IDENTIFICATION OF PEPLOMER CLEAVAGE SITE MUTATIONS ARISING DURING PERSISTENCE OF MHV-A59

James L. Gombold, Susan T. Hingley, and Susan R. Weiss

Department of Microbiology
University of Pennsylvania School of Medicine
Philadelphia, PA 19104-6076

ABSTRACT

Primary mouse glial cell cultures were infected with mouse hepatitis virus strain A59 (MHV-A59) and maintained over an 18 week period. Viruses isolated from these cultures 16-18 weeks postinfection produce small plaques on fibroblasts and cause only minimal levels of cell-to-cell fusion at times when wild type causes nearly complete cell fusion. However, when mutant-infected cultures were examined 24-36 hours postinfection approximately 90% of the cells were in syncytia showing that the fusion defect is not absolute but rather delayed. Addition of trypsin to mutant-infected cultures enhanced cell fusion a small (2- to 5-fold) but significant degree. Sequencing of portions of the spike genes of six fusion-defective mutants revealed that all contained the same single nucleotide mutation resulting in a substitution of aspartic acid for histidine in the spike cleavage signal. Mutant virions contained only the 180 kDa form of spike protein suggesting that this mutation prevented the normal proteolytic cleavage of the 180 kDa protein into the 90 kDa subunits. Examination of revertants of the mutants supports this hypothesis. Replacement of the negatively-charged aspartic acid with either the wild type histidine or a non-polar amino acid was associated with the restoration of spike protein cleavage and cell fusion.

INTRODUCTION

Mouse hepatitis virus strain A59 (MHV-A59) is a positive stranded enveloped virus with an approximately 31 kb RNA genome. Three structural proteins have been identified in MHV-A59 (1). One of these proteins, the peplomer or spike glycoprotein (S), is present on the surface of the virion and is responsible for binding to the cellular receptor. Antibodies directed at specific epitopes on S are capable of neutralizing viral infectivity. In infected cells, S is transported by the secretory system to the cell surface where it is free to interact with adjacent cells resulting in cell fusion (syncytia). Expression of S (via vaccinia virus vectors) in the absence of infection or other viral proteins is sufficient for the induction of efficient cell-to-cell fusion (2,3).

A portion of the spike in virions is cleaved into two 90 kDa fragments termed S1 (N terminal) and S2 (C terminal). In the case of MHV, the level of fusion observed correlates with the amount of cleaved S; thus, the cleaved protein rather than the precursor is believed to be fusogenic (4). Proteolytic cleavage of coronavirus spike proteins occurs adjacent to a sequence of basic amino acids on the C terminus of S1 (5). This motif is conserved among many MHV strains and in avian infectious bronchitis virus (IBV). Similar stretches of basic amino acids occur adjacent to the glycoprotein cleavage site in paramyxoviruses, many retroviruses, and influenza A viruses (6,7,8).

Coronaviruses, Edited by H. Laude and J.F. Vautherot
Plenum Press, New York, 1994

In this report, we present data showing that small plaque, fusion-defective mutants arise during persistence in primary murine glial cell cultures. Sequencing of the spike protein gene revealed that six mutants derived from two independent cultures contained the same histidine to aspartic acid substitution in the cleavage signal region. Analysis of virion structural proteins suggests that this mutation prevents proper proteolytic cleavage of the 180 kDa spike precursor. Evidence that the defect in cleavage is responsible for the fusion deficiency seen in infected fibroblasts comes from studies of revertants in which fusion, cleavage of the spike, and loss of the acidic aspartic acid residue coincide. These data confirm and extend previous reports concluding cleavage of S is required for efficient cell-cell fusion by MHV.

MATERIALS and METHODS

Virus and Cells

MHV strain A59, obtained from Dr. Lawrence Sturman (Albany, NY), was propagated in mouse L2 cells. The virus stock had been grown previously at 40C to eliminate any temperature-sensitive (ts) mutants that might be present.

Primary mixed glial cell cultures were made from dissociated brains of newborn C57BL/6 mice essentially as described (9) and were used 10-15 days after plating. These cultures were 90-95% astrocytes as determined by positive immunostaining for glial fibrillary acidic protein (data not shown). Primary glial cultures, L2 cells, and 17 Cl-1 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS).

