi: 3 rats; 6 w-pi: 5 rats; 7 w-pi: 2 rats; 8 w-pi: 3 rats; 10 w-pi: 1 rat (A8), 6 w-pi: 5 rats; 7-w-pi: 5 rats; 8 w-pi: 2 rats; 9 w-pi: 1 rat; 10 w-pi: 2 rats (R7a), 7 w-pi: 3 rats; 8 w-pi: 7 rats (R7c), 7 w-pi: 3 rats; 8 w-pi: 9 rats (R7g), 7 w-pi: 8 rats (R7e), 7 w-pi: 9 rats (R7f), 6 w-pi: 6 rats; 7 w-pi: 6 rats (R7b), 7 w-pi: 8 rats; 8 w-pi: 3 rats (R5a), 8 w-pi: 4 rats; 9 w-pi: 6 rats (Rec5), and 6 w-pi: 1 rat; 8 w-pi: 9 rats; 9 w-pi: 8 rats (57).

After exsanguination of the infected animals, the brain and spleen were homogenized in cold phosphate-buffered saline.
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). For histological analysis, the organs were immersed in 4% paraformaldehyde buffered with 0.12 M phosphate (pH 7.3) and fixed. The tissues were embedded in paraffin for histological staining with hematoxylin and eosin.

**Histology**

The degree of spongiform neurodegeneration was scored as follows: 0, no lesions; 1, less than 20 vacuoles in the total area; 2, 20 to 100 vacuoles counted in the field at 10× magnification (field (×10)); 3, clusters consisting of over 100 vacuoles spread within one field (×10); 4, clusters consisting of over 100 vacuoles scattered over 2 fields (×10); 5, clusters of vacuoles occupying over 30% of the total area. The scores were separately determined at the cortex, thalamus, cerebellum, brain stem, and spinal cord. The sum of each score in the five areas is shown in Table 1.

For detection of the viral antigen, goat anti-Rauscher MLV gp70 (Quality Biotech Incorporated Resource Laboratory) and paraffin sections were used. Non-specific antibody binding was blocked by incubation of the sections and the cells in 50% normal mixed serum (fetal calf, calf, pig, and horse) diluted in PBS. Biotinylated rabbit anti-goat IgG (ZYMED Laboratories Inc.) was used as a secondary antibody followed by treatment with avidin–peroxidase complex (ZYMED Laboratories Inc.). PBS washing was performed between each step. After primary antibody incubation, the non-specific activity of endogenous peroxidase was blocked by incubation of the cells with 0.3% H₂O₂ in methanol. For the peroxidase reaction, 0.2 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (DAB) (DOTE) in 0.1 M Tris buffer (pH 7.6) was used. The intensity of antigen expression was scored either in blood vessels or in glial cells of the central nervous system (CNS), in the selected seven areas, cerebral cortex, thalamus, cerebellar cortex, cerebellar white matter and dentate nucleus, pons, grey matter and white matter of the spinal cord. The degree of antigen expression in the blood vessels was scored as follows: 0, no antigens; 1, less than 10% antigen-positive blood vessels in the area; 2, 10–20% antigen-positive blood vessels in the area; 3, 20–50% antigen-positive blood vessels in the area; 4, more than 70% antigen-positive blood vessels in the area. The degree of antigen expression in the glial cells was scored as follows: 0, no antigens; 1, a few antigen-positive glial cells in total area; 2, more than 10 antigen-positive glial cells in the field at 10× magnification (field (×10)); 3, more than three fields (×10) containing more than 10 antigen-positive glial cells; 4, fields with more than 10 antigen-positive glial cells occupying more than 70% of the area. The sum of each score in the seven separate areas is shown in Table 2.

**Viral DNA analysis**

Genomic DNA was isolated from R7f-, Rec5-, and mock-infected F10 cells at 4 days post-infection using QIAGEN Genomic-tips (QIAGEN) according to the manufacturer’s instructions. R7f and Rec5 were infected into F10 cells at moi 0.1. For detection of total virus DNA, PCR was performed with a 50-μl reaction mixture containing 0.2 mM each of deoxyribonucleotides, 2 mM MgSO₄, 300 nM

