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Different transcriptional expression of the matrix gene of the two sibling viruses of the subacute sclerosing panencephalitis virus (Osaka-2 strain) isolated from a biopsy specimen of patient brain

Toshiyuki Seto[1,2], Minoru Ayata[1], Kaoru Hayashi[1,2], Kyoko Furukawa[1,2], Ryosuke Murata[2,3] and Hisashi Ogura[1]

[1]Department of Virology and [2]Department of Pediatrics, Osaka City University Medical School, Asahimachi 1-4-3, Abeno-ku, Osaka 545-8585; [3]Department of Pediatrics, Osaka City General Hospital, Miyakojima-hondori 2-13-22, Miyakojima-ku, Osaka 534-0021, Japan

Two sibling viruses of the subacute sclerosing panencephalitis (SSPE) virus Osaka-2 strain were isolated from a small biopsy specimen of the brain of an SSPE patient just before intraventricular interferon treatment by cocultivation with two different cell lines, Vero cells or B95a cells (Ogura *et al*, 1997). Both the virus-infected cells were found to be indistinguishable from each other in defective production of cell-free virus and in defective expression of the matrix (M) protein. The sequence analysis of the M genes predicted that they were translatable due to a lack of alteration of the translational start and stop codons for the proteins. A different pattern of the M monocistronic transcripts, however, was observed in a Northern blot analysis of the infected cells. This different pattern was confirmed further by a primer extension analysis. The undetectable expressions of the M proteins in the sibling virus-infected cells are most probably different in their molecular mechanisms. All these results indicate the possibility that the two different, replicable variants existed at Jabbour stage III of the disease's progression in a very small portion of the brain, where no lesion had yet been recognized by a magnetic resonance imaging.

Keywords: brain biopsy; SSPE virus; defective virus; M protein; viral transcription; viral translation

Introduction

Subacute sclerosing panencephalitis (SSPE) is a progressive and degenerative disease of the central nervous system (CNS) of children and adolescents. It is caused by a measles virus (MV) mutant referred to as the SSPE virus. SSPE is rare but lethal; no curable treatment has yet been established. Very few reports of SSPE virus isolation (Doi *et al*, 1972; Burnstein *et al*, 1974; Ueda *et al*, 1975; Makino *et al*, 1977; Kratzsch *et al*, 1977; Mirshamsy *et al*, 1978; Shishido *et al*, 1981; Homma *et al*, 1982; Ogura *et al*, 1997) and molecular characterization (Ayata *et al*, 1989, 1991; Cattaneo *et al*, 1987, 1988, 1989; Hirano *et al*, 1993; Komase *et al*, 1992; Wong *et al*, 1989, 1991; Yoshikawa *et al*, 1990) have appeared so far, probably because it is almost impossible to isolate the virus from sera, blood cells, and cerebrospinal fluids (CSF), but it is possible to isolate it only from infected brains. Actually, isolation even from diseased brains is markedly difficult because brain autopsy is not done in every case and isolation is labor-intensive.

Most of the isolated SSPE viruses have been found to be defective in cell-free virus production. This defective production is mainly associated with the dysfunction of the matrix (M) protein. The causes of its dysfunction differ from the isolated strains; degradation or altered antigenicity of the M protein is due to translational defect (Ayata *et al*, 1989, 1991; Cattaneo *et al*, 1986, 1988; Enami *et al*, 1989) or absence of the M protein is due to transcriptional defect (Cattaneo *et al*, 1986, 1987; Yoshikawa *et al*, 1990), all of which seem to be caused ultimately by biased hypermutation of the

Correspondence: H Ogura
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gene. Another factor which is responsible for that defective production may be alteration of the other envelope proteins hemagglutinin (H) and fusion (F), especially of the cytoplasmic domain of the F protein (Schmid *et al*, 1992; Cathomen *et al*, 1998). All these envelope proteins are thought to be involved in the assembly and budding of infectious virus (Griffin and Bellini, 1996).

Because of the extremely low efficiency of the SSPE virus isolation, another approach, direct cloning from the viral genomes in the SSPE-affected brain, has been applied to obtain more certain virological information (Cattaneo *et al*, 1986, 1988, 1989; Schmid *et al*, 1992). Direct cloning has, however, some critical problems. For example, each cloned gene from a diseased brain may not be derived from a single specified, or pathogenic SSPE virus that caused the disease. Also cloned genes may be derived from persistently infecting MV in brains that is unrelated to SSPE. This detection of MV mRNA in brains of apparently healthy individuals has recently been reported by Katayama *et al* (1995).

For the present, reliable and available data about the SSPE virus seem to be quite insufficient, and therefore not enough is understood about the pathogenesis of SSPE. It is nevertheless very important and desirable to develop a new effective method of prevention and therapy. Recently, we discussed an efficient method for isolating the SSPE virus in patient brains by consulting brain images (Ogura *et al*, 1997). According to this method, we succeeded in the isolation of the three strains, the Osaka-1, -2, and -3 strains. In this study, we characterize two sibling viruses of the Osaka-2 strain obtained from a small biopsy of a patient brain taken just prior to intraventricular treatment with interferon by cocultivation with two different cell lines, Vero cells and B95a cells. In the present study, we describe these sibling viruses as different variants of the same phenotypic defect in expression of the M protein and we also discuss its significance in SSPE pathogenesis.

