Journal of NeuroVirology (1995) 1,177–188
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# Delayed activation of altered fusion glycoprotein in a chronic measles virus variant that causes subacute sclerosing panencephalitis

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**We compared the intracellular processing of the fusion (F) glycoproteins of an acute measles virus (MV) Nagahata strain and its relative Biken strain that caused subacute sclerosing panencephalitis (SSPE). Nagahata strain synthesizes a precursor $F_0$ which acquires three asparagine (N)-linked oligosaccharide chains sequentially in 1 h. One oligosaccharide chain on the partially glycosylated $F_0$ is less accessible to endo-β-N-acetylglucosaminidase H (endo-H) but becomes accessible as the protein becomes fully glycosylated, suggesting a protein conformational change. Biken strain SSPE virus synthesizes a similarly glycosylated $F_0$. However, one oligosaccharide chain on the Biken $F_0$ remains less accessible to endo-H even after the protein is fully glycosylated. The Nagahata $F_0$ is cleaved into the $F_1$ and $F_2$ subunits with a half life of 1 h. The Biken $F_0$ is cleaved with a half life of 4 h. We cloned the F genes of Nagahata and Biken strains and showed by transfection that the defect causing delayed cleavage of $F_0$ resides in the Biken F gene. Sequence analysis predicts a mutation in the cleavage recognition sequence, a truncated carboxyl-terminus, and multiple mutations in $F_1$ of the Biken F protein. Expression of chimeric F genes showed the mutated cleavage recognition sequence and the carboxyl-terminal truncation do not delay cleavage of $F_0$. Instead, delayed $F_0$ cleavage is due to multiple mutations in the extracellular domain of $F_1$, and four amino acid substitutions near the transmembrane region impair endo-H access to the oligosaccharide chain. These results provide detailed information on the normal maturation process of the F protein of MV and additional clues to the mechanisms of MV persistence in the CNS.**

**Keywords:** measles virus; subacute sclerosing panencephalitis; fusion glycoprotein

## Introduction

Measles virus (MV) normally causes an acute systemic infection which is a major cause of morbidity and mortality in the world population (Norrby and Oxman, 1990). MV can also establish a chronic infection in the central nervous system (CNS), leading to a rare fatal degenerative neurological disease subacute sclerosing panencephalitis (SSPE) (ter Meulen *et al*, 1983; Kristensson and Norrby, 1986). Studies have shown that about half of the SSPE patients contracted acute measles when they were less than 2 years old (ter Meulen *et al*, 1983). Vaccination has greatly reduced the cases of acute measles in the developed countries. However, tens of millions of people still contract measles every year especially in developing countries. Many of the recent cases occur in non-immune individuals and infants who are difficult to be immunized effectively because of the presence of maternal anti-measles antibodies (Weiss, 1992; Tulchinsky *et al*, 1993). For these patients, development of chronic infection and SSPE remains a potential risk. A clear knowledge of the viral and host factors that influence the development of SSPE will be useful for understanding the mechanisms of MV persistence in the CNS.

MV, a morbillivirus in the paramyxoviridae family, produces at least six structural proteins: the nucleoprotein (N) which encapsidates a minus-sense RNA genome to form a nucleocapsid, the phosphoprotein (P) and polymerase (L) involved in RNA synthesis, the matrix (M) protein required for

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Received 17 October 1994; revised 30 November 1994; accepted 30 November 1994

virus assembly, the hemagglutinin (H) which serves for attachment to a cellular receptor, and the fusion (F) glycoprotein which induces membrane fusion to form multinucleated giant cells (syncytia) Barrett *et al*, 1991). Sequence comparison between acute MV and SSPE-associated viruses has revealed numerous differences in all the viral genes (Billeter and Cattaneo, 1991). However, since the direct progenitors of the viruses isolated from SSPE patients are rarely available for comparison, it is often difficult to understand the genetic differences between the acute and SSPE-associated MV strains. To better understand the genetic changes in MV during a chronic CNS infection, we have been studying the Nagahata strain MV and Biken strain SSPE virus that are evolutionarily related.

Biken strain was isolated from a patient who died of SSPE in the Osaka prefecture in Japan (Ueda *et al*, 1975). Biken strain is defective in virus assembly and it replicates in a strictly non-productive manner (Ayata *et al*, 1989). Nagahata strain is an acute MV that was prevalent in the same locale when that SSPE patient first contracted measles several years before his death (Wong *et al*, 1991). Nucleotide sequence and epidemiological data strongly suggest that the patient was infected by Nagahata strain or a closely related MV, which evolved into the Biken strain that caused SSPE (Wong *et al*, 1991). Thus, studying the genetic and biochemical differences between the Nagahata and Biken strains can shed light on the possible mechanisms of MV persistence in the CNS.

Previous studies showed that mutations have altered the structure of the Biken M protein (Ayata *et al*, 1989), making it incapable of binding to the viral nucleocapsids (Hirano *et al*, 1992; 1993). This M protein defect may explain the non-productive phenotype of Biken strain SSPE virus. In the present study, we identified a second defect in Biken strain affecting cleavage activation of the F protein. The F protein of MV is synthesized in the endoplasmic reticulum (ER) as an inactive precursor which is proteolytically cleaved into two subunits which form the functional F protein (Sato *et al*, 1988; Alkhatib *et al*, 1994). We found that the F protein precursor in Biken strain SSPE virus-infected cells was cleaved at a slower rate than the F precursor produced by Nagahata strain MV. We cloned and sequenced the Nagahata and Biken F genes, and identified the mutations that caused delayed cleavage of the Biken F protein by expression of chimera F proteins. The possible role of delayed cleavage of the F protein in chronic CNS infection is discussed.

## Results

### *The F proteins of Nagahata and Biken strains acquire three oligosaccharide chains*

The F protein of MV is synthesized as a precursor

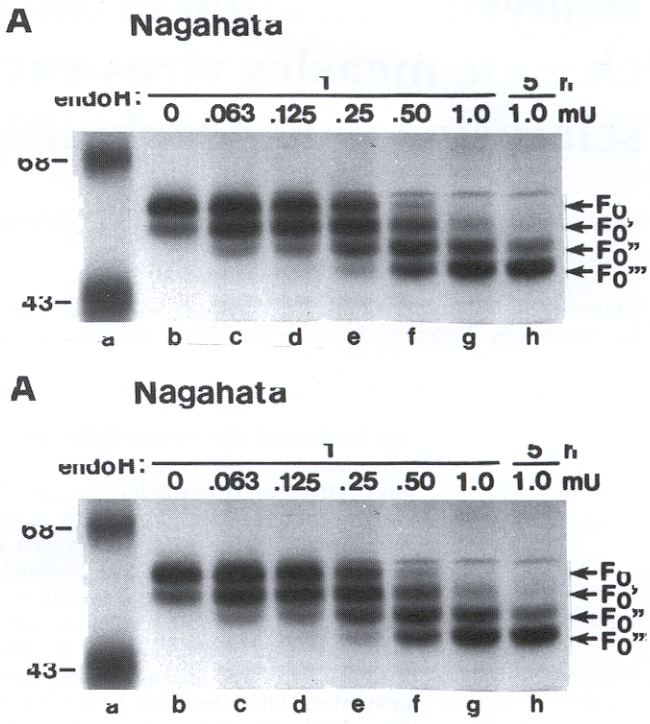



**Figure 1** Partial endo-H digestion of the F protein. Nagahata MV infected CV-1 cells (panel A) or Biken-CV-1 cells (panel B) were labeled with [$^{35}$S]methionine for 1 h. The cell lysates were immunoprecipitated and digested with endo-H for 1 h or 5 h at the concentrations indicated. Lane a shows molecular weight standards (in kD).