Infection of Glial Cells and Isolation of Virus

Three independent glial cell cultures (A,B, & C) were infected with MHV-A59 at a multiplicity of infection (MOI) of 5 and maintained at 37C. The medium was removed twice weekly and the cells refed with fresh medium. Virus was isolated from the medium by three plaque-to-plaque purifications and then grown to high titer on L2 cells at 32C. Three mutants were isolated from culture B (B10, B11, B12) after 18 weeks of infection, and three from culture C (C10, C11, C12) after 16 weeks of infection. Fusion-competent revertants were isolated following serial, low-multiplicity passages of mutants on L2 cells by plaque-purification as above.

Virus Titrations

Plaque titration of virus was done on L2 cells and at 37C in DMEM containing 2% FBS. In some cases titrations were done at 39C & 32C to identify and quantitate ts mutants. Titration of virus in the presence of 10-100 ug/ml trypsin (Sigma) was done as described above except that cell monolayers were washed twice in Tris-buffered saline prior to infection and FBS was omitted from the medium.

Viral Proteins

For analysis of the spike glycoproteins in virions, L2 or 17Cl-1 cells were infected at an MOI of 5. At 5 hpi, the cells were incubated for 30 min in DMEM lacking serum and methionine and then labelled with 50 uCi/ml of [35S] TransLabel for 6 hours. Virus in the medium was collected by centrifugation and the pellets were lysed in RIPA buffer (50 mM Tris, pH 8, 100 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 100 ug/ml PMSF). The spike protein was immunoprecipitated from virion lysates using a polyclonal goat serum (AO4) (kindly provided by Dr. K. Holmes, Bethesda, MD). Lysates were incubated with antiserum for 60 min at 4C and the antigen-antibody complexes were then precipitated with *S. aureus* (Pansorbin, Calbiochem). The pellets were washed three times, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE.

Fusion Assay

L2 cells were infected with virus at an MOI of 5. The proportion of nuclei contained

in syncytia was determined as a function of time after infection by counting three random fields using ethanol-fixed crystal violet-stained cells.

To assess the effect of trypsin on cell fusion, the cells were infected as above except that serum was omitted from the growth medium. At selected times, the cells were washed three times with PBS and then treated for 30 min at 37C with 2.5 ug or 5 ug of trypsin per ml in PBS. Afterwards, the trypsin was removed, and the cells washed once and then fed with DMEM containing 10% FBS. The degree of fusion was determined after a 90 min incubation at 37C.

RNA Sequencing

For preparation of RNA templates, L2 cell monolayers in 50 mm plates were infected with A59 or the fusion defective mutants at an MOI of 5. At 8 hpi, the cells were washed three times on ice with ice-cold PBS and lysed in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40. The lysates were digested with 200 ug of Proteinase K (Boehringer Mannheim) per ml, extracted with phenol and phenol-chloroform, and precipitated with ethanol. RNA was quantitated by measuring adsorbance at 260 nm.

Sequence information was obtained both by direct sequencing of viral RNA and by sequencing DNA by PCR using cDNAs transcribed from the viral RNA. For direct RNA sequencing, cytoplasmic RNA (50-70ug) was sequenced using reverse transcriptase and dideoxynucleotides (BMB). Primer CSN (5'-CATCGGAGTGTATGGCTC-3'), complementary to sequence approximately 30 nt downstream of the cleavage signal region (nucleotides 2185-2202 in the sequence of Luytjes et al. (5)), was purchased from Operon Technologies. Sequencing products were treated with 10-20 U of terminal transferase (BMB) to avoid ambiguities due to "strong stops".

Sequencing of the S genes of wild type and mutant viruses was determined also from DNA fragments obtained by reverse transcription-polymerase chain reaction (RT-PCR). Cytoplasmic RNA was reverse transcribed using random hexamers (BMB) and Muloney MLV reverse transcriptase (Gibco BRL). PCR amplification of the cDNA templates with Taq polymerase (BMB) and six pairs of primers (obtained from Dr. K. Holmes) yielded 6 overlapping fragments approximately 700-800 nucleotides in length spanning the entire S gene. PCR products were purified using Promega's Magic PCR Prep, then sequenced using the Promega fmol DNA sequencing kit.