Results

Biological properties of the SSPE virus Osaka-2 strain

Both Vero cells persistently infected with the Fr/V virus and B95a cells persistently infected with the Fr/B virus showed no adsorption of monkey red blood cells, although positive hemadsorption was observed in Vero cells and B95a cells lytically infected with the Edmonston (Edm) strain (data not

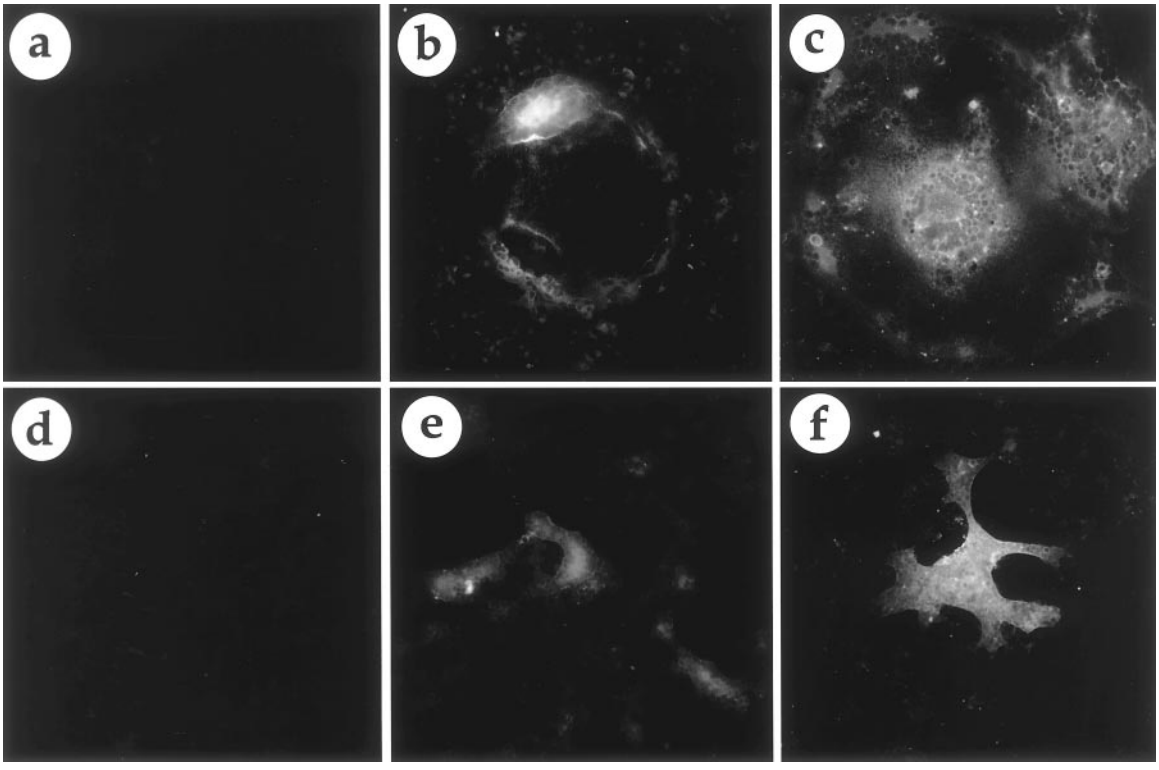

Figure 1 Membrane immunofluorescence of the H protein of the cells persistently infected with the SSPE virus Osaka-2 strain. The unfixed cells were reacted with an anti-H monoclonal antibody (L77). After acetone fixation, they were stained with FITC-conjugated anti-mouse IgG rabbit antibody. (**a**) mock-infected Vero cells; (**b**) the Vero cells lytically infected with the Edm strain at 36 h p.i.; (**c**) the Fr/V-infected Vero cells; (**d**) mock-infected B95a cells; (**e**) the B95a cells lytically infected with the Edm strain at 60 h p.i.; (**f**) Fr/B-infected B95a cells. (Final magnification × 75).

shown. Cell surface expression of the H protein was observed in the Fr/V- and Fr/B-infected cells by membrane immunofluorescence (Figure 1c and f), similar to that in the Edm-infected cells (Figure 1b and e). However, these persistently infected cells produced few infectious cell-free viruses in the culture media, resulting in less than 10 PFU/ml (data not shown), as has been observed in cases with other nonproductive SSPE strains.

Reduced patient antibody response to the M protein, and viral protein synthesis in Osaka-2 strain-infected cells

The result of RIP of the Edm-infected Vero cells with the patient's serum and CSF is shown in Figure 2a. The M protein bands in the lanes immunoprecipitated with both serum and CSF could not be detected as clearly as those immunoprecipitated with the hyperimmune monkey (GM) serum (Figure 2a, lanes 2, 5 and 8). This shows a selective reduction of the antibody against the M protein of the Edm strain in the serum and CSF. This suggested a possibility of defective M protein synthesis or altered immunogenicity of the M protein in the patient.