### Table 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Spongiosis</th>
<th>Clinical disease</th>
<th>Virus titer(log₁₀XC-PFU/g)</th>
<th>Weeks post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence (%)</td>
<td>Intensity of lesion</td>
<td>Brain</td>
<td>Spleen</td>
</tr>
<tr>
<td>A8</td>
<td>100 (14/14)</td>
<td>10.8 ± 0.6 (14)</td>
<td>7/10</td>
<td>5.1 ± 0.2 (5)</td>
</tr>
<tr>
<td>R7a</td>
<td>100 (15/15)</td>
<td>8.6 ± 0.4 (15)</td>
<td>2/15</td>
<td>4.4 ± 0.3 (6)</td>
</tr>
<tr>
<td>R7c</td>
<td>100 (10/10)</td>
<td>6.4 ± 0.5 (10)</td>
<td>0/10</td>
<td>2.6 ± 0.2 (6)</td>
</tr>
<tr>
<td>R7g</td>
<td>8 (1/12)</td>
<td>1.0 (1)⁴</td>
<td>0/12</td>
<td>2.2 ± 0.2 (4)⁹</td>
</tr>
<tr>
<td>R7e</td>
<td>100 (8/8)</td>
<td>6.7 ± 0.4 (8)</td>
<td>0/8</td>
<td>3.3 ± 0.0 (3)</td>
</tr>
<tr>
<td>R7f</td>
<td>100 (9/9)</td>
<td>6.4 ± 0.9 (9)⁴</td>
<td>0/9</td>
<td>3.1 ± 0.2 (3)⁹</td>
</tr>
<tr>
<td>R7b</td>
<td>25 (3/12)</td>
<td>2.3 ± 0.9 (3)⁴</td>
<td>0/12</td>
<td>2.8 ± 0.2 (5)⁹</td>
</tr>
<tr>
<td>R5a</td>
<td>0 (11)</td>
<td>0.0 (11)</td>
<td>0/11</td>
<td>2.3 ± 0.2 (5)⁹</td>
</tr>
<tr>
<td>Rec5</td>
<td>0 (10)</td>
<td>0.0 (10)</td>
<td>0/10</td>
<td>2.3 ± 0.1 (4)⁹</td>
</tr>
<tr>
<td>57</td>
<td>0 (18)</td>
<td>0.0 (18)</td>
<td>0/18</td>
<td>2.5 ± 0.0 (4)⁹</td>
</tr>
</tbody>
</table>

a Number of rats expressing hind-leg paralysis or weakness.

b The virus titers were determined as described in Materials and methods. Values are mean log₁₀XC-PFU/g ± SE. The numbers in parentheses represent the numbers of rats.

c The intensity of lesions was determined as described in Materials and methods. Means and SE are indented. The numbers in parentheses represents number of rats.

d Details are described in Materials and methods.

P < 0.01 vs. A8- and R7c-infected rats. P < 0.05 vs. R7f-infected rats, and P < 0.001 vs. R7b-infected rats by t test.

f P < 0.001 vs. R7a-infected rats by t test.

g Rats with spongiosis.

h Difference is not significant compared to R7f-infected rats by the t test (P > 0.1).

i P < 0.001 vs. A8-infected rats by t test. Difference is not significant compared to R7c- and R7e-infected rats by the t test (P > 0.5).

j Difference is not significant compared to R7e- and R7b-infected rats by the t test (P > 0.1).
primers for detection of the gag gene (sense; 5'-ATGACCGCTTTGATCGAGTCC-3', anti-sense; 5'-CACATTTG-GTTTCTGTCCCTGGA-3'), 600 nM primers for detection of the rat β-globin gene (sense; 5'-GATACTGTTGTGT-GACCTGCA-3', anti-sense; 5'-GGGAAACATAGCAGCAGA-AGACAA-3') as an internal control, 100 ng of template DNA, and 1 unit of KOD-Plus- DNA polymerase (TOYOBO, Co., LTD.) in PCR buffer for KOD-Plus- from the supplier. Mineral oil was overlaid, and the cycling conditions were as follows: denaturation at 94 °C, then 24 or 26 cycles of denaturation at 94 °C for 30 s, and extension at 68 °C for 1 min. Aliquots of the PCR mixture were analyzed on a 3% agarose gel, and visualized and quantified under ImageMaster VDS-CL after ethidium bromide staining.