$F_0$, which possesses three potential asparagine (N)-linked glycosylation sites (Richardson *et al*, 1986; Alkhatib *et al*, 1994). During transport to the plasma membrane, $F_0$ is cleaved by a cellular endoprotease into a glycosylated amino-proximal $F_2$ and unglycosylated carboxyl-proximal $F_1$ peptides, which are joined by disulfide bonding to form the mature F protein (Sato *et al*, 1988; Alkhatib *et al*, 1990). The F proteins of Nagahata strain MV and Biken strain SSPE virus are electrophoretically distinguishable (Hirano, 1992). To see whether the different electrophoretic mobility was due to differences in glycosylation, we examined the glycosylation patterns of the $F_0$ of Nagahata and Biken strains by limited digestion with endo-H, which cleaves high-mannose oligosaccharides (Tarentino and Maley, 1974).

African green monkey kidney (CV-1) cells lytically infected with Nagahata strain MV or chronically infected by Biken strain SSPE virus (Biken-CV-1) were labeled with [$^{35}$S]methionine for 1 h. The cell lysates were immunoprecipitated with the polyclonal F-AS antiserum which reacts with $F_0$ and $F_1$ of both Nagahata and Biken strains (Hirano, 1992). The immune-precipitates were incubated with increasing concentrations of endo-H and analyzed by SDS-PAGE. The fully glycosylated form of F pre-

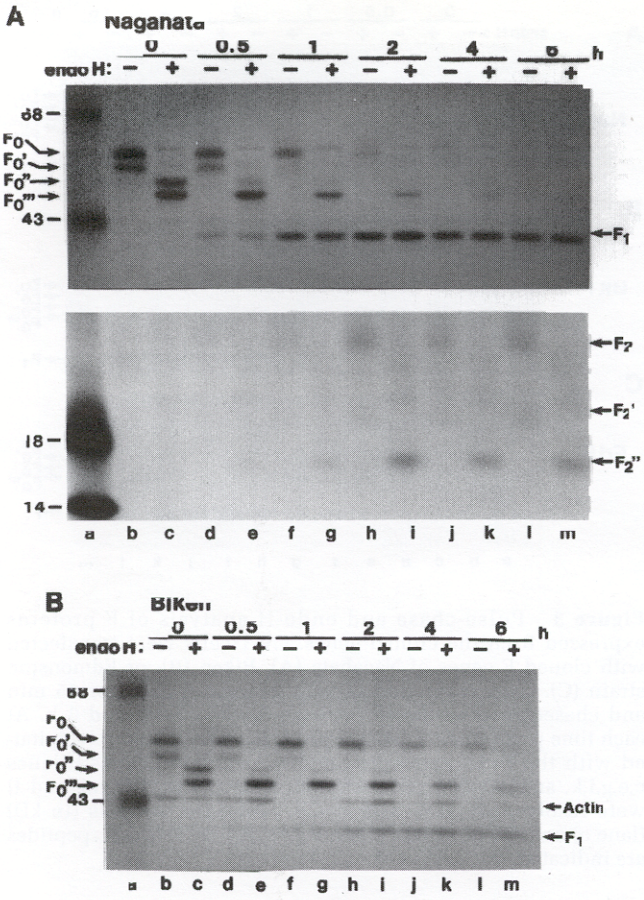



**Figure 2** Pulse-chase and endo-H analysis of F proteins in virus infected cells. Nagahata MV-infected CV-1 cells (A) or Biken-CV-1 cells (B) were labeled with [$^{35}$S]methionine for 15 min and chased in conditioned medium for 0.5, 1, 2, 4 and 6 h. At each time point, the $^{35}$S-labeled proteins were immunoprecipitated with the F-AS antiserum. Half of samples were incubated at 37 °C for 16 h with 0.1 mU of endo-H (lanes c,e,g,i,k, and m) and analyzed along with the undigested samples (lanes b,d,f,h,j, and l) by SDS-PAGE (12% acrylamide). Molecular weights (in kD) (lane a) and the positions of $F_0$, $F_0'$, $F_0''$, $F_0'''$, $F_1$, $F_2$, $F_2'$, and $F_2''$ proteins are indicated by arrows.

cursor synthesized by both Nagahata and Biken strains was $F_0$ (Figure 1A and B, lane b). Increasing concentrations of endo-H successively removed the three oligosaccharide chains, converting $F_0$ to $F_0'$, $F_0''$, and $F_0'''$ (Figure 1, lanes c–g). $F_0'$ and $F_0''$ were partially glycosylated intermediates with two or one side chain, respectively. Prolonged incubation with endo-H generated the completely deglycosylated $F_0'''$ (Figure 1A and B, lane h) which comigrated with the unglycosylated F precursor synthesized in the presence of tunicamycin (data not shown) (Sato *et al*, 1988; Alkhatib *et al*, 1994). Therefore, the Nagahata and Biken F proteins are glycosylated in a similar manner, each acquiring three N-linked oligosaccharide chains. The faint bands just above the $F_0$ band will be explained below.

### *$F_0$ of Biken strain is cleaved four times slower than the Nagahata $F_0$*

Since glycoprotein synthesis is not coordinated temporally with biosynthesis of carbohydrates, it was impossible to study the synthesis and processing of F protein by pulse-chase analysis using radioactive sugars. Therefore, we carried out pulse-chase analysis by labeling the nascent F protein with [$^{35}$S]methionine, and tracking the different glycosylated forms of F protein according to their distinct electrophoretic mobility as shown in Figure 1.

Nagahata MV-infected CV-1 and Biken-CV-1 cells were pulse-labeled with [$^{35}$S]methionine for 15 min and chased in unlabeled medium for various times. The F proteins were immunoprecipitated with the F-AS antiserum and analyzed by SDS-PAGE. During the pulse, Nagahata MV synthesized two forms of F precursor corresponding to the fully glycosylated $F_0$ and partially glycosylated $F_0'$ (Figure 2A, top panel, lane b). After 0.5 to 1 h chase, the Nagahata F precursor became fully glycosylated and cleaved into $F_1$ (Figure 2A, top panel, lanes d and f). By 2 h, most of the Nagahata $F_0$ was cleaved into $F_1$ (Figure 2A, top panel, lane h). The $F_2$ peptide was not labeled efficiently, but was detectable after the 1 h time point (Figure 2A, bottom panel, lanes f, h, j, and l).