Next, to examine the viral protein synthesis in the Fr/V-infected Vero cells or the Fr/B-infected B95a cells, lysates from infected cells were immunoprecipitated with the GM serum and various anti-MV monoclonal and polyclonal antibodies. The N, P,

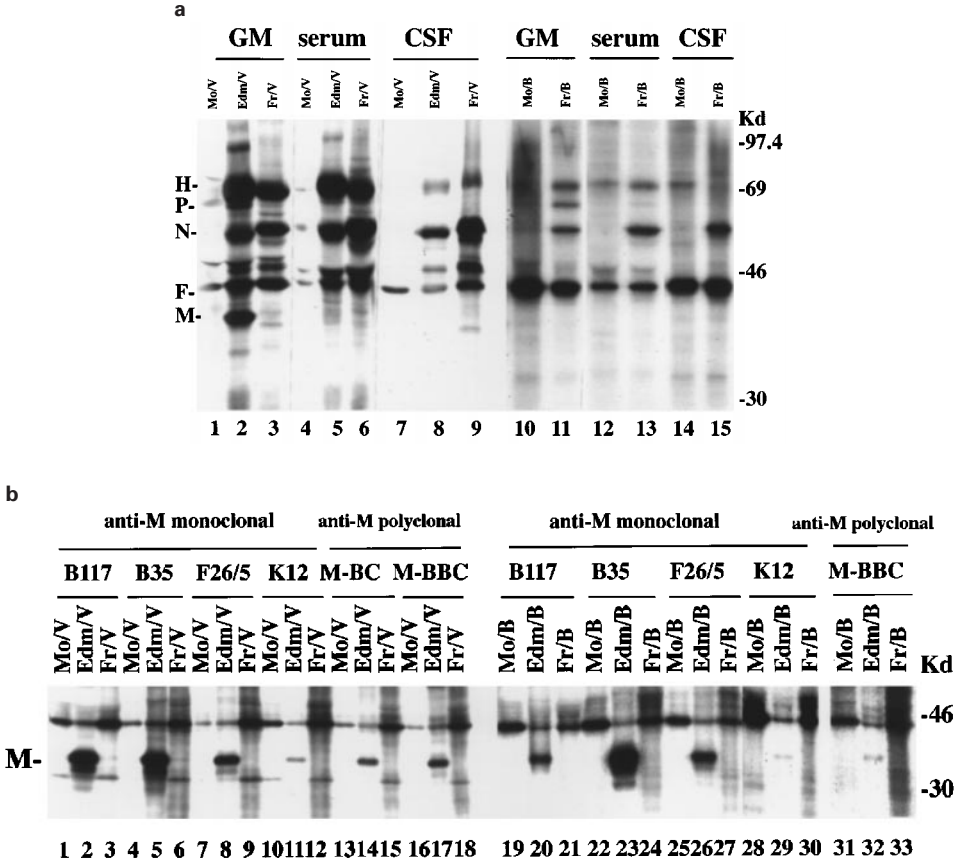

Figure 2 Antibody production in the serum and CSF of a patient with SSPE (**a**) and viral proteins in the cells persistently infected with the Fr/V virus or Fr/B virus (**b**). (**a**) The following were labeled with ^{35}S-methionine/cysteine for 8 h: mock-infected Vero cells (Mo/V; lanes 1, 4, and 7), the Vero cells lytically infected with the Edm strain at 20 h p.i. (Edm/V; lanes 2, 5, and 8), the Vero cells persistently infected with Fr/V virus (Fr/V; lanes 3, 6, and 9), mock-infected B95a cells (Mo/B; lanes 10, 12, and 14), and the B95a cells persistently infected with Fr/B virus (Fr/B; lanes 11, 13, and 15). The cell lysates were immunoprecipitated with hyperimmune monkey serum against the MV Nagahata strain (GM), with the patient's serum (serum), or with the patient's CSF (CSF). (**b**) The following were labeled with ^{35}S-methionine/cysteine for 1 h: mock-infected Vero cells (Mo/V; lanes 1, 4, 7, 10, 13, and 16), the Vero cells lytically infected with the Edm strain at 40 h p.i. (Edm/V; lanes 2, 5, 8, 11, 14, and 17), and the Vero cells persistently infected with the Fr/V virus (Fr/V; lanes 3, 6, 9, 12, 15, and 18). The following were labeled with ^{35}S-methionine/cysteine for 2 h: mock-infected B95a cells (Mo/B; lanes 19, 22, 25, 28, and 31), the B95a cells lytically infected with the Edm strain at 40 h p.i. (Edm/B; lanes 20, 23, 26, 29, and 32), and the B95a cells persistently infected with the Fr/B virus (Fr/B; lanes 21, 24, 27, 30, and 33). The cell lysates were immunoprecipitated with monoclonal antibodies against the M protein of the Edm strain (B117, lanes 1–3 and 19–21; B35, lanes 4–6 and 22–24; F26/5, lanes 7–9 and 25–27; K12, lanes 10–12 and 28–30), or with polyclonal antibodies against the M protein of the Edm strain (M-BC, lanes 13–15) and the M protein of the SSPE virus Biken strain (M-BBC, lanes 16–18 and 31–33). Immunoprecipitates were analyzed by SDS–PAGE (10% polyacrylamide). Molecular weight standards are shown as Kd (kilodaltons).

and H proteins were observed in the Fr/V-infected Vero cells and the Fr/B-infected B95a cells by the GM serum or the anti-N, P, and H monoclonal antibodies (data not shown). Despite examinations with various labeling and chase periods, the F protein could not be found in the Fr/V- and Fr/B-infected cells by the GM serum and the anti-F monoclonal antibodies, but it was found in the Edm strain-infected cells (data not shown). The M protein could not be detected at all in the Fr/V-infected Vero cells and in the Fr/B-infected B95a cells by the GM serum (Figure 2a, lanes 3 and 11), anti-M monoclonal and polyclonal antibodies (data not shown) when they were labeled for 8 h. However, it was detected in the Edm-infected Vero cells and B95a cells (data not shown). To rule out the possibility of rapid degradation of the M protein of the sibling viruses, the infected cells were pulse-labeled with ^{35}S-methionine/cysteine for 1 or 2 h and were immediately subjected to RIP assay. The M protein could not be detected in the Fr/V-infected Vero cells nor in the Fr/B-infected B95a cells, irrespective of the labeling periods (Figure 2b, lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 and 33). But it could be detected in the Edm-infected Vero cells and B95a cells (Figure 2b, lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29 and 32).