**Viral transcript analysis**

Total RNA was isolated from R7f-, Rec5-, and mock-infected F10 cells at 4 days post-infection using RNaseasy (Qiagen) according to the manufacturer’s instructions. R7f and Rec5 were infected into F10 cells at moi 0.1. A8-infected F10 cells were used as a control. Cells were washed with PBS and then lysed with 2% sodium dodecyl sulfate (SDS) and 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) in PBS on 100 °C for 5 min. Nucleic acid was sheared using a syringe with a 22-gauge needle. Sample buffer (5×) containing SDS, dithiothreitol (DTT), glycerol, and bromophenol blue in Tris–HCl (pH 6.8) was added to the lysate. After boiling for 5 min, the lysates were loaded on 15% SDS-polyacrylamide gel. The proteins were then transferred onto an Immobilon P membrane (Millipore) by electroblotting and probed with goat anti-Rauscher MLV gp70 and anti-AKR p30^gag^ (Quality Biotech Incorporated Resource Laboratory). Goat anti-actin (Santa Cruz Biotechnology, Inc.) was used as a loading control. Horseradish peroxidase-conjugated anti-goat IgG antibody (Santa Cruz Biotechnology, Inc.) was used as a secondary antibody. The membrane was developed with ECL Plus reagents (Amersham Biosciences Corp., USA), and bands were visualized and quantified with ImageMaster VDS-CL.

**Table 2**

The level of expression of Env protein in cells of CNS^a^  

<table>
<thead>
<tr>
<th>Virus (Number of rats)</th>
<th>Env-positive cells</th>
<th>Blood vessel</th>
<th>Glial cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8(3)</td>
<td>18.5 ± 0.5b</td>
<td>13.8 ± 0.4b</td>
<td></td>
</tr>
<tr>
<td>R7(3)</td>
<td>21.3 ± 1.5c</td>
<td>10.5 ± 0.9d</td>
<td></td>
</tr>
<tr>
<td>R7c(7)</td>
<td>23.4 ± 0.5ec</td>
<td>13.0 ± 1.2ec</td>
<td></td>
</tr>
<tr>
<td>Rec5(4)</td>
<td>3.0 ± 1.4c</td>
<td>1.5 ± 1.2c</td>
<td></td>
</tr>
<tr>
<td>57(3)</td>
<td>1.7 ± 0.7</td>
<td>0.3 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

^a^ The levels of antigen expression were scored as described in Materials and methods. Means and SE are indicated.

^b^ Difference is not significant compared to R7f-infected rats by the t test.

^c^ P < 0.001 vs. Rec5-infected rats.

^d^ P < 0.01 vs. Rec5-infected rats.

^e^ Difference is not significant compared to 57-infected rats by the t test.

**Results**

**Env and other viral determinants of neuropathogenicity of the A8 virus**

To determine the sequences responsible for inducing neurological disease within the 1.5-kb Clai–HindIII fragment of A8, containing the LTR and 5' leader sequence, we constructed new chimerae between A8 and 57 and injected them into newborn Lewis rats (Fig. 1). As shown in Table 1, chimera Rec5, which contains the Spel–Clai fragment of A8 on the background of 57, did not cause spongiosis, confirming our previous report. Chimera R7a, which contains the Clai–AarII fragment of A8 on the background of Rec5, caused spongiform neurodegeneration in 100% of
infected rats. The lesion intensity of R7a-infected rats was lower by 2 points compared to A8-infected rats (Table 1, \( P < 0.01 \)). After replacement of the A8 enhancer region of R7a with the 57-enhancer region, resulting in chimera R7c, spongiosis was still induced in 100% of infected rats. The lesion intensity of R7c-infected rats was lower by 2 points compared to R7a-infected rats (\( P < 0.001 \)). However, R5a, which has an A8 enhancer region on a background of Rec5, did not induce spongiosis. R7g, which contains the \( \text{ClaI–KpnI} \) fragment of A8 on the background of Rec5, did not or rarely induce spongiosis, but R7e, which contains the \( \text{KpnI–HindIII} \) fragment of A8 on the background of Rec5, caused spongiform neurodegeneration in 100% of infected rats. Chimera R7f, which contains the \( \text{KpnI–AarII} \) fragment of A8 on the background of Rec5, caused spongiform neurodegeneration in 100% of infected rats, however, the lesion intensity of R7f-infected rats was lower than that of A8-infected rats (\( P < 0.001 \)) and R7a-infected rats (\( P < 0.05 \)), but was comparable with the lesion intensity of R7c- and R7e-infected rats (\( P > 0.5 \)) (Table 1). Chimera R7b, which contains the \( \text{AarII–HindIII} \) fragment of A8 on the background of Rec5, induced spongiosis in 25% of infected rats; however, the lesion intensity was very low compared with R7a-infected rats (\( P < 0.001 \)). A8-virus infection and R7a-virus infection caused hind-leg paralysis or weakness in 7 of 10 rats and 2 of 15 rats, respectively (Table 1). No rats infected with other viruses exhibited clinical disease.

