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Characterization of a Variant Virus Selected in Rat Brains After Infection by Coronavirus Mouse Hepatitis Virus JHM

FUMIHIRO TAGUCHI,* STUART G. SIDDELL, HELMUT WEGE, AND VOLKER TER MEULEN
Institute of Virology, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany

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The intracerebral inoculation of Lewis rats with the murine coronavirus MHV-JHM leads in the majority of animals to acute encephalitis and death within 14 days. Viral RNAs isolated from the brains of animals 5 to 7 days after infection were compared by Northern blot analysis with the RNAs produced during the lytic infection of Sac(−) or DBT cells with wild-type MHV-JHM (wt virus). Reproducibly, the subgenomic mRNAs 2 and 3 but no other viral RNAs were significantly larger in the brain-derived material. All viruses isolated from infected brain material displayed and maintained this altered mRNA profile when cultivated in Sac(−) or DBT cells. A virus isolated from the infected brain material, MHV-JHM clone 2 (cl-2 virus), has been further characterized. This isolate grew in tissue culture and induced cytopathic effects comparable to those induced by wt virus. However, the mRNAs 2 and 3 produced in cl-2 virus-infected cells had molecular weights ca. 150,000 larger than those produced in cells infected with wt virus. There was no detectable difference in genome-sized RNA (mRNA 1) or subgenomic mRNAs 4, 5, 6, and 7 as determined by electrophoresis in agarose gels. T_1-resistant oligonucleotide analysis of genomic RNA revealed one additional and one missing oligonucleotide in the fingerprint of cl-2 virus compared with wt virus. The oligonucleotide fingerprints of intracellular mRNA 3 were identical for both viruses. Pulse-labeling with [^{35}S]methionine in the presence of tunicamycin showed that the primary translation product of mRNA 3, the E2 apoprotein, was ca. 15,000 larger in molecular weight in cl-2 virus-infected cells. These data show that viruses with larger mRNAs 2 and 3 (the latter encoding an altered E2 glycoprotein) are selected for multiplication in rat brains. Mechanisms for the generation of such variants and the possible nature of their selective advantage are considered.

Murine hepatitis virus (MHV) is a member of the family Coronaviridae, which are enveloped RNA viruses. The genome of MHV is a single-stranded infectious RNA of 6×10^6 molecular weight (MW), and MHV virions contain three major proteins, the nucleocapsid protein, a glycosylated transmembrane protein (E1), and a surface glycoprotein (E2). In MHV-infected cells, six subgenomic mRNAs (MWs 0.6×10^6 to 3.7×10^6) as well as genome-sized RNA (mRNA 1) are produced. These mRNAs form a 3′-coterminal nested set extending for different lengths in a 5′ direction. The in vitro translation of size-fractionated MHV mRNAs in cell-free systems or oocytes has shown that the major primary translation products of mRNAs 3, 6, and 7 are the polypeptide components of the virion surface, membrane and nucleocapsid proteins, respectively (16, 22, 24). The remaining viral mRNAs encode proteins that are thought to be components of the viral RNA polymerase or nonstructural proteins of unknown functions (26, 31).

Various strains of MHV cause different types of disease in rodents depending upon the species, age, and immune status of the animals (38). Among MHV strains, MHV-JHM is of particular interest since this virus can produce a chronic demyelinating disease in rats (12, 21, 39) and mice (6, 11, 14). The intracerebral infection of 4- to 5-week-old Lewis rats with MHV-JHM produces two distinct disease patterns. In a minority of animals, a subacute demyelinating encephalitis develops 12 to 14 days postinfection (p.i.) (12, 35, 37). The transfer of lymphocytes from rats with subacute demyelinating encephalitis to uninfected recipient animals has recently been shown to result in an experimental allergic encephalitis-like disease (35). In contrast, the majority of animals develop an acute encephalitis, which leads to death within 14 days (37).

In our initial experiments to define the viral parameters that are important in determining the disease patterns during MHV-JHM infection, we have characterized the viral mRNAs in the brains of acutely infected Lewis rats, using Northern blot analysis. We were surprised to find that specific viral mRNAs revealed reproducible size differences compared with those produced in tissue culture after infection with the wild-type MHV-JHM (wt) virus used for inoculation. In this report, we describe these observations together with a detailed analysis of a variant virus isolated from diseased brain tissue.