In order to understand the involvement of altered immunogenicity of the M protein of the Fr/V virus or the Fr/B virus, lysates from the infected cells were immunoprecipitated with the patient's serum and CSF. However, no bands corresponding to the position of the M protein were observed (Figure 2a, lanes 6, 9, 13 and 15). This suggests the possibility that the M protein is lacking in the Fr/V- and the Fr/B-infected cells.

Nucleotide sequence of the M gene of the Osaka-2 strain

The nucleotide sequences of the M genes of these two sibling viruses were determined by direct sequencing method, which allowed us to compare them with each other. The direct sequencing method also allowed us to analyze the sibling viruses' inability to translate the M mRNA. As shown in Figure 3a, seven nucleotides were different in the M genes of the Fr/V and Fr/B viruses. Six of these nucleotides were clustering in the 3′ half of the gene and they were not U to C transitions in the plus-strand sequence. The start and stop codons of the M genes of both of the viruses were conserved. Premature termination and new initiation codons were not found. These indicate that these viruses still maintain the potential for M protein synthesis in their M genes. From the nucleotide sequences of the M genes, amino acid sequences of the M protein were predicted. Replacements of 28 and 30 amino acids were detected in the M proteins of the Fr/V and Fr/B viruses, respectively, as compared with the amino acid sequence of the M protein of the Edm strain and only two amino acid substitutions were found in the two sibling viruses (Figure 3b).

Viral mRNA synthesis of the Osaka-2 strain

The transcription of the sibling viruses was analyzed by Northern blot hybridization. In the Fr/B-infected B95a cells, it was difficult to detect the P and M monocistronic mRNAs (Figure 4, lanes 10 and 15) and the N-P (Figure 4, lanes 5 and 10) and M-F (Figure 4, lanes 15 and 20) bicistronic mRNAs, although the N, F, and H monocistronic mRNAs (Figure 4, lanes 5, 20 and 25) and the P-M (Figure 4, lanes 10 and 15) and F-H (Figure 4, lanes 20 and 25) bicistronic mRNAs were present. In addition, the ratio of the amount of the P-M bicistronic mRNA to that of the P or M monocistronic mRNA was unusually too large in the Fr/B-infected cells (Figure 4, lanes 10 and 15), as compared with that in the Edm-infected cells (Figure 4, lanes 6 and 11). In contrast, all the monocistronic and bicistronic mRNAs were found in the Fr/V-infected Vero cells (Figure 4, lanes 3, 8, 13, 18 and 23), similar to those of the Edm-infected Vero cells (Figure 4, lanes 1, 6, 11, 16 and 21).

To confirm the presence or absence of the M monocistronic mRNA, the primer extension analysis of the 5′ end of the M mRNA was carried out. That of the P mRNA was also analyzed as a positive control. From the poly A$^+$ RNA of the Fr/V-infected Vero cells, fragments of 50 nucleotides from the M mRNA and fragments of 55 nucleotides from the P mRNA were synthesized by reverse transcription with the specific primers, which showed the M and P mRNA transcription from the authentic 5′ end (Figure 5, lanes 3 and 9). However, the amounts of fragments of 50 nucleotides and fragments of 55 nucleotides from the poly A$^+$ RNA of the Fr/V-infected Vero cells were much less than those from the poly A$^+$ RNA of the Edm-infected Vero cells (Figure 5, lanes 2 and 8). On the other hand, no band corresponding to the 50-nucleotide-long fragments from the M mRNA was detected in the products of reverse transcription from the poly A$^+$ RNA of the Fr/B-infected B95a cells (Figure 5, lane 4), whereas the 55-nucleotide-long fragments from the P mRNA were found (Figure 5, lane 10), supporting the results of the Northern blot analysis.

These data show that the Fr/V and Fr/B sibling viruses are different in their respective transcriptions of the M gene, despite the fact that they show the same phenotypic defectiveness in expressing the M protein.

Discussion

The Fr/V and Fr/B sibling viruses of the Osaka-2 strain were isolated from a small biopsy specimen of the brain of an SPPE patient, who was at the

eighth month after onset of the disease, by two different cell lines susceptible to MV (Ogura *et al*, 1997). In this study the two sibling viruses were characterized at molecular level. Most of the SSPE virus isolates, except for the Niigata-1 strain (Doi *et al*, 1972), were derived from brains at the terminal