RESULTS AND DISCUSSION

We have reported previously (9,10) that while infection of murine fibroblasts with MHV-A59 causes extensive fusion of the cell monolayer, infection of primary glial cell cultures causes minimal if any cytopathic effect (cpe). To better understand virus-glial cell interactions, we have begun to examine the evolution of MHV during long-term infection in these cultures. We infected at high multiplicity three separate cultures of glial cells with A59. At weekly intervals the medium was removed from the cultures and stored at -80C, and the cells refed with fresh medium.

Infectious virus in the medium was titrated by plaque assay. All three cultures produced virus continuously over the 16 to 18 week period with levels of virus varying from 10^6 to 10^7 pfu per culture (data not shown). These titrations also showed that virus present at the latest times postinfection made small, non-lytic plaques compared to wild type (data not shown). For further studies, we plaque purified viruses from the culture supernates as described in Materials and Methods. Infection of L2 cells with the plaque purified mutants caused a productive infection but the cytopathic effect normally observed with A59 was markedly reduced. At 10 hpi, a time at which A59 typically causes extensive syncytia, cultures infected with the mutants displayed 5-10 percent cpe (Fig. 1). With the exception of C10 and C11, fusion in mutant-infected cells increased over the next 12-18 hours to include 90% of the cells. Syncytia in C10- and C11-infected cells involved less than 50% of the cells at 24 hpi and this lower level of fusion may be related in part to the temperature-sensitive phenotype of these mutants (data not shown; see below). It is important to note that the mutants are not absolutely defective in their ability to induce fusion, but rather, induce fusion with slower kinetics than wild type A59.

The small plaque morphology and the delayed appearance of fusion was not due to poor growth kinetics of the clones, which, except for C10 and C11, were indistinguishable

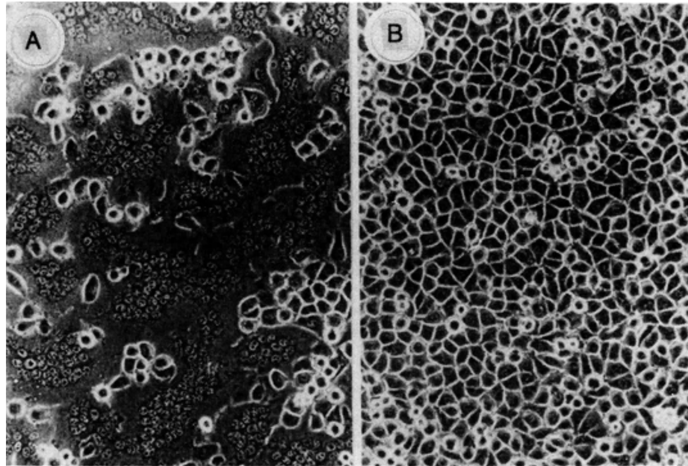

Figure 1. Mutants from persistently-infected glial cell cultures are fusion defective. Wild type A59 (A) or mutant B10 (B) were used to infect L2 cells and photographed at 10 hpi. Unlike wild type, the mutant induces minimal cytopathic effect (syncytia).

from wild type (data not shown). Furthermore, immunofluorescence staining of mutant infected cells suggested that the delayed fusion was not a result of a delay in the appearance of cell-surface spike.

Since fusion of cells during infection appears to correlate with the extent of cleavage of the spike protein (4), we addressed the possibilty that the mutants were defective in cleavage of the 180 kDa spike precursor to the 90 kDa S1 and S2 subunits. Cells were infected with either wild type or mutants at high multiplicity, and at various times after infection, the cells were incubated for 30 min in the presence of 2.5 or 5.0 ug/ml trypsin. All mutants except C10 and C11 induced higher levels of fusion in the presence of trypsin than in control cultures (data not shown). However, the increase in fusion observed in cells treated with 5 ug of trypsin per ml, though statistically significant, was generally 5-fold or less and never more than 30% of the cells fused. In addition, a wild type plaque morphology is observed when the mutants are assayed in the presence of 30 ug/ml trypsin.

Together, these data showed that trypsin was able to at least partially restore the fusion phenotype of the mutants, suggesting that the mutant spike protein was not being proteolytically cleaved into S1 and S2 subunits. To examine this directly, cells were infected with wild type or the mutants and labeled with [35S] TransLabel from 6 to 12 hpi. Virions were pelleted from the medium and the spike protein was immunoprecipitated from these virions and analyzed by SDS-PAGE. The spike protein in wild type A59 was present in both the 180 kDa and the 90 kDa forms (Fig. 2). In contrast, the mutant virions contained only the uncleaved 180 kDa spike precursor.