MATERIALS AND METHODS

Cells and viruses. For virus propagation, titration, and radioactive labeling of intracellular polypeptides, Sac(−) cells or DBT cells were used. These cells were maintained with Eagle minimal essential medium (EMEM) containing 5 to 8% fetal calf serum (FCS) as described previously (24, 32). The JHM strain of MHV, originally obtained as a suckling mouse brain suspension from L. Weiner, was cloned on Sac(−) cells and propagated on Sac(−) or DBT cells at low multiplicities of infection (MOIs). This virus is designated as wt virus. A virus isolate, cl-2, was plaque purified three times on DBT cells after isolation from the brain of a Lewis rat sacrificed 6 days p.i. The infectivities of virus stocks were determined by plaque assays on DBT cells as previously described (32).

Animals. Four- to 5-week-old Lewis rats were anesthetized with chloroform and inoculated intracerebally with 0.5×10^5 to 1×10^5 PFU of wt virus in 0.04 ml of EMEM. For the sampling of tissue for virus infectivity, animals were

* Corresponding author.

sacrificed, and the brains and spinal cords were collected asceptically and stored at −70°C.

Extraction of RNA from infected cells. DBT cells or Sac(−) cells were infected as described in the figure legends, and RNA was extracted when the cytopathic effect (CPE) had developed to ca. 60 to 80%. The extraction of RNA was performed by a method described previously (23), except that the concentration of Nonidet P-40 was increased from 0.1 to 0.65%. Extracted RNA was precipitated with ethanol, dried, dissolved in water, and stored at −70°C.

Intracellular labeling of viral mRNA. DBT cell monolayers in 4-cm petri dishes were infected with wt or cl-2 virus at an MOI of 2 to 3 and cultured with EMEM supplemented with 5% FCS for 2 to 3 h. The medium was then replaced by phosphate-free medium supplemented with 5% dialyzed FCS. At 3 to 4 h p.i., [^{32}P]phosphate (PBS 13, Amersham, Braunschweig, Federal Republic of Germany [FRG]) was added to the culture at a concentration of 200 μCi/ml. Thirty minutes before the addition of label, actinomycin D was added to the culture at a final concentration of 2 μg/ml. After 3 to 4 h of labeling, intracellular cytoplasmic RNA was isolated as described above.

Extraction of RNA from rat brains. Extraction of RNA from rat brain was done essentially by the method described by Chirgwin et al. (3). Brains were collected from infected or uninfected rats, immediately frozen in liquid nitrogen or solid CO_2, and stored at −70°C. Frozen brains were cut into small pieces, mixed with 4 M guanidium thiocyanate (5 ml of solution for 1 g of brain), and homogenized in a glass Dounce homogenizer. The homogenate was overlaid onto 2.5 ml of CsCl (1.7 g/ml) and centrifuged at 36,000 rpm for 12 h at 20°C in an SW41 rotor. The supernatant was carefully removed, and the pelleted RNA was dissolved in a small volume of 7.5 M guanidium hydrochloride, extracted with phenol-chloroform (phenol-chloroform-isoamylalcohol, 50:50:1 by volume), precipitated with ethanol, dried, dissolved in water, and stored at −70°C. In some cases, the RNA samples were subjected to affinity chromatography with polyuridylated Sepharose as described previously (17).

Electrophoresis of RNA and Northern blot hybridization. Electrophoresis of RNA was performed in agarose gels containing formaldehyde, using MOPS buffer (0.2 M morpholinopropanesulfonic acid, [pH 7.0], 50 mM sodium acetate, 1 mM EDTA) as described previously (20). Both vertical and horizontal gels were used. After electrophoresis, gels were washed several times with water and soaked with 50 mM NaOH for 30 min and then with 100 mM Tris-hydrochloride (pH 7.2) for 30 min. Gels were then soaked in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium acetate [pH 7.0]) for 30 min, and the RNA in the gel was transferred onto nitrocellulose (Schleicher and Schüll, Dassel, FRG) as described previously (20). The nitrocellulose filter was soaked in a prehybridization buffer for more than 3 h at 43°C and then was incubated in a hybridization buffer containing ^{32}P-labeled cDNA as previously described (20). Radioactive cDNA was prepared by nick translation of cDNA complementary to mRNA 7 of MHV-JHM (27). Hybridization was carried out with 2×10^6 to 10×10^6 dpm (0.2 to 0.5 μg) of cDNA at 43°C for 2 to 3 days. The filters were washed with 2× SSC containing 0.1% sodium dodecyl sulfate and 0.1× SSC containing 0.1% sodium dodecyl sulfate at 22°C, dried, and exposed to Kodak XAR 5 film at −70°C with intensifying screens.