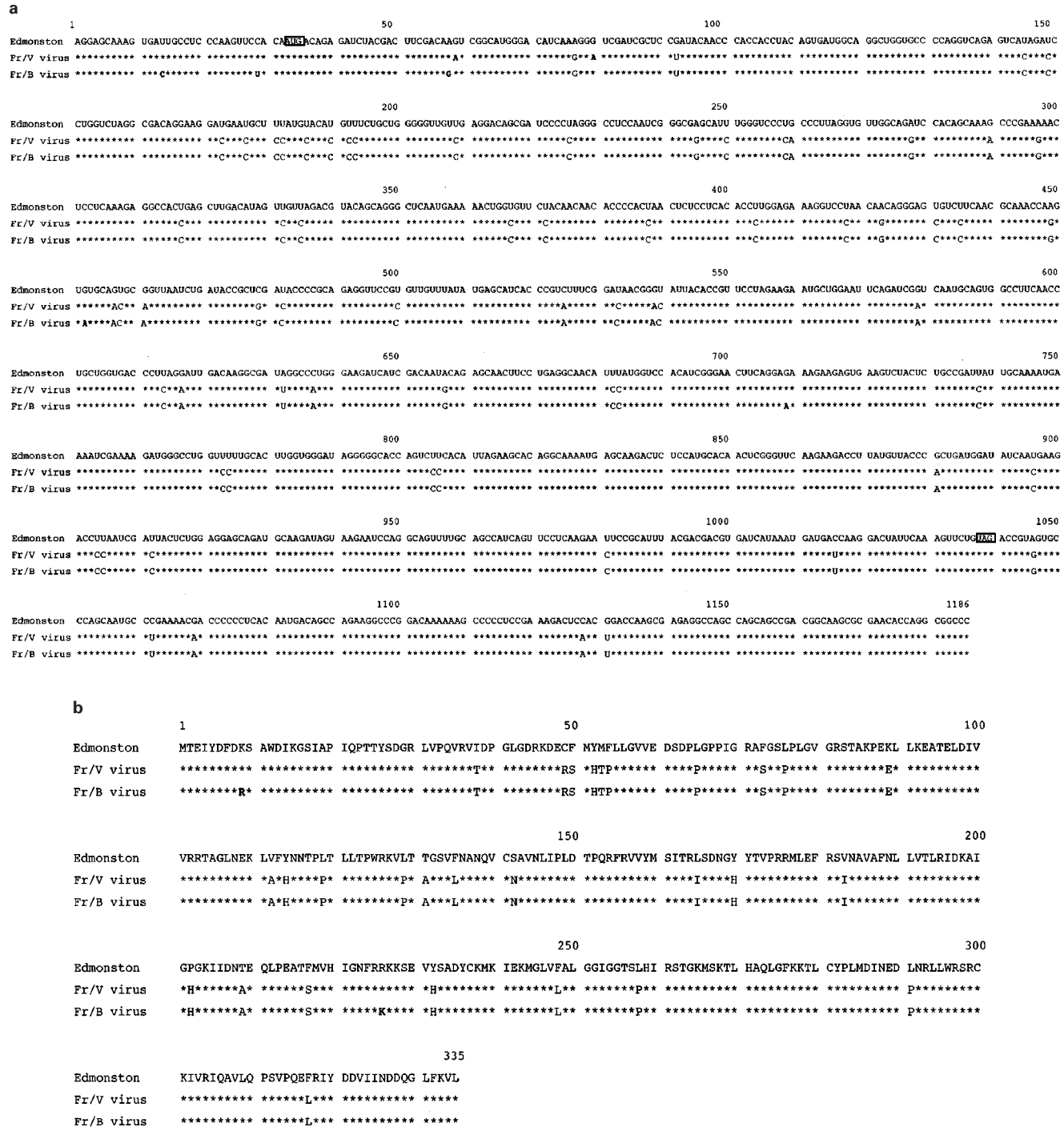

Figure 3 Alignments of the nucleotide sequences of the Osaka-2 strain M genes (**a**) and the deduced amino acid sequences of the M proteins (**b**). (**a**) The M genes of the Edm strain and the Fr/V and Fr/B viruses were compared with each other. Nucleotides are shown, excluding the region used for design of primer sequences for PCR. Asterisks (*) represent the nucleotide identical to that of the Edm strain. The translational start and stop codons are boxed. Nucleotides of the Fr/V and Fr/B viruses differing from each other are shown in bold. (**b**) The M proteins of the Edm strain and the Fr/V and Fr/B viruses were compared with each other. Asterisks (*) represent the amino acid identical to that of the Edm strain. Amino acids of the Fr/V and Fr/B viruses differing from each other are shown in bold.