To better understand the nature of the defect in these mutants, we directly sequenced the RNA encoding the S1/S2 junction in several of the clones. The sequence obtained for wild type A59 was identical to that previously published by Luytjes et al. (5). Shown in Table 1 is the highly basic amino acid sequence directly upstream from the S2 amino terminus that is thought to act as a signal for proteolytic cleavage in wild type MHV. In all six mutants, a single nucleotide change at nucleotide 2146 was observed that caused a substitution of aspartic acid for histidine. The introduction of a negatively-charged amino acid into this highly basic region may mask the signal or prevent or otherwise interfere with its usage and thereby prevents cleavage of the spike.

To identify additional mutations in the spike genes of the mutants that may effect the fusion phenotype, we sequenced in entirety gene 3 from wild type and mutants B11 and C12 as described in Materials & Methods. Wild type S varied by two nucleotides and one amino acid from the published sequence. One mutation, a A to G transition at nucleotide 3045 was silent; the other was a G to A transition at nucleotide 293 which substitutes a serine for an asparagine in S1 (S.T.H., unpublished observation; C. Ricard and L.

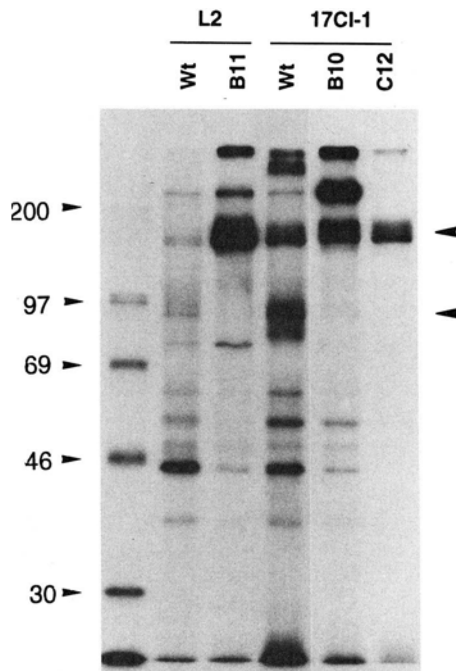

Figure 2. The 180 kDa spike protein in fusion defective mutants is not cleaved. L2 or 17Cl-1 cells were infected with wild type A59 or the fusion mutants and labeled with [35S] TransLabel. Radiolabelled virions in the medium were pelleted and the spike protein immunoprecipitated. The 90 kDa spike protein subunits are detectable only in wild type infected cells. Arrows on the right indicate the position of the 180 kDa spike precursor (upper arrow) and the 90 kDa cleavage products (lower arrow).

Table 1. Predicted amino acid sequence of the spike cleavage signal in wild type A59 and the fusion mutants.

Virus	Cleavage Signal	Fusion(a)	Cleavage of S
Wild type	RRAHR	+	+
B10	RRADR	-	-
B11	RRADR	-	-
B12	RRADR	-	-
C10	RRADR	-	-
C11	RRADR	-	-
C12	RRADR	-	-
B11R1	RRADR	-	-
C11R1	RRAAR	+	+
C12R1	RRAHR	+	+
C12R3	RRAGR	+	+

(a) Cell fusion measured at 10 hpi

Sturman, personal commmunication). The B11 and C12 mutant spike genes were identical to wild type with the exception of the cleavage signal mutations described above and an additional mutation at nucleotide 476, a Gln to Leu substitution in S1. As described below, this latter mutation does not appear to be related to the fusion phenotype of the mutants.

To test the correlation between fusion competence, cleavage of S and the sequence in the putative cleavage signal, fusion-competent revertants of mutants C11 and C12 were isolated based on their wild type plaque morphology. Infection of cells with these viruses

induces similar cytopathic effect when compared to wild type infected cells (data not shown). Immunoprecipitation of the spike from revertant virions detected both the uncleaved precursor and the cleavage subunits (Table 1). In contrast, one clone (B11R1) that was isolated but retained the mutant plaque phenotype did not revert in fusion phenotype. Cleavage of the B11R1 spike could not be detected by immunoprecipitation.