T_1-resistant oligonucleotide fingerprinting. For the fingerprinting of genomic RNA, ^{32}P-labeled RNA was recovered from virions. Sac(−) cells in a 500-ml culture bottle were infected with wt or cl-2 virus at an MOI of 0.5 to 1.0, and the cells were cultured in phosphate-free medium supplemented with dialyzed FCS for 2 to 3 h. Then [^{32}P]phosphate was added to the culture at a concentration of 200 μCi/ml. At 12 to 14 h p.i., when the CPE was more than 90% developed, the culture fluid was clarified at 4,000 rpm for 20 min. The supernatant was then overlaid onto 30% sucrose in NTE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.2], 0.001 M EDTA) and centrifuged at 25,000 rpm for 3 h at 4°C in an SW28 rotor. RNA was extracted from the pelleted virions as previously described (18) and fractionated by urea agarose gel electrophoresis (18). The ^{32}P-labeled, genome-sized RNA was extracted from the agar gel by the method of Langridge et al. (15), precipitated with ethanol, dried, and dissolved in water. The RNA was then digested with T_1 RNase (Calbiochem, Frankfurt, FRG) at a concentration of 1 U/μl at 37°C for 1 h. Two-dimensional electrophoresis of resistant oligonucleotides was performed by the method described by De Wachter and Fiers (5). The analysis of mRNA 3 from infected cells was performed in essentially the same way, except that 2 μg of actinomycin D per ml (Sigma, Taufkirchen, FRG) was added to the culture 30 min before labeling with [^{32}P]phosphate. RNA was extracted from the infected cells, using Nonidet P-40 as described above, when the CPE was 60 to 80% developed. The extracted RNA was electrophoresed through a urea agarose gel, and the portion of a gel containing mRNA 3 was detected by autoradiography and cut out; the RNA was extracted (15) and analyzed for T_1-resistant oligonucleotides as described above.

Labeling of intracellular proteins in infected cells and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. DBT cells in 4-cm petri dishes were infected with wt or cl-2 virus at an MOI of 2 to 3 and cultured with EMEM supplemented with 5% FCS for 8 to 10 h. The medium was then replaced by EMEM without methionine, containing 5% dialyzed FCS, and 30 min later, [^{35}S]methionine (SJ204; Amersham) was added at a concentration of 50 to 60 μCi/ml. After 30 min of labeling, the cells were washed with phosphate-buffered saline (pH 7.2) and lysed with phosphate-buffered saline containing 0.5% Nonidet P-40. Tunicamycin (Eli Lilly & Co., Indianapolis, Ind.) was added at 6 to 8 h p.i. at a concentration of 4 μg/ml. After 2 h, the medium was replaced with EMEM without methionine containing 5% dialyzed FCS and 4 μg of tunicamycin per ml. After 30 min, [^{35}S]methionine was added at a concentration of 100 to 120 μCi/ml, and 30 min later, the cells were lysed as described above and centrifuged at 4,000 rpm for 30 min at 4°C. The supernatants were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (25).

RESULTS

Analysis of viral mRNA in the brains of infected rats. Four- to 5-week-old Lewis rats were inoculated intracerebrally with cloned wt virus, and virus growth in the brains and spinal cords was examined. Infectious viruses could first be detected in brains 3 days p.i. Within 5 to 6 days p.i., the virus titer reached a peak of 10^4 to 10^5 PFU per brain. At this time, a significantly lower virus titer (less than 10^3 PFU per tissue) was detected in the spinal cords. The titer in the brains decreased gradually thereafter, but that in the spinal cords increased. Sixty to 70% of infected rats showed clinical central nervous system symptoms 6 to 9 days p.i., and almost all of these animals died within 2 weeks (data not shown).

