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VIROLOGY 182, 883–888 (1991)

Localization of Extensive Deletions in the Structural Genes of Two Neurotropic Variants of Murine Coronavirus JHM

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Received October 29, 1990; accepted March 8, 1991

The intracellular RNA of two neurotropic variants of the JHM strain of mouse hepatitis virus (MHV) independently isolated from the brain and spinal cord of an infected Wistar Furth rat were compared with that of the parental virus. The mRNAs corresponding to the genes encoding the peplomer (S) and the hemagglutinin-esterase (HE) proteins of the variant viruses were found to be smaller in size. The possible sequence changes were studied by oligonucleotide fingerprinting and direct RNA sequencing. Both variants have a large deletion of 246 amino acids in the carboxy-terminal end of the HE protein. However, this truncated protein was not detected in the infected cells, suggesting either a translational regulation or rapid degradation of the truncated protein in these cells. The variant virus isolated from the spinal cord has a second deletion of 147 amino acids in the amino-terminal half of the S protein. This deletion site corresponds to a hypervariable region where deletions have been frequently noted among MHV variants with different biological properties. These findings suggest that the changes in pathogenic properties of the two neural isolates are associated with drastic alterations of the viral structural glycoproteins. © 1991 Academic Press, Inc.

Mouse hepatitis virus (MHV), a member of the Coronaviridae, contains a nonsegmented positive-stranded RNA genome of 31 kb (*1–3*). The RNA genome is enclosed in a helical nucleocapsid structure formed by association with nucleocapsid proteins (N). The virions are enveloped and contain three virus-specific glycoproteins of 180 (S), 65 (HE), and 23 kDa (M) (*4, 5*). The S (spike) glycoprotein forms the projecting peplomers of the virus, contains neutralizing epitopes, binds target cells, and is required for viral infectivity (*4*). M is a transmembrane protein (*6*), the function of which is not yet clear. The HE glycoprotein contains an esterase activity (*7, 8*) and shares sequence and functional homology with the influenza C hemagglutinin (*9*). Its expression appears to be optional in murine coronaviruses. In bovine coronavirus (BCV), the HE protein has been found to contribute to viral infectivity (*10*).

The structural proteins of MHV are translated from virus-specific subgenomic mRNAs in the infected cells. These mRNAs have a 3′-coterminal nested-set structure (*11, 12*), and only their 5′-end terminal unique regions, which include more than one open reading frame in some mRNAs, are utilized for translation (*13, 14*). The S protein is translated from mRNA 3, HE from mRNA 2-1, which is synthesized only by certain strains of MHV (*5*), and M from mRNA 6. The 5′-end of the genomic RNA of MHV contains a leader sequence of approximately 70 nucleotides (*15, 16*), which is also present at the 5′-end of every subgenomic RNAs. At the 3′-end of the leader RNA, there are several repeats of a pentanucleotide sequence UCUAA, whose repeat number varies among different MHV strains (*17*). The number of UCUAA repeats appears to correlate with the efficiency of transcription of specific MHV genes. For instance, the JHM(3) strain of MHV, which contains three UCUAA repeats at the 3′-end of the leader RNA, makes only a small quantity of HE protein. In contrast, the JHM(2) strain with two UCUAA repeats makes a large quantity of this structural glycoprotein (*5, 17*).

Among many strains of MHV, JHM is of particular interest because of its capacity to cause neurological diseases in murine species (*18–20*). Intracerebral (ic) or intranasal inoculation of susceptible mice and rats with the JHM virus often results in acute, subacute, and chronic infections of the central nervous system (CNS). The symptoms of these infections depend on the age of the animal, and range from encephalitis, associated with lesions in the gray matter of the CNS, to demyelination, characterized by hindleg paralysis or paresis (*18, 21, 22*). In view of the interesting pathogenic properties of the JHM virus, extensive studies have been undertaken to understand the genetic basis for MHV-induced neurological diseases. Studies on monoclonal antibody-resistant MHV variants and on variants isolated from persistent infections have shown that mutations in the peplomer protein S are associated with marked variations in disease patterns of these virus isolates, suggesting that this structural gly-

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0042-6822/91 $3.00

coprotein plays an important role in the establishment of coronavirus infection and pathogenesis (*23, 24*). The M protein may also play a role in the development of viral disease since several monoclonal antibodies against this protein can alter the pathogenicity of the virus (*25*). The role of HE protein in MHV pathogenesis has not been studied.

