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Characterization of Coronavirus JHM Variants Isolated from Wistar Furth Rats with a Viral-Induced Demyelinating Disease

VINCENT L. MORRIS,*,[1] CHRISTINA TIESZER,* JOANNE MACKINNON,* AND DEAN PERCY†

*Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1, and †Department of Pathology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Murine hepatitis virus (MHV) can cause neurological disease when inoculated intracerebrally (ic) into mice and rats. Specifically the JHM strain of MHV (MHV-JHM) generally causes an acute encephalitis when inoculated ic into 2-day-old Wistar Furth rats. In contrast, JHM generally produces a chronic demyelinating disease with resulting posterior paralysis when inoculated ic into 10-day-old Wistar Furth rats. In addition, while JHM readily produces a productive infection in a mouse fibroblast cell line (L-2), it does not form syncytia or replicate well in a tissue cell line of glial origin (G26-24). We have isolated and characterized three MHV-JHM viral variants from the central nervous system of two Wistar Furth rats with a MHV-JHM-induced demyelinating disease. The pattern of viral-specific mRNA for all three of these variants differed from what was observed for the wild-type parental MHV-JHM that had been passaged only in tissue culture. One of these variants, ATIIf cord virus, which induced a chronic demyelinating disease in 2- or 10-day-old intracerebrally inoculated Wistar Furth rats, had a deletion in the coding region of the peplomer glycoprotein mRNA. In addition, this variant formed massive syncytia and replicated well in G26-24 cells. We have not detected this deletion in the other two JHM variants, ATIIf brain virus and ATIIe brain virus. ATIIf brain virus and ATIIe brain virus primarily produced an acute encephalitis when reinoculated into 2- or 10-day-old Wistar Furth rats. In addition, these two variants did not form syncytia and had a reduced ability to replicate in G26-24 cells.

INTRODUCTION

It has been recognized for decades that the Coronavirus, murine hepatitis virus (MHV), can cause neurological disease in murine species (Cheever *et al.*, 1949). When 2-day-old Wistar Furth rats are inoculated intracerebrally (ic) with the JHM strain of MHV, most of the rats die within one week of inoculation with an acute encephalitis (Sorensen *et al.*, 1980; Parham *et al.*, 1986). In these rats, grey matter lesions generally predominate in the central nervous system (CNS). When Wistar Furth rats are inoculated ic at 10 days of age with JHM, they generally do not develop symptoms until 2–4 weeks postinoculation (Jackson *et al.*, 1984; Parham *et al.*, 1986). These rats develop a chronic demyelinating disease characterized by hind leg paralysis or paresis (Sorensen *et al.*, 1980; Jackson *et al.*, 1984; Parham *et al.*, 1986). Those rats that survive for longer than 3 weeks postinoculation generally have predominately white matter lesions.

Wild-type MHV subgenomic RNAs produced in mouse fibroblast (L-2) cells have molecular weights of approximately 0.8, 1.1, 1.4, 1.6, 3, and 4 $\times$ 10^6 Da (Cheley *et al.*, 1981a,b). By convention, these subgenomic mRNAs are numbered consecutively with the 4 $\times$ 10^6 Da mRNA being designated mRNA 2 and the 0.8 $\times$ 10^6 Da mRNA designated mRNA 7; the genomic size mRNA is called mRNA 1 (Spaan *et al.*, 1981; Wege *et al.*, 1981). The mRNAs form a 3′-coterminal nested set extending for different lengths in a 5′ direction (Stern and Kennedy, 1980a,b; Cheley *et al.*, 1981a; Lai and Stohlman, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1983; Weiss and Leibowitz, 1983). The 5′ end of each mRNA not present in smaller mRNA species contains the coding sequence utilized during the infection (Leibowitz *et al.*, 1982; Siddell *et al.*, 1983). The mRNAs each contain a leader sequence of approximately 72 bases at the 5′ termini (Lai *et al.*, 1983, 1984; Spaan *et al.*, 1983). This leader is encoded only at the 5′ end of the genomic RNA (Lai *et al.*, 1983, 1984; Spaan *et al.*, 1983). The free leader RNA species is synthesized initially, dissociates from the negative-stranded template, and rebinds to the template at the initiation sites of the mRNAs. The leader RNA thus takes part in a leader-primed transcription (Baric *et al.*, 1983; Makino *et al.*, 1986).

Using *in vitro* translation, it has been shown that the lowest molecular weight mRNA codes for the nucleocapsid protein (Rottier *et al.*, 1981; Cheley *et al.*, 1981a). The 1.1 $\times$ 10^6 Da mRNA codes for the E1 glycoprotein, and the 3 $\times$ 10^6 Da mRNA species codes for the E2 glycoprotein (Rottier *et al.*, 1981). The nucleocapsid (N) protein has a molecular weight of approximately 56 kDa (Anderson *et al.*, 1979; Stohlman

[1] To whom requests for reprints should be addressed.