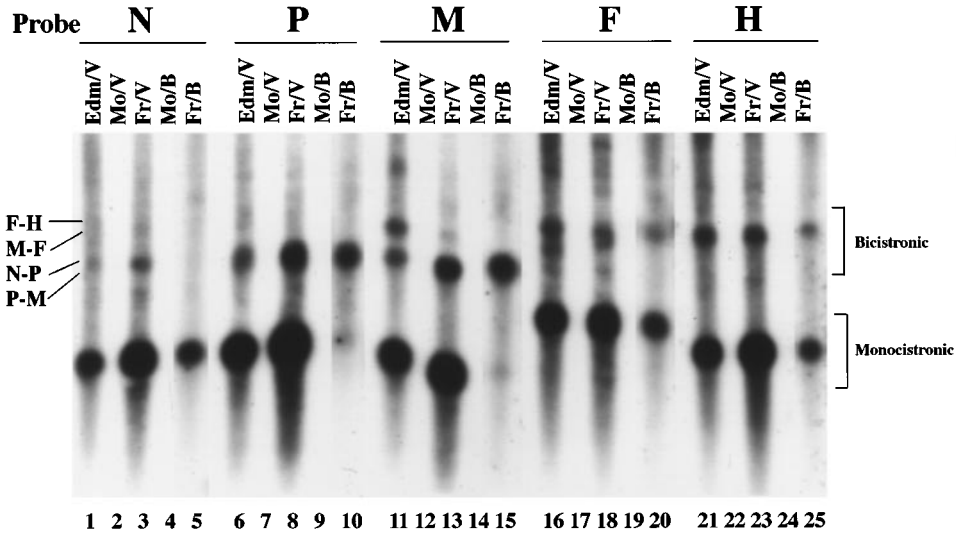

Figure 4 Northern blot hybridization of the measles virus RNA in Vero cells persistently infected with the Osaka-2 strain. Total RNA was extracted by guanidine thiocyanate followed by ultra-centrifugation through 5.7 M cesium chloride. Poly A^+ RNA was selected by the standard procedure, using oligo dT followed by electrophoresis in a 1% agarose gel containing formaldehyde and by transfer onto nitrocellulose filter paper. ^{32}P-dCTP-labeled DNA probes specific for the N, P, M, F, and H genes of the measles virus were prepared from full-length cDNA clones and were used for the hybridization. Lanes Mo/V, RNA from mock-infected Vero cells; lanes Mo/B, RNA from mock-infected B95a cells; lanes Edm/V, RNA from the Edm-infected Vero cells at 24 h p.i.; lanes Fr/V, RNA from Fr/V-infected Vero cells; lanes Fr/B, RNA from Fr/B-infected B95a cells. Note that a bicistronic mRNA species of the P and M genes (P-M) is abundant and that the N-P bicistronic (N-P) and M-F bicistronic (M-F) RNAs, as well as RNAs corresponding to the monocistronic P mRNA (P) and M mRNA (M), are undetectable in the Fr/B-infected cells, whereas the profile of the Fr/V-infected cells was quite similar to that of the Edm-infected cells. Bicistronic, bicistronic mRNAs; Monocistronic, monocistronic mRNAs.

stage of the disease. They include the following isolates: the Biken (Ueda *et al*, 1975), IP-3-Ca (Burnstein *et al*, 1974), MF (Kratzsch *et al*, 1977), Kitaken-1 (Makino *et al*, 1977), ZH (Mirchamsy *et al*, 1978), Yamagata-1 (Homma *et al*, 1982), Osaka-1 and -3 strains (Ogura *et al*, 1997), etc. This study seemed to bring more valuable information on relation of viral mutations with SSPE progression because the Osaka-2 strain was isolated from a brain at an earlier stage of the disease.

The persistently infected cells with Fr/V virus or Fr/B virus were not able to adsorb monkey erythrocytes, notwithstanding the cell surface expression of the H protein. This property is not common to SSPE virus isolates. MV standard strains such as Edm and Toyoshima, which have been passaged in monkey kidney cell lines for years, are hemadsorption-positive, whereas the recent wild-type MV strains isolated by cells of lymphoid cell origin have been reported to be negative (Saito *et al*, 1992; Sakata *et al*, 1993; Shibahara *et al*, 1994). Therefore it is unlikely that the negative hemadsorption of the Fr/V and Fr/B viruses is an altered characteristic of long-term, persistent infection in the brain. On the other hand, each sibling virus-infected cell could not produce infectious cell-free virus in the culture media, although both virus-

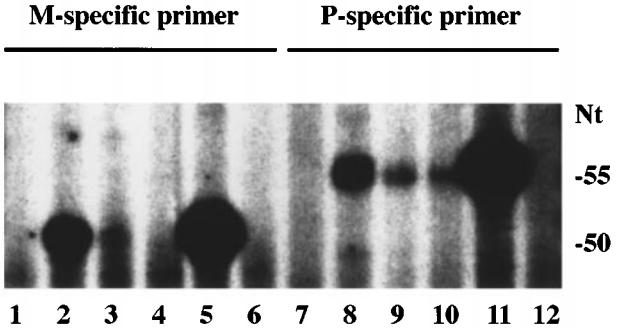

Figure 5 cDNA products obtained by extension of an M-specific primer or a P-specific primer hybridized to poly A^+ RNA from the cells persistently infected with the SSPE virus Osaka-2 strain Fr/V virus or Fr/B virus. Poly A^+ RNAs were prepared in the same way as described in Figure 4. The 5′ end of a minus-sense primer (17 mer) complementary to M mRNA situated at nucleotide position +50 to +34 or of a minus-sense primer (16 mer) complementary to P mRNA situated at nucleotide position +55 to +40 was labeled with ^{32}P-ATP. With these labeled primers, cDNA was synthesized by reverse transcription. The products were analyzed by electrophoresis in a 6% polyacrylamide-urea gel. Lanes Mo/V, RNA from mock-infected Vero cells; lanes Mo/B, RNA from mock-infected B95a cells; lanes Edm/V, RNA from the Edm-infected Vero cells at 24 h p.i.; lanes Edm/B, RNA from the Edm-infected B95a cells at 48 h p.i.; lanes Fr/V, RNA from Fr/V-infected Vero cells; lanes Fr/B, RNA from Fr/B-infected B95a cells. Nt, nucleotides in length.

infected cell types showed progressive syncytium formation. Thus, the two viruses exhibited the same biological phenotypes. Investigations of nonproductive SSPE viruses have demonstrated that aberrant alterations of the envelope proteins M, F, and H, somehow interfere with the assembly and budding of infectious viruses (Griffin and Bellini, 1996). In addition, an antibody response of our patient's serum and CSF against the M protein of the Edm strain was selectively reduced, suggesting the possibility of defective expression of the M protein of the Osaka-2 strain or of its altered antigenicity *in vivo*. Therefore, we concentrated primarily on the M protein expression of these sibling viruses.