During the 15 min pulse, Biken strain also synthesized $F_0$ and $F_0'$ (Figure 2B, lane b). The Biken $F_0'$ also became fully glycosylated $F_0$ after 0.5 1 h chase (Figure 2B, lanes d and f). However, the Biken $F_0$ was cleaved into $F_1$ more slowly, and uncleaved $F_0$ remained detectable even after 6 h chase (Figure 2B, lanes d–l). Because of the inefficient cleavage and labeling, the Biken $F_2$ was difficult to detect (data not shown). Quantitation of the $F_0$ and $F_1$ bands with a PhosphorImager (Molecular Dynamics, California) indicated that the Nagahata $F_0$ was cleaved with a half life of about 1 h and the Biken $F_0$ was cleaved with a half life of about 4 h.

### *Endo-H analysis demonstrates structural changes in the nascent $F_0$*

Cleavage of the paramyxovirus F protein is believed to occur in the trans-Golgi network (TGN) (Morrison and Portner, 1991). During glycoprotein transport through the medial Golgi, some mannose residues are replaced by other sugars including N-acetylglucosamine, rendering the oligosaccharide chains insensitive to endo-H (Arakawa and Muramatsu, 1974; Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985). Therefore, sensitivity of the oligosaccharides to endo-H can serve as an indicator for transport of glycoproteins through the medial Golgi. To see whether delayed cleavage of the Biken $F_0$ was due to a block in transport through the Golgi, half the samples from the pulse-chase experiments were digested with endo-H and analyzed along with the untreated samples.

The MV F protein never became fully resistant to

endo-H. Even after 2–6 h chase when the Nagahata $F_0$ had been cleaved into $F_1$ and $F_2$ (Figure 2A, top panel, lanes h–m), most of the oligosaccharides on $F_2$ were still digestible by endo-H to yield the deglycosylated $F_2'$ and $F_2''$ peptides (Figure 2A, bottom panel, lanes h–m). This indicates that most of the oligosaccharides on the MV F protein are not modified to an endo-H resistant form even after the F protein has reached the TGN and is cleaved into $F_1$ and $F_2$. A previous study also found no more than 20–30% of the oligosaccharides on the $F_2$ of MV became endo-H resistant (Alkhatib *et al*, 1994). Thus, endo-H sensitivity is not a reliable indicator for monitoring transport of the MV F protein through the Golgi. Because of the inefficient cleavage of the Biken F protein, we were unable to determine the endo-H sensitivity of the Biken $F_2$ peptide.

On the other hand, endo-H analysis revealed that the nascent MV $F_0$ undergoes a conformational change that accompanies N-linked glycosylation. Specifically, endo-H digestion of the Nagahata and Biken F precursors synthesized in the 15 min pulse invariably produced two bands: completely deglycosylated $F_0'''$ and $F_0''$ with one remaining oligosaccharide chain (Figure 2A and B, lane c). The residual oligosaccharides were not modified to an endo-H resistant form, because when the Nagahata F precursor became fully glycosylated after the chase, all the oligosaccharide chains were removable by endo-H to produce only $F_0'''$ (Figure 2A, top panel, lanes g, i, and k). Also, by increasing the endo-H concentration 10-fold, the F precursors synthesized during the pulse can be completely deglycosylated to $F_0'''$ (data not shown). Therefore, one oligosaccharide chain appears to be less accessible to endo-H when the nascent $F_0$ is partially glycosylated but becomes accessible when the protein is fully glycosylated. The Biken F protein appears to undergo this maturation process slower than the Nagahata F protein. Even after the Biken $F_0$ became fully glycosylated after the chase, endo-H still produced the incompletely deglycosylated $F_0''$ in addition to $F_0'''$ (Figure 2B, lanes g, i, k, and m).

These data indicate that the nascent Nagahata $F_0$ acquires two or three oligosaccharide chains in 1 h. One oligosaccharide chain on the partially glycosylated $F_0$ appears to be less accessible to endo-H until $F_0$ is fully glycosylated, suggesting a structural change in the F protein. Two faint bands are often seen just above $F_0$. The upper band, which is also produced by the cloned F genes and increases in intensity during the chase (Figure 3), may be due to modifications of the oligosaccharides, since that band is removed by endo-H (Figure 2A, lanes h and i). The second faint band, which is resistant to endo-H (Figure 2A and B, lanes c, e, g, i, and k) and is not seen in transfection experiments (Figure 3), comigrates with the MV N protein which is often non-specifically precipitated by rabbit sera (Wong *et al*, 1987).

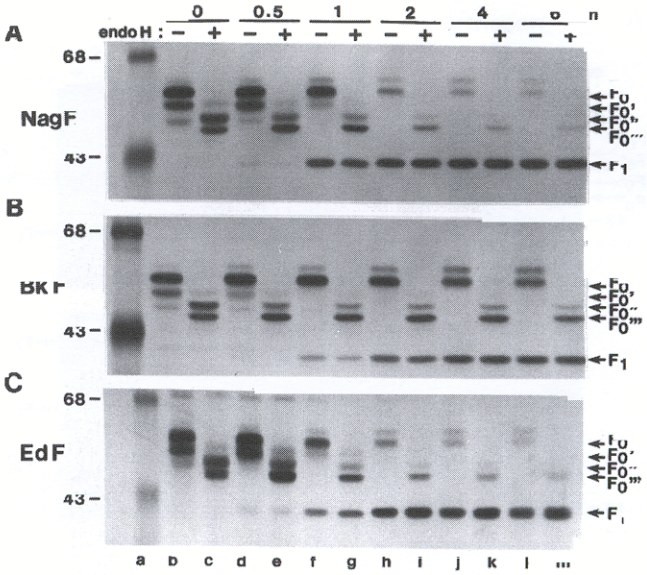



**Figure 3** Pulse-chase and endo-H analysis of F proteins expressed from the cloned genes. CV-1 cells were transfected with cloned F genes of Nagahata (A), Biken (B), or Edmonston strain (C). Cells were labeled with [$^{35}$S]methionine for 15 min and chased in conditioned medium for 0.5, 1.2,4, and 6 h. At each time point, the $^{35}$S-labeled proteins were immunoprecipitated with the F-AS antiserum, and the endo-H digested (lanes c,e,g,i,k, and m) and undigested proteins (lanes b,d,f,h,j, and l) were resolved by 12% SDS-PAGE. Molecular weights (in kD) (lane a) and the positions of the $F_0$, $F_0'$ $F_0''$, $F_0'''$, and $F_1$ peptides are indicated.

*Genetic changes in the Biken F gene*

To investigate the structural basis of the aberrant mobility and cleavage of the Biken F protein, we cloned and sequenced both the Nagahata and Biken F genes. The Biken F gene differed from Nagahata F gene by 20 nucleotides in the F protein-coding region. By comparison, the Biken F gene differed from the revised Edmonston F sequence (Richardson *et al*, 1986; Cattaneo *et al*, 1989) by 26 nucleotides in the same region (Figure 4). Twelve of the Biken nucleotides that differed from the Edmonston F gene were identical to the Nagahata sequences [Figure 4, nucleotides (nt) 743, 829, 1042, 1102, 1240, 1306, 1318, 1351, 1540, 1672, 2035, and 2149], while only six of the Biken nucleotides that differed from the Nagahata F gene were the same as the Edmonston sequences (Figure 4, nt 1158, 1309, 1987, 1997, 2155, 2202). Thus, the Biken F sequence is more closely related to the Nagahata than the Edmonston F sequence, consistent with the epidemiological relationship of these virus strains (Ueda *et al*, 1975; Wong *et al*, 1991).