Brain material containing high levels of infectious viruses,

i.e., material obtained 5 to 7 days p.i., was analyzed directly for viral RNAs by Northern blot hybridization. In all cases in which MHV-specific mRNAs were detected (10 animals of 14 examined), the mRNA profile differed from that produced during a lytic infection of DBT or Sac(−) cells with the wt virus used for inoculation. Whereas no differences in the electrophoretic mobilities of genome-sized RNA (mRNA 1) and subgenomic mRNAs 4, 5, 6, and 7 could be detected, mRNAs 2 and 3 were reproducibly and significantly larger in the brain-derived material (Fig. 1).

Isolation of cl-2 virus. To facilitate biochemical analysis of the viruses that replicated in the brains of infected animals, we plaque purified four independent isolates from infected brain material. One isolate, cl-2 virus, which is representative of this group in all respects that we have examined, was studied in detail. When cl-2 virus was grown in tissue culture, the mRNA profile produced corresponded to the profile derived from the population of viruses that replicate in the rat brain, and differed from the wt virus used for inoculation (Fig. 2A). Both mRNAs 2 and 3 are larger, by ca. 150,000 MW, than the corresponding mRNAs produced by wt virus. There was no detectable difference in the size of any other viral mRNA, although a number of subgenomic RNA species that had not been found in the RNA isolated from brain material were apparent in both the wt and cl-2 tissue culture infections. Since it could be argued that size differences would become less apparent in RNAs larger than mRNAs 2 and 3, i.e., genome-sized RNA (mRNA 1), we labeled virus-specific RNAs with [^{32}P]phosphate in the presence of actinomycin D and subjected these RNAs to extended electrophoresis. Even under these conditions, no difference in genome-sized RNA (mRNA 1) from cl-2 and wt viruses could be detected (Fig. 2B).

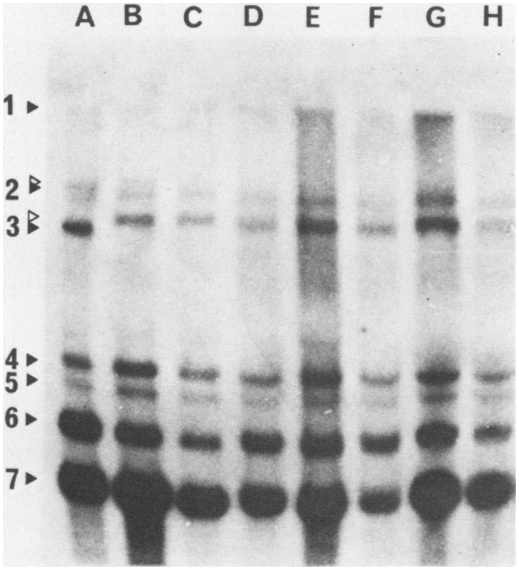

FIG. 1. Northern blot analysis of RNA from infected rat brains. RNA was isolated, electrophoresed, blotted, and hybridized to MHV-JHM-specific ^{32}P-labeled cDNA as described in the text. Lanes: A, RNA (2 μg) from wild-type virus-infected DBT cells (MOI, 1); B and C, RNA (5 μg) from DBT cells infected with a 10% homogenate (MOI, ca. 0.01) of rat brain obtained 6 days p.i.; D and E, polyadenylated RNA (4 and 7 μg, respectively) from rat brain 6 days p.i.; F, G, and H, RNA (75 μ) from rat brains 6 days (F and H) or 7 days (G) p.i.