Recently, two JHM variant viruses At11f have been isolated from the brain and the spinal cord, respectively, of a single Wistar Furth rat with a JHM-induced demyelinating disease. The virus isolated from the spinal cord was named At11f cord, while the other, At11f brain, was independently isolated from the brain of the same rat (*26*). The At11f cord variant differs from the parental JHM virus in its pathogenic properties in that it induces a chronic demyelinating disease in Wistar Furth rats when inoculated intracerebrally, independently of their age. Also, unlike the parental JHM virus, the At11f cord variant forms massive syncytia and replicates extensively in the murine oligodendroglioma cell line G26-24. In contrast, the At11f brain isolate produced an acute encephalitis when inoculated into 2- and 10-day-old Wistar Furth rats. Both the brain and the spinal cord isolates displayed a different pattern of virus-specific mRNAs from the parental JHM virus in the infected cells (*26*). However, the brain isolate did not replicate as well as the At11f cord variant in the G26-24 cell line and did not form syncytia (*26*). The parental JHM virus and the At11f cord virus were plaque-purified three times and passaged less than ten times in L-2 cells. Both the plaque-purified and unpurified stocks of the At11f brain variant were used in this study. The unpurified At11f brain variant used had been passaged in G26-24 and L-2 cells less than ten times, and had the same passage history as the one used previously for its biological characterization (*26*).

To obtain a better understanding of the genetic changes that may contribute to the altered biological and pathogenic properties of the At11f brain and cord isolates, we have begun to characterize the genomic structure of these viruses. In this paper, we have identified large deletions within the genes encoding the S and HE proteins of these variant viruses.

To examine the virus-specific RNA patterns of these variants and the parental JHM strain, virus-infected DBT cells treated with actinomycin D (2.5 μg/ml) were labeled with [^{32}P]orthophosphate between 5 and 9 hr p.i. At the end of this period, RNA was extracted from the infected cells, denatured, and analyzed by gel electrophoresis according to the published method (*27*). Because some mRNAs of the parental JHM virus were synthesized only in a very small quantity, intracellular RNA from another well-characterized MHV, JHM(2), was included for comparison. JHM(2) was derived from

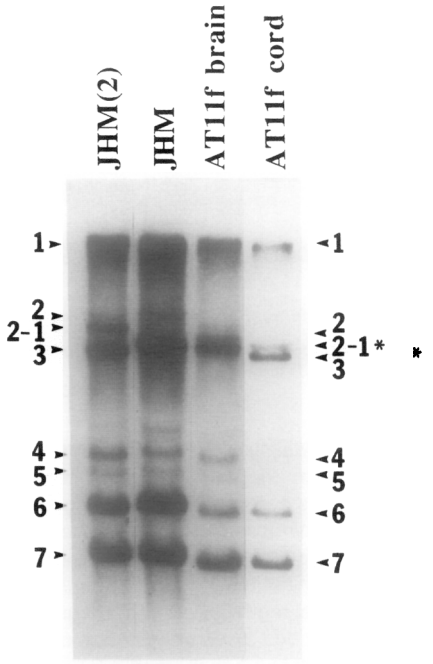

FIG. 1. Electrophoretic analysis of virus-specific intracellular RNAs of MHV strains JHM(2), the parental JHM and the neural isolates At11f brain and cord. The viruses were grown in mouse astrocytoma cell line DBT (*34*) and labeled with [^{32}P]orthophosphate between 5 and 9 hr p.i. in the presence of actinomycin D (2.5 μg/ml). RNA was then extracted from the infected cells, denatured with glyoxal and DMSO, and analyzed by gel electrophoresis on a 1% agarose gel as described (*27*). This photograph is a composite of different time exposures of the same gel.

a JHM strain maintained in laboratories in Japan (*17, 29*). As shown in Fig. 1, all the virus strains examined synthesized seven to eight mRNA species in varying quantity. The size of several of these mRNAs appeared different; most notably, the mRNA 3 of the At11f cord isolate was smaller than those of the parental JHM strain and of the brain isolate but similar to that of JHM(2), which has a deletion of 423 nucleotides in the S gene (*28*). In addition, the sizes of mRNA 2 and mRNA 2-1 of both neural isolates were smaller than those of the corresponding mRNA synthesized by the JHM(2) strain; the parental JHM virus synthesizes only negligible quantity of mRNA 2-1 (see below). As expected from the nested-set structure of MHV mRNAs, the mRNA 2 of the parental JHM strain is larger than that of JHM(2) because of the presence of the deletion in gene 3 of JHM(2) (*28*). The amounts of mRNA 2 in both neural isolates were low; however, a longer exposure clearly showed that mRNA 2 of both neural isolates was smaller than that of the parental JHM strain (data not shown). This result suggests that there are internal deletions in genes 2-1, 3, and possibly also gene 2 of the neural JHM variants. However, these size differences could also be due to aberrant transcrip-