The 12 nucleotides where both the Biken and Nagahata F genes differed from the Edmonston strain were silent mutations. The nucleotide differences between the Biken and Nagahata F genes resulted in 12 amino acid changes; 10 of these were also observed when the Biken F gene was compared

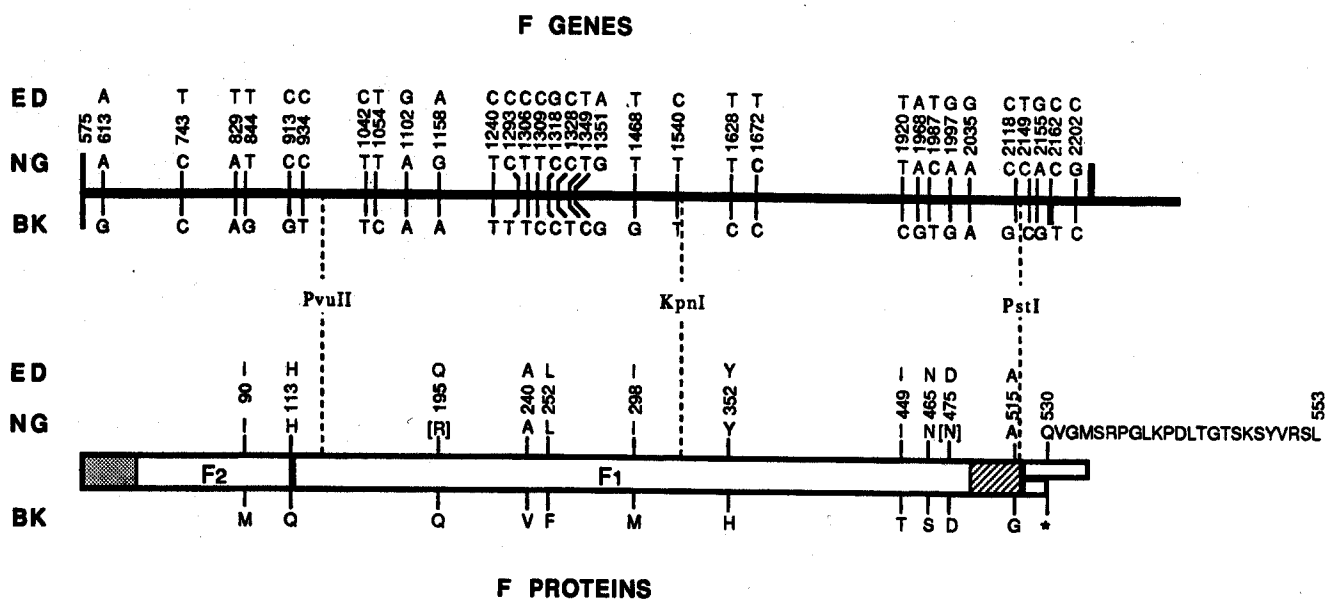



**Figure 4** Sequence comparison of the F genes of MV Edmonston (ED), Nagahata (NG), and SSPE virus Biken (BK) strains. The first ATG codon (nt 575 from the 5′ end of the F mRNA) is denoted as the translation start site (Cattaneo *et al*, 1989). Top horizontal line shows the nucleotide sequence. Thick vertical lines represent translation start and stop codons. Thin vertical lines represent mutations. Bottom horizontal bar represents the predicted amino acid changes shown in single letter code. Stippled and cross-hatched areas represent the signal peptide and transmembrane anchor, respectively. Asterisk represents a premature translation stop codon, deleting amino acid residues 530–553. Mutations in Nagahata strain are shown in brackets. Restriction enzyme sites used for the construction of the chimera F genes are indicted by dotted lines.

to the Edmonston F gene [amino acids (aa) 90, 113, 240, 252, 298, 352, 449, 465, 515, and 530]. Two changes (aa 195 and 475) were found only in the Nagahata F gene and not in the Biken or Edmonston F gene (Figure 4), indicating that these two changes occurred in Nagahata strain MV after Biken strain diverged from it. Thus, these two amino acid changes in the Nagahata F protein are probably irrelevant to the Biken F protein defect. The fact that the F proteins of the Nagahata and Edmonston strains isolated more than two decades apart in different continents differ only by two amino acid residues confirms the remarkable conservation in the F proteins of acute MV strains (Rota *et al*, 1992). In sharp contrast, the F protein of Biken SSPE virus isolated in the same area 4 years after the Nagahata strain has undergone significant changes. Interestingly, a His-to-Gln mutation (aa 113) changed the putative cleavage recognition sequence in the Biken F protein to Arg-Gln-Lys-Arg, identical to that in Newcastle disease virus (NDV) (Chambers *et al*, 1986; McGinnes and Morrison, 1986). Except for this His-to-Gln mutation and a Ile-to-Met substitution at position 90 in $F_2$, most of the changes in the Biken F protein occurred in the $F_1$ peptide. A mutation at nucleotide 2162 created a new termination codon that deleted 24 amino acid residues from the carboxy-terminus (Figure 4).

*Genetic changes cause delayed cleavage of the Biken $F_0$*

To see whether delayed cleavage of the Biken $F_0$ is due to the mutations in the Biken F gene, we expressed the F genes of Nagahata, Biken, and Edmonston strains in CV-1 cells by using the vTF7-3 recombinant vaccinia virus vector and studied the F protein cleavage by pulse-chase analysis. During a 15 min pulse, all three F clones produced the fully glycosylated $F_0$ and the partially glycosylated $F_0'$ with two oligosaccharide chains. Because expression from the recombinant vaccinia virus vector was higher than in MV-infected cells, the $F_0''$ precursor with one oligosaccharide chain was also detected (Figure 3A, B and C, lane b). These precursors became fully glycosylated in 1 h (Figure 3A, B and C, lanes d and f). The $F_0$ precursors of Nagahata and Edmonston strains were cleaved into $F_1$ with a half life of about 1 h (Figure 3A and C, lanes f, h, j, and l), whereas the Biken $F_0$ was cleaved with a half life of about 4 h (Figure 3B, lanes f, h, j, and l). The nascent $F_0$ peptides synthesized from the Nagahata and Edmonston F genes during the pulse were digested by endo-H to produce $F_0''$ and $F_0'''$ (Figure 3A and C, lane c). After the chase, endo-H digestion yielded mainly $F_0'''$ (Figure 3A and C, lanes i, k, and m). By contrast, endo-H could not remove completely an oligosaccharide chain on the Biken $F_0$,