### Virus titers in the brains of rats infected with neuropathogenic and non-neuropathogenic viruses

To investigate the correlation between neuropathogenicity and virus titers in the brain of rats infected with chimeric viruses containing neuropathogenic A8-Env, we measured virus titers in the brain of rats infected with neuropathogenic and non-neuropathogenic viruses. The virus titer in the brains of rats infected with R7c was lower by two orders of magnitude compared to rats infected with R7a (\( P < 0.001 \)), whereas R7c was still able to induced spongiform neurodegeneration (Table 1). The virus titers in the brains of rats infected with R7c were similar to those infected by no- or low-neuropathogenic viruses, R7g, R7b, R5a, Rec5, and 57 (\( P > 0.1 \)). The virus titer in the brains of rats infected with neuropathogenic R7f were comparable to those of rats infected with low-neuropathogenic R7b and neuropathogenic R7e (\( P > 0.1 \)). In the spleen, virus titers were similar among R7a, R7c, R7g, R7e, R7f, R5a, and Rec5 (Table 1). The titers in the spleen of rats infected with A8, R7b, and 57 were not determined.

### Comparison of the expression of viral proteins, viral DNA and viral transcripts in cultured cells infected with R7f and Rec5

The neuro-pathogenicity of Rec5 was dramatically altered when the 0.3 kb \( \text{KpnI–AarII} \) fragment was replaced with that from A8, resulting in R7f, as shown in Table 1. In order to examine the effects of the A8 0.3-kb \( \text{KpnI–AarII} \) fragment on viral replication, we compared the amount of viral DNA, viral transcripts and the viral proteins in R7f- and Rec5-infected cultured cells.

The relative amount of viral DNA in F10 cells infected with Rec5 and R7f was determined by PCR using primers specific for the gag gene. The intensity of the gag amplification product in R7f-infected F10 cells was identical to that in Rec5-infected F10 cells (Fig. 2). The intensity ratio of the band in R7f- and Rec5-infected F10 cells after normalization to the intensity of the band of \( \beta \)-globin gene was 0.9. In independent experiments, the same ratio was obtained (data not shown).

The relative amount of transcripts of proviral DNA in F10 cells infected with Rec5 and R7f was determined by RT-PCR. The full-length transcripts were detected by a primer pair that amplifies a 236-bp fragment spanning the 3’ end of the pol gene to the 5’ end of the env gene (Fig. 3A). The intensity ratio of the band in R7f- and Rec5-infected F10 cells after normalization to the intensity of the band of \( \beta \)-globin RNA was 1.2 (Fig. 3B). A second independent experiment was performed, and the ratio was 1.1. The spliced transcripts were detected by a primer pair that amplifies a 254-bp fragment from the 3’ end of LTR to the 5’ end of the env gene (Fig. 3A). The intensity ratio of the band of the spliced transcripts in R7f- and Rec5-infected F10 cells as represented in their non-permutated form, flanked at their ends by the LTR sequence. In chimeric viral genomes, solid regions are sequence-derived from the A8 virus and open regions are sequence-derived from 57 viruses.
after normalization to the band intensity of 18S ribosomal RNA was 1.2 in two independent experiments (Fig. 3B). In addition, viral reverse transcriptase activities of the culture supernatant of R7f- and Rec5-infected F10 cells were equivalent (data not shown).

Viral envelope protein is synthesized as a precursor polyprotein (gpr85) consisting of N-terminal SU glycoprotein (gp70) and C-terminal transmembrane protein (TM). Proteins gpr85 and gp70 were detected by Western blotting using anti-Env antibody in cultured glial cells F10 infected with A8 (Fig. 4A). Protein gp70 was mainly observed in F10 cells infected with Rec5 and R7f. Protein gpr85 was also detected in these cells by long exposure. The intensity of the gp70 band in R7f-infected F10 cells was 3-fold higher than in Rec5-infected F10 cells. Gag precursor protein (p65Gag) and p30Gag was detected by Western blotting using anti-p30Gag antibody (Fig. 4B). In F10 cells infected with Rec5 and R7f, the expression of Gag precursor protein (p65Gag) and p30Gag was similar. Two independent experiments were performed and similar results were obtained.