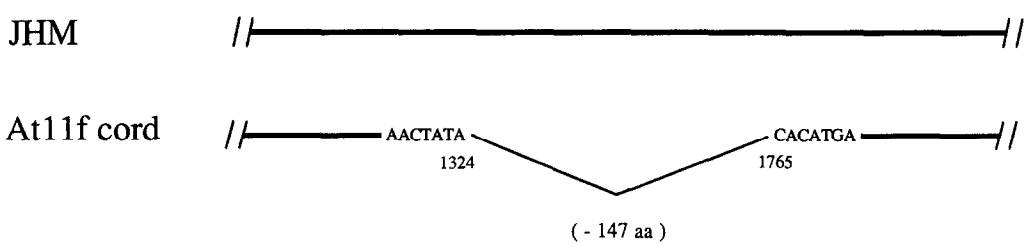

FIG. 2. Diagrammatic representation of gene 3 of the parental JHM and of the At11f cord isolate. The deleted region is represented by the thin line, the neighboring nucleotides are indicated. Nucleotides are numbered according to Banner *et al.* (*28*) and Parker *et al.* (*31*).

tional initiation. Furthermore, because MHV mRNAs have a nested-set structure, the genetic alterations affecting the size of mRNA 2 could be the result of deletions or insertions in the coding sequences of mRNAs 2-1 or 3.

To characterize the structure of mRNA 3 of the At11f cord isolate, we first determined by T1-oligonucleotide fingerprinting analysis whether the coding region and leader sequence of mRNA 3 of this variant virus contained any missing or altered sequence. The oligonucleotide map showed that the cord isolate lacked oligonucleotide spots numbered 4, 9, and 19, which have previously been mapped contiguously to the middle portion of this gene (*27*) (data not shown). This result suggests that the smaller size of mRNA 3 of At11f cord isolate is most likely caused by a deletion in the coding sequence of gene 3. The site of the deletion was determined by direct sequencing (*28*) of the purified genomic RNA of both the parental JHM virus and the variant viruses. After identifying the approximate site of the deletion by preliminary sequencing, the region from nucleotides 842 to 1830 of gene 3 was completely sequenced. Figure 2 shows that, when compared with the sequence of the parental JHM, gene 3 of the variant virus has a deletion of 441 bases extending from nucleotide 1324 to 1765. The reading frame downstream of the deletion remains unaltered. The rest of the gene 3 sequence is identical with that of the parental virus. Therefore, the smaller size of mRNA 3 of the At11f cord variant is the consequence of a deletion within the coding sequence of gene 3. The loss of 441 nucleotides from base 1324 to 1765 of the gene 3 sequence results in a smaller peplomer protein lacking an internal 147 amino acids.

We next examined the size variation of mRNA 2-1. When compared with JHM(2), which makes a large quantity of mRNA 2-1 (*5*), both the brain and spinal cord isolates appeared to have a smaller mRNA 2-1 (Fig. 1). However, because of the nested-set structure of MHV mRNAs, it could not be determined with certainty whether the coding region of gene 2-1 had any deletion, since the size comparison of mRNA 2-1 was complicated by the size differences in mRNA 3 between these viruses. We therefore performed PCR amplification of the gene 2-1 of the parental JHM and the two variant viruses, using two specific primers corresponding to both ends of the gene. The results showed that the gene 2-1 of the parental JHM has exactly the same size as that of the published JHM-2 (Fig. 3) (*5*). In con-