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et al., 1983). The E1 glycoprotein has a molecular weight of approximately 24 kDa and likely functions as a matrix protein (Cheley and Anderson, 1981; Sturman and Holmes, 1983). The E2 glycoprotein is a heterodimer with a molecular weight of 180 kDa (Sturman *et al.*, 1985). This molecule forms the projecting peplomers of the virus and its functions likely include attachment to cells, induction of cell to cell fusion, and elicitation of neutralizing antibodies (Collins *et al.*, 1982; Siddell *et al.*, 1982; Fleming *et al.*, 1983; Sturman and Holmes, 1984).

MHV has been shown to have a high rate of recombination (Lai *et al.*, 1985). The recombinants are derived at a high rate by a mixed infection of DBT cells with temperature-sensitive mutants of MHV-A59 and MHV-JHM at the nonpermissive temperature (Lai *et al.*, 1985). Recombinant virus also arises at a high frequency in mouse CNS tissue that is infected with a mixture of ts mutants of A59 and JHM (Keck *et al.*, 1988a). We have previously reported a truncated version of the E2 glycoprotein mRNA is present in the CNS of Wistar Furth rats with a JHM-induced demyelinating disease (Jackson *et al.*, 1984). Further work demonstrated that JHM E2 glycoprotein can be detected in individual cells of JHM-infected CNS tissue; however, the ratio of detectable E2 antigen to nucleocapsid antigen in the total CNS tissue of infected rats is reduced by more than 13-fold compared with JHM-infected tissue culture cells (Parham *et al.*, 1986). In these studies, virus was not isolated from these rats and characterized, and we did not explore the possibility that changes in the isolated virus could be correlated with the biological properties of this virus.

In this paper we report the isolation of JHM viral variants from the CNS of Wistar Furth rats with a JHM-induced demyelinating disease. We found that differences in the subgenomic mRNAs produced by the viral variants and wild-type JHM were accompanied by differences in the biological properties of these viruses.

MATERIALS AND METHODS

Cells and virus

The JHM strain of mouse hepatitis virus (MHV) was obtained from the American Type Culture Collection (Rockville, MD). The JHM virus was plaque purified three times. Virus was propagated at 37° in 1× Eagle's minimum essential media (EMEM) in 5% fetal calf serum on L-2 murine fibroblast cells (Rothfels *et al.*, 1959) or on the G26-24 murine oligodendroglioma cell line (Sundarraj *et al.*, 1975; Lucas *et al.*, 1977; Bignami and Stoolmiller, 1979). Two- or ten-day-old Wistar Furth rat pups (Sprague-Dawley, Indianapolis, IN) were inoculated with approximately 5×10^4 PFU of virus (JHM or viral variants) in a 20-μl ic inoculation.

Recovery of JHM viral variants

A 10-day-old Wistar Furth rat pup (designated ATIIf) was inoculated ic with a cloned isolate of the murine hepatitis virus (MHV) strain JHM. At 14 days postinoculation, the rat was severely runted and developed hind leg paresis. Virus was recovered independently from the brain and spinal cord and designated ATIIf brain virus and ATIIf cord virus, respectively. We thus could directly compare two virus isolates recovered from a single inoculated rat pup. A littermate of ATIIf (designated ATIIe) was also inoculated ic at 10 days of age with the same cloned stock of JHM virus. At 13 days postinoculation, rat ATIIe showed symptoms similar to those observed in ATIIf and was killed. Virus was isolated from the brain of rat ATIIe and was designated ATIIe brain virus. Results obtained with ATIIe brain virus were generally similar to those obtained with ATIIf brain virus. MHV-JHM viral variants were isolated from the brain or spinal cord of the inoculated Wistar Furth rats using a modified procedure of Sorensen *et al.* (1980). Specifically the tissue was minced into 1× EMEM supplemented with 10% fetal calf serum to form a 20% (w/v) suspension. This suspension was homogenized with a motorized Dounce (G. K. Heller Corp., Floral Park, NY), and passed first through an 18-gauge needle, and then through a 28-gauge needle. The cell debris was pelleted by centrifugation at 915 *g* for 10 min. The virus was then pelleted from the supernatant at 64,800 *g* for 1 hr. The pellet was resuspended in 1× EMEM with 10% fetal calf serum. ATIIf cord virus was three times plaque purified. Similar results were obtained with virus preparations before or after plaque purification.

The viral variant strains appeared to be stable in culture since the pattern of viral-specific mRNA and proteins remained constant with passage in culture. When the CNS tissue of a mock-infected littermate of ATIIf and ATIIe was homogenized and used to inoculate cell cultures using the same procedures as was described to isolate the viral variants, no virus was recovered.

Preparation of tissue and extraction of RNA

Rats were killed and the brain and spinal cord were removed; samples were taken for histopathology as previously described (Jackson *et al.*, 1984). RNA was extracted from tissue or from tissue culture cells using an urea–LiCl extraction procedure (Auffray and Rougeon, 1980).

Labeling of cloned DNA and Northern transfer analysis

Plasmid g344 with a 1800-bp MHV-specific insert (Budzilowicz *et al.*, 1985) was provided by Dr. S. Weiss

(University of Pennsylvania, Philadelphia, PA). The cloned DNA maps from approximately 200 bp into the nonstructural gene 4 to 200 bp into gene 7 (nucleocapsid) (Budzilowicz *et al.,* 1985). This DNA was labeled by the procedure of Feinberg and Vogelstein (1984). The labeled DNA was purified using a spun column procedure (Maniatis *et al.,* 1982). Northern transfer analysis was performed using the procedures of Thomas (1980).