The patient's serum and CSF as well as the various specific monoclonal and polyclonal antibodies against the MV M protein were unable to recognize the M protein in the Fr/V- and Fr/B-infected cells, in spite of the pulse-labeling of the infected cells. This seemed to be unlike the case of the IP-3-Ca strain (Sheppard *et al*, 1986). Our data indicate that both of the sibling viruses are defective in M protein expression.

The mechanisms of the defect of M protein expression of SSPE viruses vary with the cases. Rapid degradation and altered antigenicity of the aberrant M protein have both been observed (Ayata *et al*, 1989, 1991; Cattaneo *et al*, 1986, 1988; Enami *et al*, 1989), in addition to the absence of M mRNA (Cattaneo *et al*, 1986, 1987; Yoshikawa *et al*, 1990). Northern blot analysis of the Osaka-2 strain unexpectedly showed different results with the Fr/V and Fr/B viruses. These differences were further confirmed by primer extension analysis. An absence of P and M monocistronic mRNAs and an abundance of P-M bicistronic mRNA were observed in the Fr/B-infected cells. This indicated a transcriptional alteration with the read-through of RNA polymerase at the intercistronic junction, as has already been observed in the MF strain (Cattaneo *et al*, 1987), case K (Cattaneo *et al*, 1986), the IMR-32 cell-adapted Yamagata-1 strain (Yoshikawa *et al*, 1990), and the Osaka-1 strain (Ayata *et al*, 1998a). The molecular mechanism of this inability of RNA polymerase to reinitiate at the junction has not yet been addressed. The gene end and gene start motifs situated upstream and downstream from the intercistronic gene junctions, respectively, might be involved. Recently, the sequence of the gene end motif has been indicated to be involved in transcription termination and reinitiation of respiratory syncytial virus (Kuo *et al*, 1996) and vesicular stomatitis virus (Barr *et al*, 1997; Hwang *et al*, 1998). The analysis of the mechanism in the overproduction of P-M bicistronic RNA by Fr/B virus and the Osaka-1 strain is now under investigation; the minigenome expression system is used for such analysis. The Fr/V virus was found to transcribe the M mRNA almost normally when compared to the Edm strain, and it was similar to the Biken (Avata *et al*, 1989), Niigata-1 (unpublished observation), and Yamagata-1 (unpublished observation) strains. In the sequence analysis of the M gene of the Fr/V virus, no replacements in the start and stop codons and no premature termination were detected, which suggests that the M mRNA of the Fr/V virus is translatable. The aberrant M proteins, caused by accumulated mutations in the open reading frames in the Niigata-1 strain (Ayata *et al*, 1991), in the Biken strain (Ayata *et al*, 1989), and in the Yamagata-1 strain (Ayata *et al*, 1989), have been discussed. Even by short-time labeling of the Fr/V-infected cells, the M protein could not be detected in the RIP assay. However, the possibility cannot be completely ruled out that as a result of the biased hypermutation of its gene, rapid degradation or altered immunogenicity of the M protein is responsible for the defective M protein expression of the Fr/V virus. The M protein of the Fr/V virus is not detected in the infected cells, despite the presence of its translatable M mRNA; this phenomenon should be investigated further.

The reason for the undetectable F protein in this study is also unknown. As the Osaka-2 strain-infected cells showed progressive syncytium formation, the H and F proteins must have been present both in the cytoplasm and on the cell surface (Wild *et al*, 1991). However, because of degradation, the amount of the F protein might have been below the detection limit of RIP assay.

At present, we cannot fully rule out the possibility that the variations in transcription and sequences of the two sibling viruses may have developed by *in vitro* passages on Vero cells or B95a cells. However, the SSPE virus Osaka-1 strain Fr/V and Oc/V sibling viruses, which were isolated from the frontal lobe and the occipital lobe of an SSPE brain, respectively (Ogura *et al*, 1997), were found to be identical to each other in the M gene sequences, nevertheless both the viruses were passaged on Vero cells (Ayata *et al*, 1998a). Each sibling virus of the Osaka-2 strain could have been selected depending on the cell types for virus isolation used, because MV field isolates from clinical specimens by Vero cells can grow in B95a cells, whereas MV isolates by B95a cells is unable to grow in Vero cells (Kobune *et al*, 1990). Recent reports have suggested that recent MV isolates capable of growing in marmoset B cells may use a molecule other than CD46 as the cellular receptor, or they may require another coreceptor in order to infect cells (Hsu *et al*, 1998; Tanaka *et al*, 1998). Barz *et al* (1998) also suggested that MV may alternatively, and strain-specifically, use CD46 and an unknown molecule as receptors. It is not yet clearly known if the isolation of our two different sibling viruses of the Osaka-2 strain depends on using a different cell type for the isolation. The syncytium forming ability of the Fr/V and Fr/B viruses in each counterpart cell is being investigated.

In the present study, it is quite noteworthy to have shown that at least two different infective variant viruses with a common defective phenotype in M protein expression were present 8 months after disease onset in a small biopsy specimen from the intact area shown in MR images. This study also presents the first strong indication that different pathogenic viruses continue to control the progression of SSPE in the infected brain, even at Jabbour III stage, as can be speculated from the clinical observation of the disease's regression. It is, therefore, of much importance and of much interest to obtain further clinical and virological information from this patient; clonal expansion of the sibling viruses in the patient brain, as has reported by Baczko *et al* (1993) using directly cloned M genes from an SSPE brain, and effect of interferon treatment on the viruses should be investigated in future.