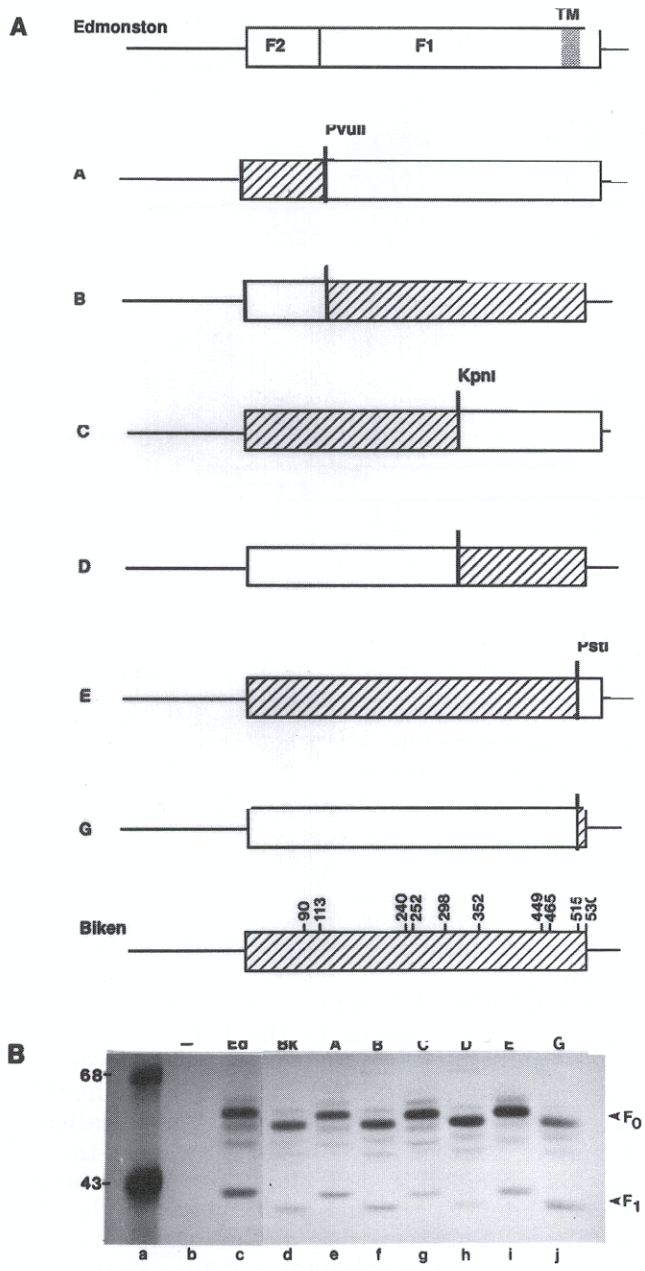



**Figure 5** Construction and expression of chimera F genes. (A) Chimera F genes were constructed from the Edmonston and Biken F genes by use of the *Pvu*II, *Kpn*I, and *Pst*I restriction enzyme sites. Position of the mutations are shown in the Biken F gene. (B) CV-1 cells were transfected with the Edmonston (Ed), Biken (Bk), and various chimera F genes. The cells were labeled with [$^{35}$S]methionine for 3 h. The $^{35}$S-labeled proteins immunoprecipitated with the F-AS antiserum and resolved by 12% SDS-PAGE. Molecular weights (in kD) (lane a) and the positions of the $F_0$ and $F_1$ peptides are indicated.

producing a partially deglycosylated $F_0''$ even after 4–6 h chase (Figure 3B, lanes k and m). Therefore, as in the virus-infected cells, an oligosaccharide chain on the $F_0$ expressed from the Nagahata and Edmonston F genes appears to be less accessible until $F_0$ becomes fully glycosylated. An oligosaccharide chain on the $F_0$ expressed from the Biken F gene remains less accessible to endo-H even after the protein is fully glycosylated.

These results show that delayed cleavage of the Biken $F_0$ and impaired access to an oligosaccharide chain are due to mutations in the Biken F gene. In experiments not shown, we found that vaccinia virus did not alter the rate of cleavage of the MV F protein. The same results were obtained by expression of the Edmonston and Biken F genes in African green monkey kidney (COS) cells via a simian virus 40 promoter. The endo-H-sensitive F-related protein just above $F_0$ (Figure 3A, B and C, lanes d, f, h, j, and l) is possibly due to modifications of the oligosaccharides as discussed above (Figure 2A, lane h).

*Construction of chimeric F genes carrying different Biken mutations*

A series of chimeras were constructed to localize the mutations in Biken strain responsible for the delayed cleavage of its $F_0$. Since the kinetics of $F_0$ cleavage in Nagahata and Edmonston strains MV are indistinguishable, and since the two mutations unique to the Nagahata F protein are probably irrelevant to the Biken F protein defect (see explanation for Figure 4), we constructed chimeras using the F genes of Biken and Edmonston strains. Chimera A contains the Ile-to-Met substitution (aa 90) in $F_2$ and the His-to-Gln mutation (aa 113) in the cleavage recognition sequence (Figure 5A). Chimera B contains all the mutations in the Biken $F_1$, Chimera C contains the Biken $F_2$ plus three mutations (aa 240, 252, 298) in the amino half of the Biken $F_1$ peptide. Chimera D contains the mutations (aa 352, 449, 465) in the carboxy-proximal region of the Biken $F_1$, the Ala-to-Gly mutation (aa 515) in the transmembrane region, and the 24 amino acid deletion at the carboxy-terminus. Chimera E contains all the Biken mutations but a wild-type carboxy-terminus. Chimera G is wild-type except for the truncated carboxy-terminus (Figure 5A).

To ensure the chimera protein-coding frames were intact, the junction regions were sequenced, and the chimeras were expressed in CV-1 cells by use of the vTF7-3 recombinant vaccinia virus vector. The chimera F proteins were immunoprecipitated and analyzed by SDS-PAGE. Chimera proteins A, C, and E which contained the carboxy-terminus of the Edmonston F protein comigrated with the Edmonston F protein (Figure 5B, lanes c, e, g, and i). Chimera proteins B, D, and G which contained the carboxy-terminus of Biken F protein had the faster mobility of the Biken F protein (Figure 5B, lanes d, f, h, and j). The uppermost bands are possibly due to differential modification of the oligosaccharides as explained above. These results confirm that the increased mobility of the Biken F protein is due to its carboxy-terminal truncation.