Labeling and extraction of protein

Two 100-mm petri dishes of L-2 cells were infected with virus (multiplicity of infection, 1.8 PFU). At approximately 100% syncytia formation, the culture was labeled with [^{35}S]methionine (10 μCi/ml) for 30 min (Cheley and Anderson, 1981). Tunicamycin (4 μg/ml of media) was added at 10 or 50% syncytia and left on until 100% syncytial formation (Duksin and Mahoney, 1982); similar results were obtained when the drug was added at 10 or 50% syncytia. The viral specificity of the 180 kDa envelope glycoprotein for the wild-type JHM virus, ATIIe brain viral variant, and ATIIf brain viral variant and the 165 kDa protein for the ATIIf cord viral variant were confirmed by immunoprecipitation (Francoeur and Mathews, 1982) with polyvalent JHM-specific antiserum (Parham *et al.,* 1986).

RESULTS

Characterization of viral variants

Viral variants (ATIIf cord, ATIIf brain, and ATIIe brain) were isolated from the brains and spinal cord of Wistar Furth littermates with a murine hepatitis virus (strain JHM)-induced hind leg paresis (see Materials and Methods for details). All of the viral variants and wild-type parental JHM were capable of forming massive syncytia in mouse fibroblast L-2 cells (Fig. 1; data not shown for JHM virus). However, ATIIf brain virus, ATIIe brain virus, and wild-type JHM virus-infected oligodendroglioma cells (G26-24) resembled uninfected G26-24 cultures except individual cells "rounded up" and lifted off from the monolayer (Fig. 1; data not shown for JHM virus). These infected cultures only rarely contained viral-induced syncytia. In contrast, ATIIf cord virus formed massive syncytia equally well in mouse L-2 and G26-24 cells (Fig. 1). Starting with a single stock of ATIIf cord virus, the ratio of the titer in G26-24 cells to the titer in L-2 cells was 0.472 (Table 1). However, the same ratio for ATIIf brain virus was 0.008, for ATIIe brain virus was 0.011, and for wild-type JHM was 0.010 (Table 1). Therefore, the ratio of the viral titer in G26-24 cells compared with L-2 cells was approximately 50-fold higher for ATIIf cord virus than for the brain virus variants and wild-type JHM (Table 1). Our data with the wild-type JHM are in good agreement with previously published results (Lucas *et al.,* 1977).

Intracerebral inoculations using viral variants

Ten-day-old Wistar Furth rats were inoculated intracerebrally with the viral variants. Generally some littermates were inoculated with one viral variant while the remaining littermates were inoculated with a different viral variants for comparison. In general, the ATIIf cord viral variant produced a different pattern of disease than was observed with ATIIf brain virus and ATIIe brain virus. In 17 rats injected with the ATIIf cord virus, a more chronic demyelinating disease typified by hind leg paralysis developed; these rats died in an average time of 20 days (Fig. 2). In 19 injected rats, ATIIf brain virus generally produced a rapid encephalitis, which killed the rats in an average time of 9 days (Fig. 2). Results similar to those observed with ATIIf brain virus were obtained in eight rats injected with ATIIe brain virus.

In addition, a litter of Wistar Furth rats was inoculated ic at 2 days of age with either ATIIf brain virus or ATIIf cord virus. The three ATIIf brain virus-injected rats died in an average time of 5 days; this time course is similar to what has been reported for wild-type JHM virus (Sorensen *et al.,* 1980). For the four littermates injected with ATIIf cord virus, the average time of death was 13 days. The uninjected control rat showed no symptoms.

Histopathological examination indicated that, in general, the white matter lesions were more extensive in the spinal cord and brain stem region (metencephalon and mesencephalon) in rats inoculated at 10 days with ATIIf cord virus when compared with rats inoculated at 10 days with ATIIf brain or ATIIe brain virus (Table 2). Forty-six percent of ATIIf cord virus-inoculated rats had moderate white matter lesions in the spinal cord; in contrast, 93% of ATIIf brain virus-infected rats had either no lesions or only minimal lesions in the white matter of the spinal cord. The number of ATIIf cord virus-injected rats with moderate or marked white matter lesions in the metencephalon and mesencephalon was at least twice that observed in ATIIf brain virus-injected rats. The prosencephalon, which includes the cerebral hemispheres and is the most anterior portion of the central nervous system, was the only part of the brain in which the severity of the white matter lesions was generally greater in ATIIf brain virus-injected rats than in rats injected with ATIIf cord virus.