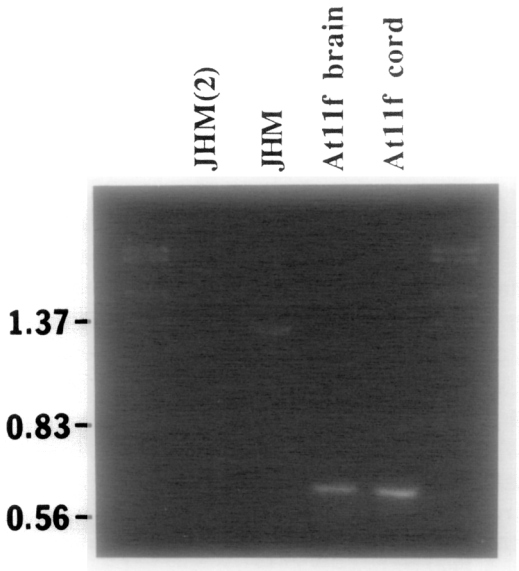

FIG. 3. PCR amplification of the gene 2-1 of MHV strains. RNA from MHV-infected DBT cells was extracted at 9 hr p.i. as previously described (*27*) and used for cDNA synthesis and PCR amplification according to the protocol of Fuqua *et al.* (*35*). Briefly, 2 μg of intracellular RNA were resuspended in 100 μl of reaction buffer containing 3 m*M* $MgCl_2$, 10 m*M* Tris–Cl (pH 8.4), 50 m*M* KCl, 0.01% gelatin, 0.5 m*M* each of the four deoxyribonucleoside triphosphates, and 100 ng each of primer 226 and 229R. Oligo-226 (5′-CTAACACCGCTATTCCGTCAT-3′) is complementary to nucleotides 1309–1328 of gene 2-1. Oligo-229R (5′-CGTACCGTATGCAGAATGAAG-3′) is homologous to nucleotides 781–801 of gene 2 located 36 nucleotides upstream from the intergenic site of gene 2-1 (*5*). After an initial denaturation at 94° for 2 min, 20 units of AMV reverse transcriptase (Seigakaku) were added and the samples were incubated at 45° for 30 min. The cDNA products were then amplified by addition of 2.5 units of *Taq*I DNA polymerase (Perkin–Elmer–Cetus, Norwalk, CT). Each cycle of amplification consisted of a 1.5-min denaturation at 94°, followed by 2-min annealing at 58°, and 3-min extension at 72°. After 35 cycles, the final product was extended for 10 min. 20-μl aliquots of the PCR reaction were electrophoresed on a 1.4% agarose gel.

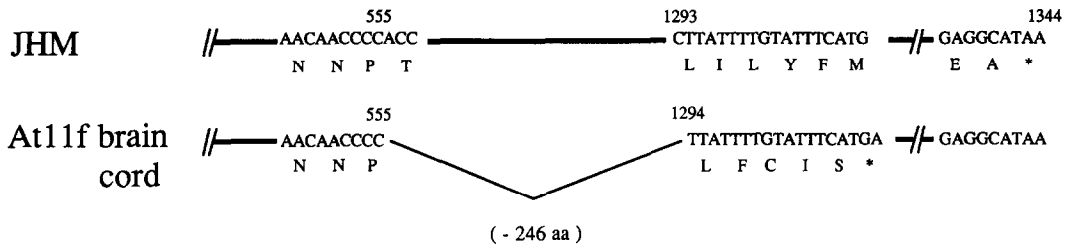

FIG. 4. Diagrammatic representation of the structure of the gene 2-1 of At11f brain and cord isolates. The deleted region (nucleotides 555 to 1294) is represented by a thin line, and the neighboring nucleotides are indicated. The translation of the ORF is shown in a single letter code. Nucleotides are numbered, starting from the first nucleotide of the intergenic site of gene 2-1 (*5*).

trast, the PCR-amplified gene 2-1 of both variants are smaller than that of the parental JHM, suggesting that the gene 2-1 of these two variants has a deletion. The sites of the deletion were determined by DNA sequencing of the PCR products and also direct RNA sequencing of the genomic RNAs. The results showed that both variants contain a deletion of 739 nucleotides extending from nucleotide 555 to 1294 of gene 2-1, which translates into a deletion of 246 amino acids in the gene 2-1 of both viruses (Fig. 4). Furthermore, because the reading frame was changed after the deletion, the ORF of this gene in both variant viruses has a capacity to encode a protein of only 182 amino acids. This truncated protein product would be expected to lack the carboxy-terminal transmembrane domain.

The finding that the parental JHM did not synthesize an appreciable amount of mRNA 2-1, while both variants do (Fig. 1), is reminiscent of our previous finding with JHM(2), in which there is a strong correlation between the amount of mRNA 2-1 and the number of UCUAA repeats in the leader RNA (*5*). Therefore, the leader sequences of these viruses were determined. The results showed that the parental virus has three UCUAA repeats while the variants have two (data not shown). This result is in agreement with the previous findings concerning the transcriptional regulation of this gene (*5, 17*).