Materials and methods

Cells and viruses
Vero cells were cultured at 35°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% fetal calf serum and 4% newborn calf serum, and B95a cells (Kobune *et al*, 1990) were at 35°C in RPMI-1640 medium supplemented with 5% fetal calf serum. The SSPE virus Osaka-2 strain Fr/V and Fr/B viruses are sibling viruses isolated by Vero cells and B95a cells, respectively, from a biopsy specimen of the frontal lobe of a patient with SSPE (Ogura *et al*, 1997). The persistent infection with each sibling virus, which has ever been maintained in cell population, was established just when the sibling viruses were isolated from the brain. The persistent infected cells were cultured in the same manner as the parental cells and were passaged every 4–5 days. The MV Edm strain was used as a standard strain and was propagated in Vero cells and in B95a cells.

Hemadsorption assay
A 0.2% suspension in serum-free DMEM of African green monkey red blood cells was added to the Edm strain- or the Osaka-2 strain-infected cell cultures. After 1 h of incubation at 37°C, the cultures were washed three times with phosphate-buffered saline, fixed, and stained with hematoxylin and eosin.

Immunofluorescence assay
Unfixed cells on coverslips were incubated with an anti-H monoclonal antibody (L77, 1 : 500) at 4°C for 30 min. After washing they were fixed with acetone and were stained with an anti-mouse IgG rabbit antibody conjugated with FITC at 37°C for 30 min.

Cell-free infectivity assay
Culture media of the Osaka-2 strain infected cells were centrifuged at 3000 r.p.m. for 15 min at 4°C. The upper parts of the cell-free supernatants were inoculated into Vero cell monolayers. The cultures were incubated at 35°C and the number of syncytium foci were counted daily.

Radioimmunoprecipitation (RIP) assay and SDS-polyacrylamide gel electrophoresis (PAGE)
To examine the antibody response of the patient with SSPE, Vero cells lytically infected with the Edm strain at 20 h postinfection (p.i.), the Fr/V-infected Vero cells, and the Fr/B-infected B95a cells were labeled with ^{35}S-methionine/cysteine (>37.0 TBq Ci/mmol, EXPRESS™ Methionine/Cysteine Protein Labeling Mix, [^{35}S]-Easy Tag, NEN, USA) for 8 h after 1 h-incubation with methionine/cysteine-free DMEM. The labeled cells were subjected to immunoprecipitation without chase. To examine viral protein synthesis, Vero cells and B95a cells lytically infected with the Edm strain at 48 h p.i., the Fr/V-infected Vero cells, and the Fr/B-infected B95a cells were labeled with ^{35}S-methionine/cysteine for 1 or 2 h after 1 h-incubation with methionine/cysteine-free DMEM. Without chase, the labeled cells were subjected to immunoprecipitation and SDS–PAGE, as described previously (Ogura *et al*, 1987). For the RIP, the following were used: hyperimmune monkey serum (GM), monoclonal antibodies against the N (F227 and Edm21), P (NAG3 and PKo), H (L77), F (A5046 and A5047), and M (B117, B35, F26/5, and K12) proteins of the Edm strain, and polyclonal antibodies against the M protein of the Edm strain (M-BC) and against the M protein of the SSPE virus Biken strain (M-BBC).

RNA extraction
Total RNA was extracted from mock-infected cells or the cells infected with the Edm strain or the Osaka-2 strain as previously described (Ogura *et al*, 1987). Poly A$^+$ RNA was further selected by oligotex dT30 <Super> (Roche, USA).

Nucleotide sequencing
M-gene specific cDNA was amplified through reverse transcription and polymerase chain reaction (RT–PCR) of the total RNA from the infected cells with a set of primers. The products were purified using a Qiaex II gel extraction kit (Qiagen GmbH, Germany) and were directly sequenced with a Dye Terminator cycle sequencing kit (Applied Biosystems, USA) as previously described (Ayata *et al*, 1998b).

Analysis of viral specific RNA
Agarose gel electrophoresis of the poly A$^+$ RNA from the infected cells and Northern blot hybridization were carried out as previously described (Ogura *et al*, 1987), except for the following: radioactive cDNA probes were prepared by random priming

method using fragments of the N, P, M, F, and H genes, random primer (9mer, Takara, Japan), dNTP, and α-^{32}P-dCTP (111TBq/mmol, NEN, USA).

Primer extension
To analyze the 5′ end of the M and P mRNAs, the 5′ termini of 17 mer oligonucleotides complementary to the M mRNA situated at position +50 to +34 and 16 mer oligonucleotides complementary to the P mRNA situated at position +55 to +40 were labeled with γ-^{32}P-ATP (111TBq/mmol, NEN, USA) by incubation with T4 polynucleotide kinase (New England Biolabs, USA) and were purified by Probequant G-50 microcolumns (Pharmacia Biotech, Tokyo). Using the labeled primers and Superscript II RNase H^- reverse transcriptase (Life Technologies, USA), poly A^+ RNA from the infected cells was reverse transcribed and the products were analyzed by electrophoresis with a 6% polyacrylamide urea gel to measure the length of the synthesized cDNA.

Nucleotide sequence accession numbers
DDBJ accession number for the M genes of the Osaka-2 strain Fr/V and Fr/B viruses are AB002690 and AB016238, respectively.

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