Lesions in the optic nerve of injected rats were mainly minimal. In addition, the gray matter lesions were predominantly minimal in most samples. The histopathology on samples from Wistar Furth rat pups in-


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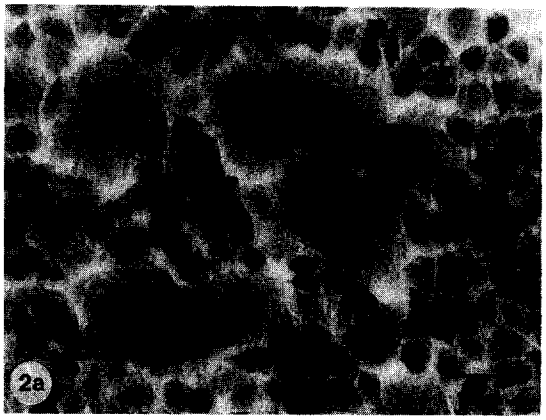
1b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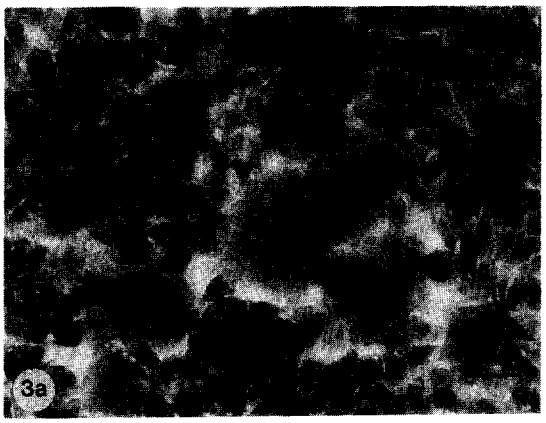
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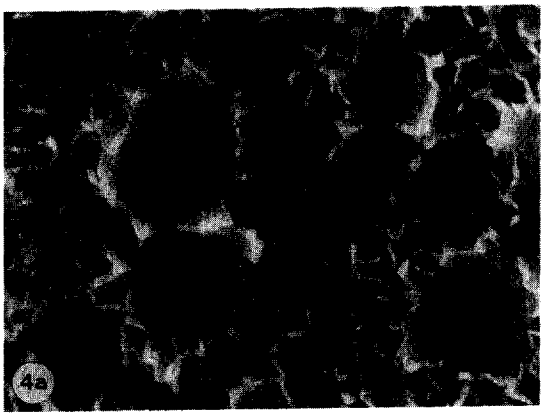
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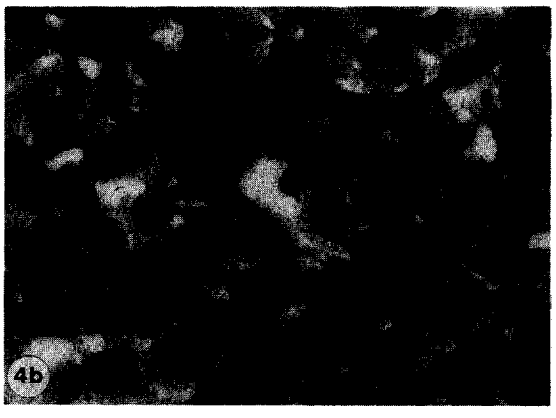
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TABLE 1

TITERS OF VIRAL VARIANTS AND WILD-TYPE JHM

Virus[a]	Cell line[b]	Titer (PFU/ml)	Titer in G26-24/ titer in L-2 cells
ATIIf cord	G26-24	8.5×10^6	0.472
ATIIf cord	L-2	1.8×10^7	
ATIIf brain	G26-24	7.0×10^4	0.008
ATIIf brain	L-2	9.0×10^6	
ATIIe brain	G26-24	8.5×10^4	0.011
ATIIe brain	L-2	7.5×10^6	
JHM	G26-24	4.9×10^5	0.010
JHM	L-2	4.9×10^7	

[a] For each virus the identical virus preparation was used to inoculate both L-2 and G26-24 cells.

[b] L-2 cells are a mouse fibroblast cell line. G26-24 are an oligodendroglioma cell line.

jected with ATIIe brain virus was similar to the results obtained with the ATIIf brain virus-injected rats. The white matter lesions were characterized by vacuolation, hypertrophy, and hyperplasia of astroglial cells, and minimal to moderate mononuclear cell infiltration. Gray matter lesions were characterized by destruction of neurons and astroglial cells, proliferation of endothelial cells lining capillaries, and mononuclear and polymorphonuclear cell infiltrations.

Comparison of mRNA and proteins synthesized by JHM variants

Since the JHM variants caused different patterns of neurological diseases in Wistar Furth rats, we examined the mRNA synthesized by these viruses. Northern transfer analysis indicated that viral mRNAs 4, 5, 6 (E1 envelope glycoprotein), and 7 (nucleocapsid) comigrated for all the viral variants and JHM wild-type viruses (Fig. 3; data not shown for ATIIe virus). However, the ATIIf cord virus produced a truncated mRNA 3 (E2 envelope glycoprotein) with a molecular weight of 2.85 $\times 10^6$ Da; the ATIIf brain, ATIIe brain, and wild-type JHM viruses all produced E2 glycoprotein mRNA of 3.0 $\times 10^6$ Da which comigrate. In addition, ATIIf brain and ATIIe brain viruses each produced two novel mRNA species (3.3 and 3.6 $\times 10^6$ Da). These mRNA species were distinct from the 4.0 $\times 10^6$ Da mRNA species seen with the wild-type JHM virus. We observed a uniform deletion of approximately 1.5 $\times 10^5$ Da from the E2 glycoprotein and higher molecular weight subgenomic mRNAs of ATIIf cord virus when compared with ATIIf and ATIIe brain viruses. Because the E2 mRNA was the lowest molecular weight mRNA with a deletion and because of the nested set arrangement of the coronavirus mRNAs (Stern and Kennedy, 1980a,b; Cheley *et al.*, 1981a; Weiss and Leibowitz, 1983), one can conclude that the deletion occurred in the coding region of the E2 glycoprotein mRNA of the ATIIf cord virus. The genomic size RNA from wild-type JHM and JHM variants appeared to comigrate (Fig. 3). Therefore no size change was detected in this RNA species. This result is in agreement with the JHM variants isolated from Lewis rats (Taguchi *et al.*, 1985).