To determine the size of the proteins encoded by the structural genes of these variants, we examined the intracellular virus-specific proteins. Infected cells were pulse labeled with ^{35}S-Translabel (Amersham) for 15 min, and immunoprecipitated with various antibodies. Figure 5A shows that the S protein of the At11f cord variant is smaller than that of the JHM parental virus, but similar in size to that of the JHM(2), which has a deletion of 153 amino acids (*28*). This result is consistent with the presence of a deletion in the S gene of the At11f cord virus. Figure 5A also shows that the JHM parental virus produced a small amount of HE protein, which has the same size as that produced by the JHM(2) virus, indicating that the mRNA 2-1 of the parental JHM virus does not contain a deletion. In contrast, neither of the two neural variants produced this protein. The reason for the failure to detect the HE protein in the variant viruses is not yet clear. This could be due to rapid degradation of the truncated proteins or the failure to initiate translation. We have also examined whether a truncated protein immunoprecipitable with anti-HE antibodies could be detected in purified virions or infected cell media since the truncated protein is expected to lack a C-terminal transmembrane domain; no protein was detected (data not shown). All of the viruses examined synthesized a 30-kDa polypeptide, which was precipitated by an antiserum prepared against the TrpE–MHV fusion protein containing 118 amino acids of the predicted gene 2 product (Fig. 5B). Thus, all of these viruses have an intact gene 2; the differing size of the mRNA 2 in these viruses is due to deletions in the downstream genes.

These results showed that the neural variants have undergone multiple changes from the parental virus. Both of them have a large deletion of 739 nucleotides in the HE gene, and the cord variant had an additional deletion of 441 nucleotides in the S gene. In addition, both of them have two UCUAA repeats at the 3′-end of the leader RNA, resulting in the high expression of mRNA 2-1, in contrast to the parental virus, which has three UCUAA repeats and, thus, a small amount of mRNA 2-1. However, the truncated gene product (HE) of mRNA 2-1 was not detected in cells infected by either of the variant viruses, while the truncated S protein was expressed by the At11f cord variant (Fig. 5). It is possible that these changes have contributed to the changes of biological and pathogenic properties of these viruses.

Variation in the size of the S glycoprotein has been observed during both *in vivo* and *in vitro* passages of MHV. The previously published JHM sequence (*30*) has been shown to lack 423 nucleotides in the same region of the S gene as the At11f cord variant, although the exact deletion sites are slightly different (*28, 31*). A59 virus and several other MHV isolates are also missing from 156 to 474 nucleotides in the same area (*28*). Thus, this region appears to be prone to deletions