Env expression in the brains of rats infected with neuropathogenic and non-neuropathogenic viruses

To examine the expression levels of Env protein in the CNS of rats infected with neuropathogenic and non-neuropathogenic viruses, the intensity of antigen expression was scored either in blood vessels or in glial cells of the brain at 14 days after infection. The intensity of antigen expression was scored either in blood vessels or in glial cells of the brain at 14 days after infection. The intensity of antigen expression was scored either in blood vessels or in glial cells of the brain at 14 days after infection.
Fig. 4. Expression of Env protein (A) and Gag protein (B) in rat glial cells (F10) infected with R7f and Rec5. F10 cells infected with A8 virus were used as a control for the expression of proteins. Lysates of the infected cells were analyzed by immunoblotting with anti-Env (gp70) and anti-Gag (p30) antibodies. After re-probing, the blot was subsequently stained using an anti-β-actin antibody to control for loading differences. This figure is representative. Two independent experiments were performed, and similar results were obtained.

CNS in seven selected areas: cerebral cortex, thalamus, cerebellar cortex, cerebellar white matter and dentate nucleus, pons, grey matter and white matter of the spinal cord. The sum of each score in the seven separate areas is shown in Table 2. The level of expression of Env protein in both blood vessel and glial cells of R7c-infected rats was higher than that of Rec5-infected rats ($P < 0.001$), although the virus titers in the brains of R7c- and Rec5-infected rats were similar (Table 1). There was no difference between the expression level of Env protein of Rec5-infected rats and that of 57-infected rats ($P > 0.1$). The expression level of Env protein in both blood vessel and glial cells of R7f-infected rats was comparable to that of R7c- and A8-infected rats, but was higher than that of Rec5-infected rats (blood vessel: $P < 0.001$, glial cell: $P < 0.01$) (Table 2).

Discussion

We previously reported that the LTR and 5′ leader sequence of A8 is essential for the induction of spongiform neurodegeneration in addition to the env gene, a primary determinant for neuropathogenicity (Takase-Yoden and Watanabe, 1997). In order to identify the genetic determinant within the ClaI–HindIII fragment of A8, we constructed additional chimeras between A8 and non-neuropathogenic 57 and examined the pathogenicity of these viruses. Rec5 did not cause spongiosis, consistent with our previous report. R7a caused spongiform neurodegeneration with lesions of comparable intensity to A8-infected rats (Table 1). However, R7b exhibited low neuropathogenicity compared with R7a, indicating that the determinant for neuropathogenicity excluding A8-env is located within the ClaI–AarII fragment of A8. Compared with R7g, R7f caused spongiform neurodegeneration but R7g induced little or no spongiosis. These results indicated that the 0.3-kb KpnI–AarII fragment containing 0.04-kb of R, U5, and the 5′ half of the 5′ leader of A8 is essential for the induction of spongiform neurodegeneration. There were 17 differences in the nucleotide sequence of this fragment between A8 and 57 (Fig. 5).

We examined whether the titer of the virus in the brain correlated with the induction of spongiosis. Initially, we looked for the region of the A8 gene that determined viral titers in the brain. Viral titers in the brains of rats infected with R7a were reduced by two orders of magnitude in R7c-infected rats due to replacement of the BplI–HphI fragment of A8-LTR containing the nuclear factor-binding regions, FVa, FVα, and FVα, with the BplI–BsiEI fragment of 57-LTR containing the nuclear factor-binding regions FVa, FVb2/ NF1, FVα (Fig. 1 and Table 1). This finding indicated that the enhancer region of A8-LTR is important in determining viral titers in the brain. The titer of R5a was comparable to that of Rec5 and the titer of R7g was comparable to that of Rec5 and 57. Furthermore, titers of R7e and R7f in the brain were higher by one order of magnitude than that of Rec5. Therefore, additional determinants of the viral titer in the brain are located within the KpnI–AarII fragment of A8, which contains the R-U5-5′ leader region. The BplI–HphI fragment containing the nuclear factor-binding regions and the KpnI–AarII fragment are also determinants of viral titer in the thymus as previously reported (Takase-Yoden and Watanabe, 2002, 2004). In contrast, titers in the spleen were equivalent for all viruses tested (Table 1). Therefore, it is possible that there are tissue-specific events, including regulation by a tissue-specific enhancer, in viral replication in the target tissues. Kai and Furuta (1984) reported that the titers of several neuropathogenic and non-neuropathogenic viruses derived from Fr-MLV in the spleen of rats were equivalent but those in the brain were different. We then focused on the correlation between neuropathogenicity and viral titers in the brain. Although the titers in the brain of R7c-infected rats were lower by two orders of magnitude than the titers of R7a-infected rats (Table 1), both R7a and R7c were neuropathogenic. The titers in the brains of R7c-infected rats were comparable to those of rats infected with non-neuropathogenic R7g, R7b, R5a, Rec5, and 57 (Table
1). Furthermore, R7e and R7f were neuropathogenic, and the titers of these viruses in the brain were lower by one or two orders of magnitude than those of neuropathogenic A8 and R7a viruses. These findings indicate that the amount of infectious virus in the brain does not determine whether spongiform neurodegeneration is induced. Compared among A8, R7a, and R7c in the histopathological scores, a correlation between lesion intensity and viral titers in the brain was found (Table 1). Furthermore, a correlation between clinical disease and viral titers in the brain was observed. Therefore, it is suggested that the amount of infectious virus in the brain influences the development of lesions and disease.