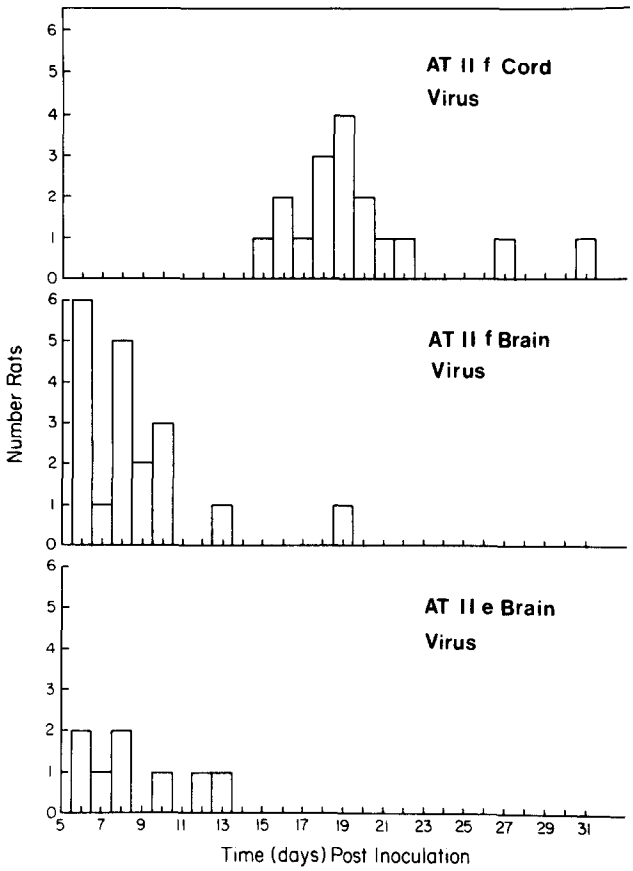

FIG. 2. Time of death resulting from ic inoculation of 10-day-old Wistar Furth rats with variants of murine hepatitis virus JHM. Rats inoculated with ATIIf brain and ATIIe brain virus generally developed an acute encephalitis while rats inoculated with ATIIf cord virus generally developed a chronic demyelinating disease.

FIG. 1. Cytopathic effect (CPE) resulting from infecting cells in culture with murine hepatitis virus JHM variants. Panels labeled with (a) represent mouse L-2 cells; panels labeled (b) show murine oligodendroglioma cells (G26-24). Panels 1a and 1b are uninfected. Panels 2a and 2b are infected with ATIIe brain virus. Panels 3a and 3b are infected with ATIIf cord virus. Panels 4a and 4b are infected with ATIIf brain virus. Note the syncytia present in G26-24 cells infected with ATIIf cord virus (3b) but absent in G26-24 cells infected with ATIIf brain virus (4b) or ATIIe brain virus (2b).

TABLE 2

EXPERIMENTAL ENCEPHALITIS IN THE RAT

Virus used	Severity of lesion	Percentage of rats with indicated severity of lesions[a]								
		Prosencephalon		Mesencephalon		Metencephalon		Spinal cord		Optic nerve
		GM[b]	WM[c]	GM	WM	GM	WM	GM	WM	WM
ATIIf cord	Marked	15%	0%	0%	8%	0%	0%	0%	0%	0%
	Moderate	8%	15%	38%	54%	23%	92%	15%	46%	0%
	Minimal	69%	85%	62%	38%	62%	8%	85%	46%	75%
	No lesion	8%	0%	0%	0%	15%	0%	0%	8%	25%
Number samples:		13	13	13	13	13	13	13	13	8
ATIIf brain	Marked	31%	0%	0%	0%	0%	0%	0%	0%	0%
	Moderate	15%	31%	8%	23%	31%	46%	23%	7%	0%
	Minimal	54%	69%	92%	77%	38%	46%	69%	62%	56%
	No lesion	0%	0%	0%	0%	31%	8%	8%	31%	44%
Number samples:		13	13	13	13	13	13	13	13	9
ATIIe brain	Marked	17%	0%	0%	0%	0%	0%	0%	0%	0%
	Moderate	17%	50%	33%	17%	0%	40%	0%	0%	20%
	Minimal	66%	33%	50%	66%	40%	40%	83%	66%	40%
	No lesion	0%	17%	17%	17%	60%	20%	17%	34%	40%
Number samples:		6	6	6	6	5	5	6	6	5

[a] Ten day old Wistar Furth rats were injected with the virus indicated.
[b] Gray matter lesion.
[c] White matter lesion.