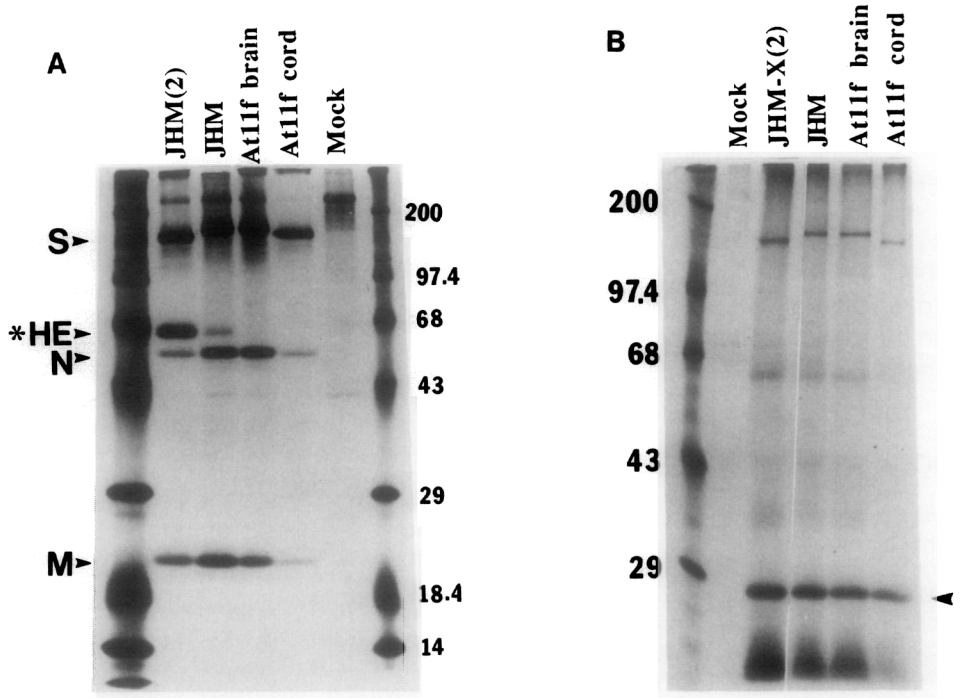

FIG. 5. Immunoprecipitation of MHV-specific intracellular proteins. Virus-infected cells were radiolabeled with ^{35}S-Translabel at 9 hr p.i. for 15 min. Cell lysates were prepared as previously described (*7*), immunoprecipitated according to the protocol of Shin and Morrison (*36*), and analyzed by electrophoresis on a 7.5 to 15% gradient polyacrylamide gel containing 1% SDS. (A) Immunoprecipitation with anti-JHM(2) rabbit antiserum (*7*). (B) Immunoprecipitation with anti-p30 rabbit antiserum. Arrow indicates the immunoprecipitated p30 protein.

under a variety of different conditions. Parker *et al.* (*31*) have reported similar deletions in the S protein of several JHM variants which had been selected for resistance to neutralization with monoclonal antibodies (*23*). These viruses also display altered pathogenicity in mice similar to that of the At11f cord isolate. Also, Taguchi *et al.* have reported the isolation, from both the brain of Lewis rats and primary rat neuronal cultures, of neurotropic JHM variants, which have a larger mRNA 3 (*32, 33*). Thus, the variation of the sequence in this region of the S gene may be related to the alteration of the pathogenicity of these viruses.

The deletion observed in the HE protein of MHV has not previously been described. We have reported the increased synthesis of the HE glycoprotein after *in vitro* passages, which correlated with the decrease in the number of UCUAA repeats at the 3′-end of the leader (*5*). Here we observed a similar variation of the UCUAA repeat number during the passage of the virus in the CNS of rats. However, the HE protein was not synthesized as a result of the deletions. It is unlikely that the observed deletions and increased mRNA expression in the neural variants originated from the passaging of these viruses in tissue culture after isolation. In fact, no change in the biological and physical properties of the viruses has been observed during passage of these neural isolates in L-2 and G26-24 cells. Furthermore, we have not detected any changes in the RNA pattern and protein profile of the parental JHM virus after passaging under our culture conditions.

It should be noted that the At11f brain isolate initially used in this and a previous study (*26*) was not cloned because of technical reasons. However, the HE sequence reported here was obtained by direct sequencing of genomic RNA, suggesting that the major population of this variant virus is characterized by a deletion in the HE glycoprotein. We have now confirmed this deletion with several plaque-purified At11f brain isolates (data not shown). Furthermore, the fact that both the At11f brain and cord variants have an identical HE sequence suggests that this HE deletion was selected early during JHM infection of the rat (*26*).

The biological significance of this deletion in gene 2-1 is more difficult to assess since the gene in both variants probably is not expressed, and these viruses are characterized by strikingly different biological and pathogenic properties. However, the variation in the expression of HE glycoprotein has been associated with the changes in the pathogenic properties of the virus. The JHM(2) virus, which expresses a larger amount of the HE protein, is more neurovirulent than, but does not replicate as well in glial cells as the JHM(3) virus, which synthesizes a small amount of HE (Yokomori *et al.*, unpublished data). Thus, the amount of this

structural glycoprotein may in some way influence the viral pathogenicity, depending on the genetic background of the virus.

Whether the truncated mRNA 2-1 of both the At11f brain and cord variants is actually translated *in vivo* is not clear at this time. The sequence analysis of the truncated 2-1 gene suggests that the mRNA 2-1 should be functional. However, the failure to detect such a protein in infected cell lysates may be due to a short half-life of the protein product. Alternatively, there is a possibility that the translation of this gene may be regulated by additional factors in the virus-infected cells.

ACKNOWLEDGMENTS

We thank Lee Tan for helpful suggestions during the course of this project. We also thank Kyoko Yokomori for helping in the preparation of virus stocks, and Daphne Shimoda for editorial assistance. This work was supported by U.S. Public Health Research Grant NS 18146 and a National Multiple Sclerosis Society Grant RG 1449 awarded to M.M.C.L., and a Multiple Sclerosis Society of Canada Grant awarded to V.L.M. N.L.M. is a Postdoctoral fellow of the National Multiple Sclerosis Society. M.M.C.L. is an Investigator of HHMI.

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