Sequence analysis of the revertants in the region of the cleavage site revealed that restoration of cleavage was associated with the loss of the aspartic acid residue seen in the mutants (Table 1). One clone reverted to the wild type histidine residue, while the others substituted the aspartic acid with a small non-charged amino acid. The Gln to Leu mutation in subunit S1 of the mutants S1 was retained in the revertants (data not shown) suggesting that this mutation is not associated with the fusion phenotype. We conclude that efficient fusion requires cleavage of the spike protein, and that alterations in the cleavage signal, such as the incorporation of negatively-charged amino acids, can prevent its recognition by the (cellular) protease. These results support a previously published report that efficient fusion by MHV-A59 requires cleavage of S (4) and suggest that the fusion-defective phenotype of the mutants is a direct result of the inablility of the spike protein to be processed into its mature form.

We report here the characterization of viruses isolated from persistent infections of murine glial cells. In general, these viruses display normal growth kinetics in murine fibroblasts and achieve titers similar to the parental A59 virus from which they were derived. However, fusion in mutant-infected fibroblasts is markedly delayed and plaques are small and non-lytic. Two clones, C10 and C11, are slightly different. These mutants are temperature-sensitive and replicate with slower kinetics at 37C. However, even though they achieve titers comparable to the other mutants, they do not induce the same level of fusion in cells, even if the assays are done at 32C (the permissive temperature for C10 and C11). Although this suggests that the fusion defect is not due directly to the ts defect, the slow growth kinetics of C10 and C11 may indirectly affect the level of fusion in infected cells.

All six mutants that were examined contain a histidine to aspartic acid substitution within the basic amino acid cleavage signal thought to be responsible for the proper proteolytic processing of the peplomer. Analysis of the spike protein present in virions shows that these fusion mutants do not contain significant amounts of cleaved spike, but rather incorporate the uncleave 180 kDa precursor. Restoration of fusion-competence in revertant viruses is associated with the replacement of the acidic aspartic acid residue with either the wild type histidine or a non-charged amino acid. We suggest that incorporation of aspartic acid within the dibasic signal prevents cleavage either by preventing its recognition by or its interaction with the protease responsible for cleavage. Sawicki (11) isolated from persistently-infected 17Cl-1 cells a small plaque mutant of MHV-A59 that is similar to the mutants described here. Purified virions of this mutant contained only the uncleaved 180 kDa spike protein; furthermore, 100-fold more trypsin was required to convert the 180 kDa protein to the 90 kDa form. Interestingly, this mutant contains a substitution of the histidine residue in the cleavage signal for an asparagine (S. Sawicki, personal communication).

Two other classes of fusion defective mutants of MHV have been described previously. Gallagher et al. (2) showed that a mutants of MHV-4 recovered from a persistently-infected neural cell line contained mutations within the heptad repeats of the S2 subunit of the spike protein. The heptad repeat regions are believed to be important in the oligomerization of protein monomers (12). These mutations caused a change in the pH-dependence of fusion such that an acid pH was required by the mutant to induce syncytia in infected cells. Gallagher et al. (13) also reported that neutralization-resistant mutants of MHV-4 selected with a monoclonal antibody to S were defective in their ability to induce syncytia. These mutants contain deletions in the S1 subunit in the "hypervariable" region (13,14). A JHM isolate from the spinal cord of a demyelinated rat (15) was also found to contain a deletion in S1 (16). While the precise mechanism by which any of these mutations effect fusion in these MHV-4 variants in unknown, the identification of these variants clearly demonstrates that different regions in the protein are crucial for proper function of the protein. Here, we have identified a third region of S, the cleavage signal region, that affects efficient cell-to-cell fusion by the spike.