To further investigate the possibility of a deletion in the E2 glycoprotein mRNA of the ATIIf cord virus, we examined the E2 protein synthesized by variant viruses and wild-type JHM. The E2 glycoprotein produced by ATIIf cord virus had an apparent molecular weight of 165 kDa while the E2 glycoprotein made by ATIIf brain, ATIIe brain and wild-type JHM virus had an apparent molecular weight of 180 kDa (Figs. 4A and B). To confirm the difference between the ATIIf cord virus E2 glycoprotein and that of the wild-type JHM virus, we mixed protein samples from cells infected with each virus and subjected them to electrophoresis. The difference in the electrophoretic mobility of the 180 and the 165 kDa protein was quite apparent (Fig. 4A).

Since the apparent molecular weight difference could be due to differences in glycosylation, we also compared the sizes of the E2 polypeptides synthesized in the presence of tunicamycin. The differences in the sizes of the E2 polypeptides for ATIIf cord virus and the JHM virus and brain virus variants were still apparent even when the virus was grown in the presence of tunicamycin (Fig. 4C; data not shown for ATIIe brain virus). Again the differences were confirmed by mixing experiments, this time between protein extracted from ATIIf brain virus and ATIIf cord virus-infected cells (Fig. 4C). The ATIIf cord virus thus had a deletion in its E2 mRNA of approximately 150,000 Da (Fig. 3) and a deletion of approximately 15,000 Da from the E2 protein (Figs. 3 and 4). These deletions corresponded to a loss of approximately 130 amino acids from the E2 glycoprotein and approximately 390 nucleotides from the E2 mRNA. These results thus support the idea that the E2 glycoprotein mRNA produced by the ATIIf cord virus contains a deletion in its coding region.

DISCUSSION

MHV-JHM is capable of inducing neurological disease with two possible outcomes when inoculated intracerebrally into Wistar Furth rats. If rat pups are inoculated at 2 days of age, a rapid, acute encephalitis generally occurs within 1 week of inoculation (Sorensen *et al.*, 1980; Parham *et al.*, 1986). Gray matter CNS lesions are usually more extensive in these animals. However, when Wistar Furth rats are inoculated at 10 days of age, they generally develop a chronic demyelinating disease characterized by hind leg paralysis at approximately 2–4 weeks postinoculation (Sorensen *et*

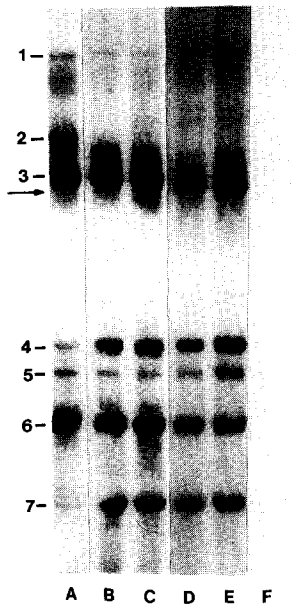

FIG. 3. Northern transfer analysis of RNA extracted from virus-infected and uninfected tissue culture cells. RNA was extracted from infected and uninfected cells, denatured with glyoxal, separated by electrophoresis in 1.1% agarose gels, transferred to nitrocellulose paper, and hybridized with a JHM-specific probe. The JHM homologous RNA species were visualized by autoradiography. Lane A, JHM-infected L-2 cell RNA; lane B, ATIIf brain virus-infected oligodendroglioma (G26-24) cell RNA; lane C, ATIIf brain virus-infected L-2 cell RNA; lane D, ATIIf cord virus-infected L-2 cell RNA; lane E, ATIIf cord virus-infected G26-24 cell RNA; lane F, uninfected L-2 cell RNA. Uninfected G26-24 cell RNA gave results similar to those seen in lane F. In ATIIf cord virus-infected cells (lanes D and E), the 3.0×10^6 Da E2 glycoprotein mRNA species is replaced by a 2.85×10^6 Da mRNA species (see arrow).

al., 1980; Jackson *et al.*, 1984; Parham *et al.*, 1986). In this report, we investigated viral variants that arose in the CNS of rats with a JHM-induced demyelinating disease and studied the effect of alterations in their mRNAs. When 10-day-old rats were inoculated with ATIIf brain virus or ATIIe brain virus, the rats developed a rapid encephalitis instead of the more chronic demyelinating disease that has previously been seen with wild-type parental JHM virus (Sorensen *et al.*, 1980; Jackson *et al.*, 1984) and was observed with ATIIf cord virus-injected rats. In contrast, when 2-day-old rats were inoculated with ATIIf cord virus, the more chronic CNS disease resulted instead of the rapid encephalitis that has been reported for wild-type JHM (Sorensen *et al.*, 1980; Parham *et al.*, 1986) and was observed with the ATIIf brain virus variant. Therefore, the alterations observed in the mRNAs of viral variants appeared to be important in determining the course of the viral-induced CNS disease.