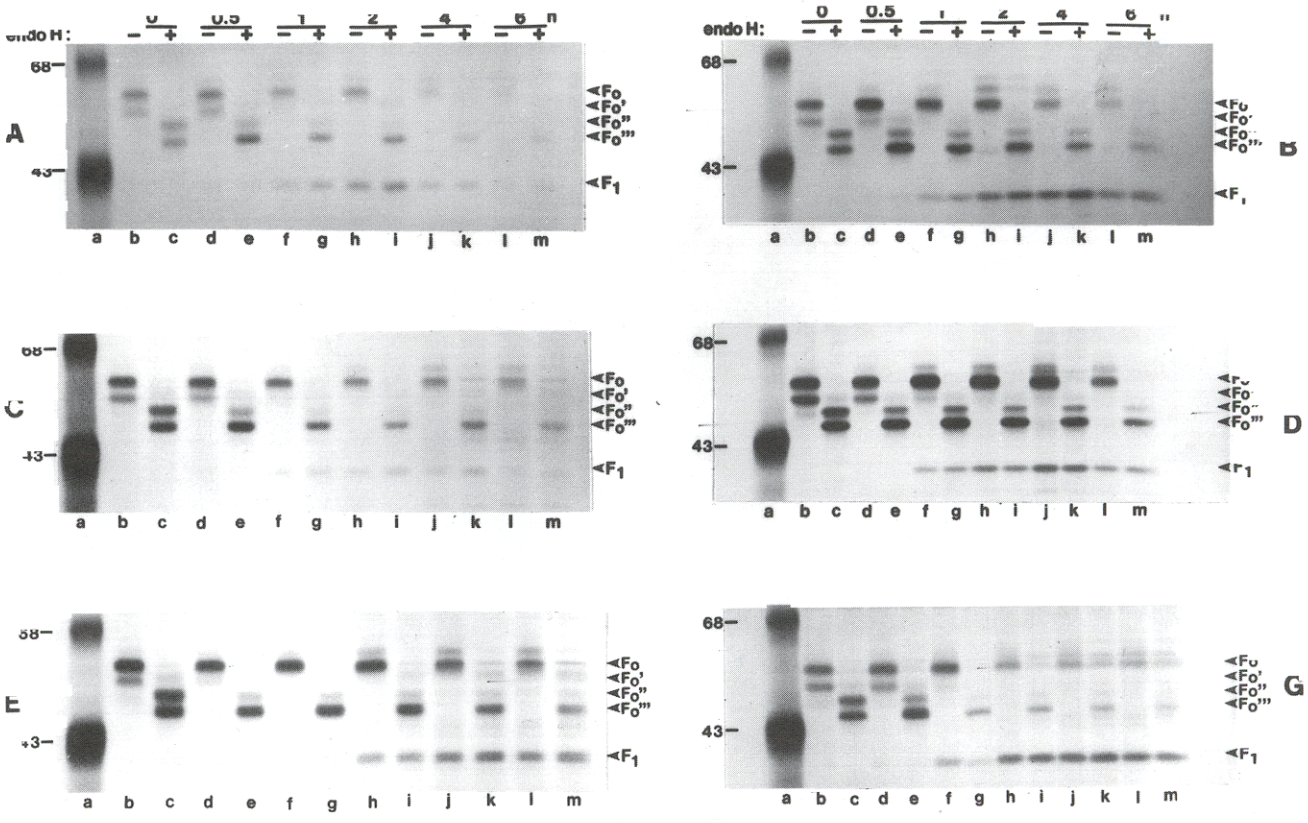



**Figure 6** Pulse-chase and endo-H analysis of chimera F proteins. CV-1 cells were transfected with the chimera F genes and labeled with [$^{35}$S]methionine for 15 min and chased in conditioned medium for 0.5, 1,2,4, and 6 h. At each time point, the $^{35}$S-labeled proteins were immunoprecipitated with the F-AS antiserum, and the endo-H digested (lanes c,e,g,i,k, and m) and undigested proteins (lanes b,d,f,h,j, and l) were resolved on 12% SDS-PAGE. Molecular weights (in kD) (lane a) and the positions of different forms of the $F_0$, $F_0'$, $F_0''$, $F_0'''$, and $F_1$ proteins are indicated.

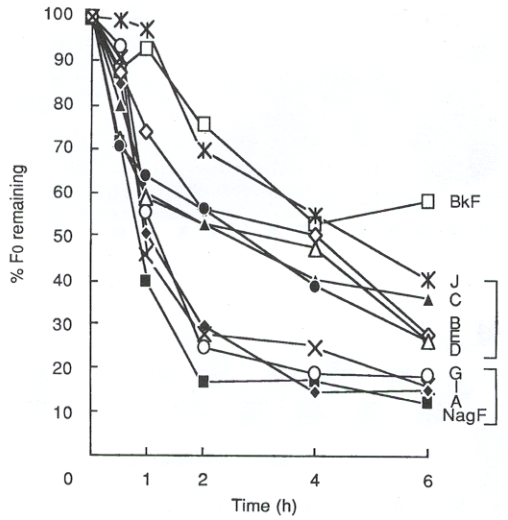
*Processing of chimera F proteins carrying different Biken mutations*

The F chimeras expressed in CV-1 cells were studied by pulse-chase analysis. During a 15 min pulse, all the chimera F genes produced the $F_0$ and $F_0'$ precursors which became fully glycosylated after 1 h chase (Figure 6A–G, lanes b, d, and f), confirming there is no defect in N-linked glycosylation. However, the fully glycosylated chimera $F_0$ peptides were cleaved at markedly different rates. The $F_0$ peptides of chimeras A and G were cleaved as efficiently as the Edmonston $F_0$, with half lives of about 1 h (Figure 6A and G; Figure 7). The $F_0$ of chimeras B, C, D, and E had half lives ranging from 2.5 to 4 h (Figure 6B, C, D, and E; Figure 7). Interestingly, the $F_1$ peptides of chimeras A and C which shared the Biken $F_2$ mutations failed to accumulate after 6 h chase (Figure 6A and C, lanes l and m). Since both the Biken and wild-type $F_1$ peptides are stable (Figure 3), this suggests that chimera F proteins containing Biken $F_2$ and wild-type $F_1$ are unstable.

The $F_0$ of chimeras B, D, and E were not efficiently deglycosylated by endo-H, producing more $F_0''$ during the chase (Figure 6B, D, and E, lanes e, g, i, k, and m; Figure 7B). The $F_0$ of chimera C, which was cleaved into $F_1$ more slowly, was efficiently deglycosylated by endo-II (Figure 6C, lanes e, g, i, k, and m; Figure 7B). Thus, the four mutations [Tyr-to-His, Ile-to-Thr, Asn-to-Ser, and Ala-to-Gly (aa 352, 449, 465, and 515)] present in the carboxy-half of $F_1$ in chimeras B, D, and E but not in chimera C must render the oligosaccharide chain less accessible. To test this hypothesis, two more chimeras carrying the mutations in the amino-(chimera I) or carboxy-half (chimera J) of the extracellular domain of Biken $F_1$ were constructed and studied by pulse-chase analysis in CV-1 cells (Figure 8A). Chimera I produced $F_0$ that was cleaved as efficiently as the Nagahata $F_0$ (Figure 8B, lanes f–m; Figure 7A), and was deglycosylated completely by endo-H once the protein became fully glycosylated (Figure 8B, lanes f–m). By contrast, the $F_0$ of chimera J was cleaved into $F_1$ inefficiently and was deglycosylated inefficiently even after it was fully glycosylated (Figure 7A; Figure 8B, lanes f–m).

These results show that the mutations in $F_2$ including the putative cleavage recognition

A




B

| | NagF | EdF | BkF | A | B | C | D | E | G | I | J |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fo cleavage | | | | | | | | | | | |
| efficient | ● | ● | | ● | | | | | ● | ● | |
| inefficient | | | ● | | ● | ● | ● | ● | | | ● |
| endoH cleavage | | | | | | | | | | | |
| efficient | ● | ● | | ● | | ● | | | ● | ● | |
| inefficient | | | ● | | ● | | ● | ● | | | ● |

**Figure 7** (A) Kinetics of $F_0$ cleavage. The percentage of $F_0$ remaining at different times was determined from the gels shown in Figures 6 and 8 by a PhosphorImager. ■, Nagahata; □, Biken; ◆, chimera A; ◇, chimera B; ▲, chimera C; △, chimera D; ●, chimera E; ○, chimera G; ×, chimera I; *, chimera J. (B) Processing characteristics of Nagahata, Edmonston, Biken, and Chimera F proteins.