Proteolytic processing of envelope proteins is common in many viruses, and for some is a requirement for cell-to cell-fusion as well as virus-cell fusion or infectivity. The fusion proteins of paramyxoviruses, orthomyxoviruses and retroviruses are cleaved into two subunits by cellular proteases (6,7,8) and, like MHV, the site of cleavage is preceded

by a group of basic amino acids. In paramyxoviruses, cleavage of the fusion protein, F0, apears to be most efficient when at least 4 of the 5 amino acids in the cleavage signal are basic (7). The importance of cleavage of F0 stems from the observation that processing of the fusion protein is necessary for infectivity and virulence, showing that mutations within the signal sequence can be attenuating. Cleavage of the hemagglutinin of influenza virus also is influenced by the content of basic amino acids near the cleavage site (17) and is associated with virulence (6). The coronaviruses differ from these other classes of viruses in some ways: (1) Cleavage of the spike protein of porcine transmissible gastroenteritis virus and feline infectious peritonitis virus does not occur. Since these viruses cause fusion of infected cells, it is clear that cleavage of S is not a strict requirement for fusion for all coronaviruses. However, while the mutations within the MHV cleavage signal do not prevent fusion, they do appear to reduce the efficiency of fusion. (2) Cleavage of the paramyxovirus, myxovirus and retrovirus fusion envelope glycoproteins exposes a hydrophobic fusion peptide at the newly generated amino terminus; this is not true of the cleaved coronavirus spike protein. A hydrophobic fusion peptide has not yet been conclusively identified for any of the coronaviruses. Furthermore, the mutants described here do not cleave the spike protein, but are still infectious in cultured cells and cause a significant amount of cell-to-cell fusion at late times after infection. The phenotype of the mutants described here suggest that cleavage of S is a prerequisite for efficient cell-to-cell fusion. The fusion observed following prolonged incubations could be due to low and undetectable levels of cleaved S that accumulate over time on the cell surface. Alternatively, fusion could be due to high levels of uncleaved spike that inefficiently induces cell fusion.

ACKNOWLEDGMENTS

We thank Ms Marianne Allessio and Ms Xiurong Wang for technical assistance, Dr. Ehud Lavi for helpful discussions and Dr. K. Holmes for the AO4 antiserum. This work was supported by Public Health Service Grant NS-21954. JLG and STH were supported in part by training grant NS-07180 and NS-11037.

REFERENCES

1. Spaan, W. J. M., Cavanagh, D., Horzinek, M. C. J. Gen. Virol. 69:2939-2952, 1988.
2. Gallagher, T. M., Escarmis, C., Buchmeier, M. J. J. Virol. 65:1916-1928, 1991.
3. Vennema, H., Heijne, L., Zijderveld, A, Horzinek, M. C., Spaan, W. J. M. J. Virol. 64:339-346, 1990.
4. Frana, M. F., Behnke, J. N., Sturman, S., Holmes, K. V. J. Virol. 56:912-920, 1985.
5. Luytjes, W., Sturman, L., Bredenbeck, P. J., Charite, J., van der Zeijst, B. A. M., Horzinek, M. C., Spaan, W. J. M. Virology 161:479-487, 1987.
6. Bosch, F. X., Garten, W., Klenk, H. D., Rott, R. Virology 113:725-735, 1981.
7. Glickman, R. L., Syddall, R. J., Iorio, R. M., Sheehan, J. P., Bratt, M. A. J. Virol. 62:354-356, 1988.
8. Perez, L. G., Hunter, E. J. Virol. 61:1609-1614, 1987.
9. Gombold, J.L., Weiss, S.R. Microb. Path. In Press.
10. Lavi, E., Suzumura, A., Hirayama, M., Highkin, M. K., Dambach, D. M., Silberberg, D. H.,Weiss, S. R. Microb. Path. 3:79-86, 1987.
11. Sawicki, S. G. Adv. Exp. Med. Biol. 218:169-174, 1987.
12. DeGroot, R. J., Luytjes, W., Horzinek, M. C., van der Zeijst, B. A. M., Spaan, W. J. M., Lenstra, J. A. J. Mol. Biol. 196:963-966, 1987.
13. Gallagher, T. M., Parker, S. E., Buchmeier, M. J. J. Virol. 64:731-741, 1990.
14. Parker, S.E., Gallagher, T. M., Buchmeier, M. J. Virology 173:664-673, 1989.
15. Morris, V.L., Tieszer, C., MacKinnon, J., Percy, D. Virology 169:127-136, 1989.
16. LaMonica, N., Banner, L., Morris, V. L., Lai, M. M. C. Virology 182:883-888, 1991.
17. Kawaoka, Y.,Webster, R. G. Proc. Natl. Acad. Sci. 85:324-328, 1988.