It has been reported that recombinant virus can be recovered from the brains of mice that were infected with two different strains of MHV (Keck *et al.*, 1988a). The recombination frequency was very high and recombination occurred at multiple sites on the viral RNA genome (Keck *et al.*, 1988a). Furthermore, Keck and co-workers (1988b) have shown that the replacement of A59 genetic sequences at the 5′ end of the E2 glycoprotein gene with the fusion-negative MHV-2 sequences do not affect the fusion ability of the recombinant viruses. They thus suggest that the 3′ end of the E2 glycoprotein may be crucial for the fusion-inducibility of the virus. Our variant virus strains may be useful in exploring this question, since we found that a deletion in the E2 glycoprotein mRNA of ATIIf cord virus was associated with the ability of the variant to induce fusion in a cell line of glial origin (G26-24).

Even though we inoculated the rats with cloned virus, we were able to recover viral variants from the CNS of these inoculated rats which differ in their patterns of

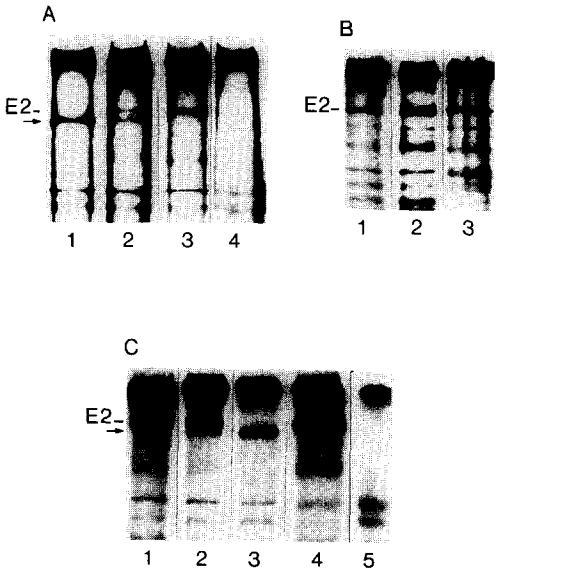

FIG. 4. SDS–polyacrylamide gel electrophoresis analysis of [^{35}S]methionine-labeled proteins extracted from L-2 cells infected with parental JHM virus or JHM viral variants as follows: (A) Lane 1, ATIIf cord virus-infected cells; lane 2 mixture of proteins from ATIIf cord virus-infected cells and parental JHM virus-infected cells; lane 3, wild-type JHM virus-infected cells; lane 4, uninfected L-2 cells. (B) Lane 1, JHM virus-infected cells; lane 2, AT11f brain virus-infected cells; lane 3, ATIIe brain virus-infected cells. (C) Labeled proteins extracted from L-2 cells infected with parental JHM virus or JHM viral variants and treated with tunicamycin. Lane 1, ATIIf brain virus-infected cells; lane 2, mixture of proteins from ATIIf cord virus-infected cells and ATIIf brain virus-infected cells; lane 3, ATIIf cord virus-infected cells; lane 4, wild-type JHM virus-infected cells; lane 5, uninfected, tunicamycin-treated L-2 cells. The position of the 180 kDa JHM-specific E2 envelope glycoprotein is indicated. The 165 kDa protein which is produced in place of the 180 kDa protein in ATIIf cord virus-infected cells is indicated by the arrow. The molecular weights of these proteins was determined using molecular weight standards (Bio-Rad, Richmond, CA).

mRNAs from the wild-type parental virus. In addition, even though a rat (designated ATIIf) was inoculated at one site intracerebrally, we were able to isolate two separate variants from the CNS of this rat. The virus isolated from the brain (ATIIf brain virus) produced acute encephalitis when reinoculated ic into Wistar Furth rats, and the virus isolated from the spinal cord (ATIIf cord virus) produced a chronic demyelinating disease with predominantly white matter lesions. Thus the site of infection in the CNS may result in a selection of variants with different physical and biological properties.

In our experiments, the alterations in the mRNA of the variants occurred during the infection of the CNS by the virus and did not involve any *in vitro* selection by antibodies or other means. The major difference that was apparent between these variants was the deletion in the E2 glycoprotein mRNA that was present in the ATIIf cord virus. The ATIIf cord viral variant induced a chronic demyelinating disease in 2- or 10-day-old intracerebrally inoculated Wistar Furth rats. The other variants (ATIIf brain virus and ATIIe brain virus) produced an acute encephalitis in either 2- or 10-day-old intracerebrally inoculated Wistar Furth rats. These results confirmed and expanded our previous results that the JHM RNA species present in the CNS of Wistar Furth rats with a JHM-induced demyelinating disease differ from what is seen in tissue culture cells infected with wild-type JHM virus (Jackson *et al.*, 1984). Our earlier work also suggests that a truncated version of the JHM E2 glycoprotein mRNA is present in rats with a JHM-induced demyelinating disease (Jackson *et al.*, 1984). Work by Fleming uses monoclonal antibodies to the E2 viral glycoprotein to select antigenic variant viruses that escape neutralization *in vitro* (Fleming *et al.*, 1986). Variants selected with one of the E2 monoclonal antibodies are highly virulent and causes an encephalitis in inoculated mice. A second selected variant predominantly causes a subacute paralytic disease clinically and extensive demyelinating histology. Thus the E2 glycoprotein appears to be important in determining JHM pathogenesis in different systems. However, one cannot rule out that other factors or undetected genomic changes could also be involved. Future work will investigate this question.