In our previous studies, it was revealed that viral replication in F10 cells has well-correlated neuropathogenicity (Takase-Yoden and Watanabe, 1997), and we therefore used F10 cells in our in vitro studies. There was a higher expression of Env protein in F10 cells infected with neuropathogenic R7f than in those infected with non-neuropathogenic Rec5 (Fig. 4A). The accumulation of uncleaved envelope precursor protein (gpr85) in NIH3T3 cells or the presence of Env protein accompanied with differential processing of N-linked sugar in glial cells in neuropathogenic MLVs has been reported (Lynch and Sharpe, 2000; Lynch et al., 1994; Szurek et al., 1990a, 1990b). In this study, no abundance of gpr85 in R7f-infected F10 cells was observed (Fig. 4A). The amount of spliced transcripts of the virus, from which Env protein is translated, in R7f-infected F10 cells was comparable to that in Rec5-infected F10 cells (Fig. 2). The amount of spliced transcripts of the virus, from which Env protein is translated, in R7f-infected F10 cells was comparable to that in Rec5-infected F10 cells (Fig. 3). The reason the R-U5-5' leader region has different effects on Env expression and Gag expression is not yet clear. The distance between the 5' end and AUG codon differs between the mRNA for Env and the mRNA for Gag, because the splicing donor site is located in the middle of the 0.3-kb KpnI–AatII fragment (Fig. 5). These differences in the structure of Gag mRNA and Env mRNA may induce different effects of the R-U5-5' leader region in post-transcriptional events.

As shown in Table 2, the expression level of Env in the brains of rats infected with neuropathogenic R7c was higher compared with that in the brains of rats infected with non-neuropathogenic Rec5, although the titers of these viruses in the brain were equivalent (Table 1). The viral titer in the brains of rats infected with neuropathogenic R7f was lower by two orders of magnitude than that in the brains of rats infected with neuropathogenic A8, but the expression level of Env was equivalent (Table 2). These findings indicated that the expression level of Env protein in the brain correlated with neuropathogenicity. Reasons for the discrepancy between expression levels of Env protein and titers of infectious virus are unknown. However, the capacity to produce infectious viruses in the brain may be independent

---

**Fig. 5.** Alignment of the KpnI–AatII fragment of A8 (accession no.D88386) and 57 (accession no. X02794). Asterisks represent the sequence identity. polyA: polyadenylation signal, PBS: primer binding site, SD: splice donor site, glyco-Gag start; the start codon of glycosylated-Gag protein.
of Env protein expression in the cells, but become dependent at later stages, including viral assembly, genome packaging, and budding.

Acknowledgments

The authors thank Katsumi Goto for performing histological studies and Misaho Wada for performing PCR, Western blot and assays of virus titers. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT, 2004–2008.

References


Szurek, P.F., Floyd, E., Yuen, P.H., Wong, P.K., 1990a. Site-directed mutagenesis of the codon for Ile-25 in gPr80env alters the neurovirulence of ts1, a mutant of Moloney murine leukemia virus TB. J. Virol. 64, 5241–5249.

Szurek, P.F., Yuen, P.H., Ball, J.K., Wong, P.K., 1990b. A Val-25-to-Ile substitution in the envelope precursor polyprotein, gPr80env, is responsible for the temperature sensitivity, inefficient processing of gPr80env, and neurovirulence of ts1, a mutant of Moloney murine leukemia virus TB. J. Virol. 64, 467–475.