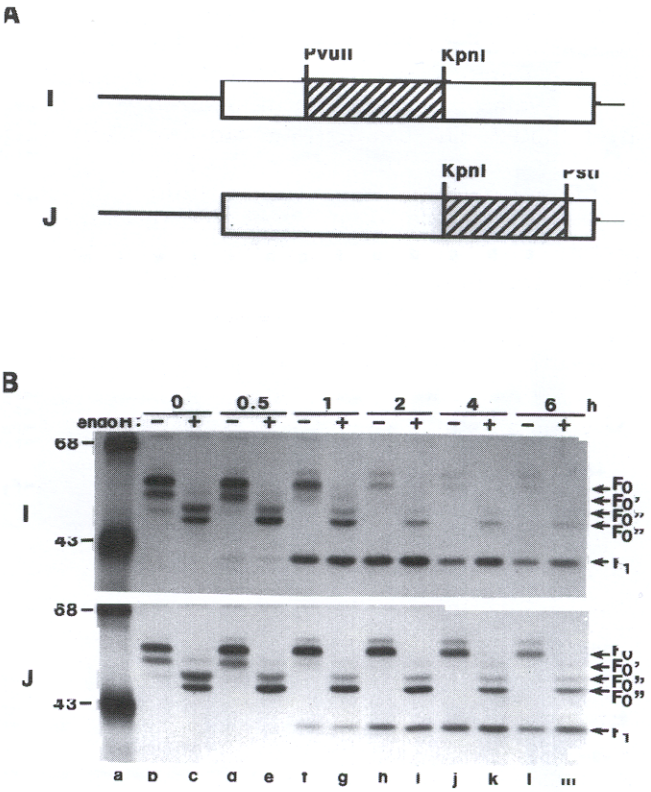



**Figure 8** Localization of mutations impairing $F_0$ cleavage and deglycosylation by endo-H. Chimera F gene 'I' was constructed from chimeras B and C, and chimera F gene 'J' from chimeras D and E (panel A). Chimeras I and J were transfected into CV-1 cells and the proteins were analyzed as described in the legend to Figure 6.

sequence (chimera A) or the carboxy-terminal truncation (chimera G) have no effects on the rate of F protein cleavage. Instead, delayed cleavage of $F_0$ is due to multiple mutations in the extracellular domain of the Biken $F_1$, and four mutations (aa 352, 449, 465, and 515) in the carboxy-proximal region of $F_1$ near the transmembrane anchor impair access to an oligosaccharide chain by endo-H.

## Discussion

A striking characteristic of SSPE is the presence of intracellular inclusion bodies containing MV nucleocapsid-like structures in the brain cells (Herndon and Rubinstein, 1968). Although MV antigens are readily detectable in SSPE brains by biochemical or immunofluorescence techniques (ter Meulen *et al*, 1969; ter Meulen *et al*, 1983), SSPE brains rarely show the extensive syncytia which are a hall-mark of acute MV infection (Norrby and Oxman, 1990). MV lacks mechanisms to integrate the viral genome into the host chromosomes. Persistence of MV relies on continuous virus replication. However, MV infection normally kills the host cells rapidly. How MV can persist in the CNS without causing extensive cell death and cell fusion has been one of the key questions in understanding chronic MV infection. Studies show that MV persistence is achieved by profound changes in the virus–host relationship. Instead of maximizing production of viral gene products as in an acute infection, the persisting MV produces reduced levels of viral gene products, and some of the products are different from those produced in an acute infection (Billeter and Cattaneo, 1991).

Quantitative changes in MV gene expression in chronic CNS infection have been documented at the transcription as well as translation levels. *In situ* hybridization and RNA blot analyses have shown a marked restriction in MV gene expression in the SSPE brain (Haase *et al*, 1985). In particular, transcription from the viral genes near the 5′-end of the genome, including the F and H genes, is severely reduced (Cattaneo *et al*, 1987). Host factors can play a role in the changes in MV gene expression at both the transcription and translation levels (Schneider-Schaulies *et al*, 1994). Chronic CNS infection has also been associated with qualitative changes in the

MV gene products. In some SSPE virus strains, mutations in the M gene disrupt the translation reading frame (Cattaneo *et al*, 1986; Enami *et al*, 1989; Ayata *et al*, 1991). In others, the M proteins are mutated. These mutations can drastically alter the structure and functions of the M proteins. For example, the M protein of Biken strain is antigenically altered (Ayata *et al*, 1989), and the altered M protein does not bind to the viral nucleocapsids in Biken virus-infected cells or in a cell-free nucleocapsid-binding reaction (Hirano *et al*, 1992). This functional defect is common among the M proteins of SSPE virus strains (Hirano *et al*, 1993; Suryanarayana *et al*, 1994). Since M protein serves for virus assembly (Peeples, 1991), the M protein defects likely play a central role in the non-productive mode of MV replication in chronic CNS infection.

In addition to the M protein defects, mutations have also been found in the F proteins of SSPE virus strains (Billeter and Cattaneo, 1991). All the F proteins of SSPE virus strains analyzed to date show deletions or mutations in the carboxy-terminal region (Schmid *et al*, 1992). However, the F proteins expressed from cDNA clones from several SSPE cases do not show gross abnormalities in the cell fusion function (Cattaneo and Rose, 1993). Our present study shows that mutations in the F gene can delay cleavage activation of the F protein. We have tested whether the mature Biken F protein can induce cell fusion by transfection into cultured cells. Just as the F proteins of acute MV and other SSPE virus strains tested, the Biken F protein can induce cell fusion when it is coexpressed with the MV H protein (Korte-Sarfaty *et al*, unpublished data). Our findings provide interesting comparison with a recent study, which showed that a cell fusion-deficient MV strain neurotropic in hamsters produces mostly bicistronic M-F RNA impairing translation of the F protein, but the F protein itself of that MV strain is not defective in cell fusion function (Hummel *et al*, 1994). Therefore, the cell fusion function is conserved in MV strains that cause CNS infections, but the cell fusion activity may be down-regulated at a transcriptional, translational, or post-translational processing level. The down-regulation of cell fusion activity may be important for persistence of MV in the CNS.