ATIIf brain virus and ATIIe brain virus produced two novel high-molecular-weight RNAs (3.3×10^6 and 3.6×10^6 Da) which were not present in the wild-type JHM. These RNA species might represent deletions in mRNA 2. However, since this mRNA codes for a nonstructural protein whose antiserum is not available, we have not been able to determine if the protein coded by mRNA 2 is altered. In addition, the nucleotide sequence of the coding region for mRNA 2 has not been published; when this information is available it will assist in determining the nature of the novel RNAs produced by the brain virus variants.

Polymorphism has been observed in the E2 glycoprotein of coronaviruses (Talbot and Buchmeier, 1985). Sequencing analysis has revealed that 89 amino acids are present in MHV strain A59 but are absent in JHM (Schmidt *et al.*, 1987; Luytjes *et al.*, 1987). This difference is similar to the number of amino acids that are deleted from the E2 glycoprotein of the variant ATIIf cord virus when compared with either wild-type JHM or ATIIf brain or ATIIe brain virus. Since the E2 protein is involved in the adsorption of the virus to cells and the induction of cell to cell fusion (Collins *et al.*, 1982; Siddell *et al.*, 1982; Fleming *et al.*, 1983; Sturman and Holmes, 1984), it seems logical that changes in this protein could alter the ability of the virus to infect certain cell types. Since ATIIf cord virus infection was associated with greater white matter involvement and chronic demyelinating disease, one would predict that the variant ATIIf cord virus would have an increased ability to infect glial cells when compared with ATIIf brain and ATIIe brain viral variants. In fact, we observed that ATIIf cord virus showed a marked increase in syncytiogenesis in an oligodendroglioma cell line (G26-24). Furthermore, the ratio of the titer in G26-24 cells over the titers in L-2 cells was approximately 50-fold higher for ATIIf cord virus than it was for ATIIf brain or ATIIe brain virus.

Viral variants have also been recovered from Lewis rats with a JHM-induced acute encephalitis (Taguchi *et al.*, 1985). Taguchi and co-workers have reported that these variants produce mRNAs 2 and 3 which are approximately 500 bases larger than is reported for wild-type JHM. These variants also produce an envelope glycoprotein that is 15,000 Da larger than is seen with wild-type JHM. However, no alterations are detected in the genomic size RNA. The wild-type JHM and one of the variant viruses (cl-2) produce cell fusion in a continuous cell line of mouse origin (DBT). The titer for the wild-type virus was approximately 10-fold higher than for cl-2. Both JHM and cl-2 produce acute encephalitis in intracerebrally inoculated rats; however, less infectious cl-2 virus is required to produce an acute encephalitis. Some similarities and differences can be seen between these experiments and those reported in this paper. Our JHM variants were isolated from a Wistar Furth rat with a viral-induced demyelinating disease instead of an encephalitis. Variant ATIIf cord virus contains an apparent deletion in the coding region of mRNA 3 instead of an insertion of extra nucleotides. Curiously, the size of the insertion in mRNA 3 for the variant cl-2 is very similar to the size of the deletion in mRNA 3 for ATIIf cord virus. The relative location of these alter-

ations in mRNA 3 may help determine the significance of this observation. In addition, cl-2 and the variant viruses we have isolated all induce cell fusion and replicate well in continuous cell lines in which JHM also induces syncytia and produces a high titer of virus. However, we have extended these observations to show that ATIIf cord virus can replicate much better in a cell line of glial origin (G26-24) than does wild-type JHM. Cl-2 and ATIIf brain virus and ATIIe brain virus do not contain deletions in their E2 envelope glycoprotein and produce encephalitis when injected into rats. In contrast, ATIIf cord virus does have a deletion in the E2 glycoprotein and produces a demyelinating disease in rats. These results are consistent with the hypothesis that a deletion in the viral E2 glycoprotein is associated with the ability of the virus to produce a demyelinating disease in rats. Further work with additional variants will be necessary to test this hypothesis. Finally, no alterations are detectable in the genomic size RNA produced by the variants isolated by both Taguchi and coworkers (Taguchi *et al.,* 1985) and ourselves; however, all of the variants produce alterations which are readily detectable in mRNA 3 and/or mRNA 2. The lack of detectable alterations in the genomic size RNA raises the possibility that the variants may not arise via a simple deletion or insertion of bases in the viral genome. The viral mRNAs are generated by a leader-primed transcription which involves the fusion of noncontiguous transcripts (Baric *et al.,* 1983; Makino *et al.,* 1986). Therefore, a possible mechanism for the generation of the variants involves an alteration in either the leader coding sequences or in one or more of the primer binding sites. Recent studies suggest that the binding of leader RNA to template RNA during the synthesis of subgenomic mRNA may not be precise even for wild-type JHM (Makino *et al.,* 1988). Therefore, small alterations in either the primer or primer binding sites could result in changes in the size of subgenomic mRNAs. Nucleotide sequencing studies should help determine how these variants arise.

The presence of JHM variants in ic inoculated rats appears to be a general phenomenon. It is hoped our work and the work of others will determine their role in viral-induced CNS disease.

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