The mechanism of delayed cleavage of the Biken $F_0$ is of interest. The MV $F_0$ is mainly cleaved by furin (Watanabe *et al*, 1995), a cellular endoprotease which recognizes the minimal consensus seqence Arg-X-X-Arg (Hatsuzawa *et al*, 1992; Molloy *et al*, 1992). The His (aa 113) in the cleavage recognition sequence Arg-His-Lys-Arg in the Biken $F_0$ has been mutated to Gln (Figure 4). We initially suspected that this mutation impairs cleavage of the Biken $F_0$. However, chimeric F protein containing the His-to-Gln mutation (chimera A) is cleaved as efficiently as the wild type F protein, whereas chimeras B, C, D, E, and J containing mutations far from the cleavage site are not cleaved efficiently (Figures 6 and 7). Therefore, delayed $F_0$ cleavage is due to multiple mutations in the extracellular domain of $F_1$ rather than a mutated cleavage recognition sequence. Notably, the mutated cleavage recognition sequence in the Biken F protein still conforms to the minimal consensus sequence recognized by furin. Previous studies showed that unglycosylated $F_0$ of MV synthesized in the presence of tunicamycin is not cleaved (Sato *et al*, 1988; Alkhatib *et al*, 1990) and mutations that destroy the glycosylation sites impair cleavage of the MV $F_0$ (Alkhatib *et al*, 1994). N-linked glycosylation is necessary for glycoproteins to interact with calnexin, a cellular chaperone in the ER which may play a role in glycoprotein folding (Doms *et al*, 1993; Hammond *et al*, 1994). However, we found no mutations at the predicted N-linked glycosylation sites, and the Nagahata and Biken F proteins acquire the same number of oligosaccharide side chains with essentially the same kinetics (Figures 1 and 2). Another possibility is that the Biken F protein may not be transported efficiently to the TGN where furin resides (Molloy *et al*, 1994). Some mutations in the cytoplasmic domain affect intracellular transport of the glycoprotein of vesicular stomatitis virus (Rose and Bergmann, 1983). However, chimera MV F protein with a truncated cytoplasmic region (chimera G) is cleaved efficiently (Figures 6 and 7), indicating the deletion in the cytoplasmic domain does not affect transport of the MV F protein to the TGN. Indeed, the uncleaved $F_0$ is transported to the plasma membranes of Biken-CV-1 cells (Hirano *et al*, 1992). Therefore, the most likely mechanism for the delayed cleavage of the Biken $F_0$ is that multiple mutations alter the conformation of $F_0$ and hinder access to the proteolytic cleavage site. Similarly, the four mutations near the transmembrane region of $F_1$ likely impair endo-H access to the oligosaccharide chain by influencing the protein conformation (Figures 2 and 3).

The role of the deletion at the carboxy-terminus of the Biken F protein (Figure 4) is presently unknown. Similar deletions and mutations have been found in the F proteins of other SSPE-associated MV mutants analyzed (Schmid *et al*, 1992). Sanderson and coworkers showed that both the F and M proteins of Sendai virus are retained in the perinuclear region when cells are subjected to low temperature or monensin treatment (Sanderson *et al*, 1993), and the F or H protein facilitates membrane association of the M protein (Sanderson *et al*, 1994). Whether this involves a direct interaction between the F and M proteins, and whether the carboxy-terminus of the F protein is involved in this interaction remains to be established. Finally, it should be pointed out that although *in vitro* expression systems such as the vaccinia virus vectors provide a convenient means to study the molecular

changes in the MV proteins, these systems may not reflect the environment in a chronic MV infection in human brain. In particular, we cannot rule out the possibility that host cell factors may also play a role in down-regulation of the fusion activity. It has been shown that the membrane cofactor protein CD46 which serves as a receptor for MV is required for MV-induced cell fusion (Naniche *et al*, 1993a; Dorig *et al*, 1993). In MV-infected cells, CD46 is down-regulated (Naniche *et al*, 1993b). Taken together, the accumulating evidence indicates that chronic infection by MV in the CNS is accompanied by profound changes in the virus–host interaction, which can be due to altered expression or functions of viral proteins involved in virus maturation, attenuation of cell fusion activity due to changes in the F protein, or down-regulation of cellular factors required for cell fusion.

## Materials and methods

*Cells and viruses*
African green monkey kidney CV-1 cells infected with Edmonston strain (Enders *et al*, 1962) and Nagahata strain MV (Wong *et al*, 1991), and Biken-CV-1 cells persistently infected with Biken strain SSPE virus (Ueda *et al*, 1975; Hirano, 1992) were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum. Recombinant vaccinia virus vTF7-3 (Fuerst *et al*, 1986) was propagated in human cervical carcinoma (HeLa) cells in MEM containing 5% newborn calf serum and non-essential amino acids.

*Cloning of F and H genes*
Full-length cDNAs of the F genes of Edmonston and Nagahata strains were synthesized from poly(A)$^+$ RNA from virus-infected CV-1 cells and cloned into the pCD vector (Okayama and Berg, 1983) as previously described (Wong and Hirano, 1986). These F cDNAs were subcloned into the pTZ18RX vector (Ayata *et al*, 1989). Full-length cDNAs of the Biken F gene were synthesized from poly(A)$^+$ RNA from clonal Biken-CV-1 cells by use of a F-specific primer and cloned directly into the pTZ18RX vector as previously described (Ayata *et al*, 1989).

*Construction of chimera F genes and sequence analysis*
Chimera F genes were constructed by swapping the different fragments of the Edmonston and Biken F cDNAs delineated by the restriction enzyme sites *Pvu*II, *Kpn*I, and *Pst*I (Figure 5). Sequence analysis of the Edmonston, Nagahata, and Biken F genes was carried out by the dideoxynucleotide-induced chain termination (Sanger *et al*, 1977) or double-stranded DNA sequencing method (Toneguzzo *et al*, 1988). Chimera F genes were confirmed by sequencing the regions including the junctions. All the cloned genes were tested for protein expression by *in vitro* transcription and translation in reticulocyte lysates before being used for transfection in cultured cells.

*Antisera*
The *Avr*II to *Sal*I fragment of the Edmonston F cDNA was inserted into the pATH vector to produce a trpE-F fusion protein containing the amino region of $F_1$. The trpE-F fusion protein was purified by gel electrophoresis and used to immunize a New Zealand White rabbit, to produce the F-AS antiserum as previously described (Wong *et al*, 1987).

*Expression of F protein by transfection*
Most transfection experiments were performed using the vaccinia virus-T7 RNA polymerase system (Fuerst *et al*, 1986). CV-1 cells were infected with the vTF7-3 recombinant vaccinia virus encoding the T7 RNA polymerase at a multiplicity of infection (MOI) of 10 or 20, and the cells were transfected with 10 or 20 μg of the various pTZ18RX-constructs by the calcium-phosphate precipitation method as described (Gombart *et al*, 1993). Some experiments were repeated by transfection with the pCD-constructs into COS cells, as described previously (Wong and Hirano, 1986).

*Metabolic labeling and protein analysis*
Virus-infected cells were labeled when 80–90% of the cells showed cytopathic effects, and transfected cells were labeled at 5 h post-transfection. Pulse-chase experiments were carried out by labeling the cells with [$^{35}$S]methionine (Express; NEN/Dupont) for 15 min, after which the cells were washed with medium containing 10 mM L-methionine and chased in conditioned medium for 0.5, 1,2,4 and 6 h. At each time point, cells were lysed in RIPA buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% sodium dodecyl sulfate (SDS)]. $^{35}$S-labeled proteins immunoprecipitated with the F-AS antiserum were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For digestion with endo-β-N-acetylglucosaminidase H (endo-H) (Boehringer Mannheim), the immunoprecipitated proteins were washed with water to remove SDS and then incubated at 37°C for 16 h in digestion buffer containing 60 mM sodium acetate pH 5.2, and 0.5 mM phenylmethylsulfonyl fluoride. The reaction was stopped by adding sample loading buffer. The bands on the gels were quantified with a PhosphorImager (Molecular Dynamics).

## Acknowledgements

We thank Greg Wipf for preparing the F-AS antiserum. This work was supported by research grants NS31427 from the National Institute of Health and VM-60G from the American Cancer Society.

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