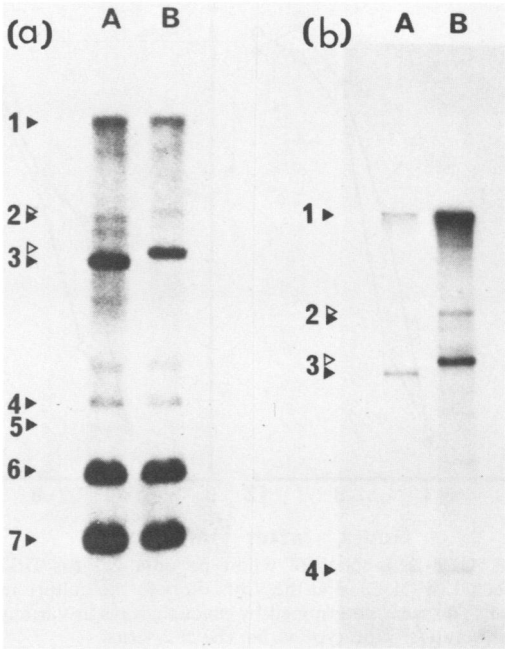

FIG. 2. (a) Northern blot analysis of RNA from wild-type or cl-2 virus-infected DBT cells. The experimental procedures are described in the text. Lane A, RNA (5 μg) from wild-type virus-infected cells; Lane B, RNA (5 μg) from cl-2 virus-infected cells. (b) Electrophoresis of virus-specific intracellular RNA. DBT cells were infected with wild-type virus (MOI, 1) or cl-2 virus (MOI, 1) and labeled with $^{32}P_i$ in the presence of actinomycin D. RNA was isolated and electrophoresed, and the dried gel was exposed to X-ray film as described in the text. Lane A, RNA (10,000 dpm) from wild-type virus-infected cells; lane B, RNA (20,000 dpm) from cl-2 virus infected cells.

We also examined whether the mRNA profile produced by cl-2 virus was stable during further passage in tissue culture or in animals. During eight passages in DBT cells, the mRNA profile characteristic of cl-2 virus remained constant. Also, the virus population that preferentially replicated in the brains of animals inoculated with cl-2 virus, as well as virus subsequently reisolated from such animals, all showed mRNA profiles identical to that of cl-2 virus (data not shown).

Growth of wt and cl-2 virus in cultured cells. DBT cells were infected with wt or cl-2 virus at an MOI of 2 PFU per cell, and virus titers in the supernatant and the cells were examined. The virus titer increased in the supernatant and the cells from 4 h p.i. and attained a peak at 10 to 12 h p.i., both in wt and cl-2 infection (Fig. 3). There was no significant difference between intracellular and extracellular titers, except at 6 h p.i., when the intracellular titer was 1 to 1.5 $\log_{10}$ higher in both infections. The wt virus reached a titer ca. 1 $\log_{10}$ higher than that of cl-2 virus. The fusion of infected cells, a characteristic of CPE, was first observed at 5 h p.i. (involving ca. 10 to 15% of the cells), and almost all cells were involved in fusions at 8 to 10 h p.i. In this respect, no major difference between the wt and cl-2 virus infection was evident. Preliminary experiments indicate that in vivo cl-2 virus is more "virulent" than wt virus, i.e., less infectious virus is required to produce an acute encephalitis under defined conditions.

T_1-resistant oligonucleotide fingerprinting of genomic RNA and mRNA 3 from wt and cl-2 virus. Genomic RNA labeled

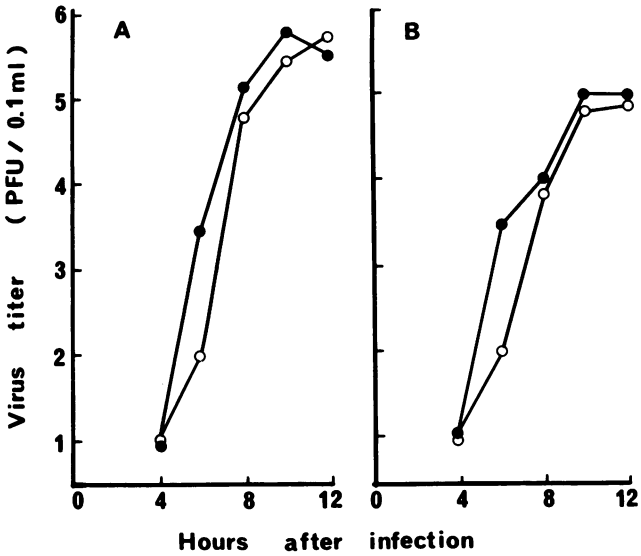

FIG. 3. Growth kinetics of wild-type or cl-2 virus DBT cells were infected (MOI, 2), and the virus titers in the culture medium (○) or cells (●) were determined by plaque assays at various times after infection. (A) Wild-type virus; (B) cl-2 virus.

in vivo with [^{32}P]phosphate was extracted from wt and cl-2 virions, selected by urea agarose gel electrophoresis, and analyzed by T_1-resistant oligonucleotide fingerprinting. We could identify more than 35 T_1-resistant oligonucleotides in the genomic RNA of both viruses (Fig. 4). The fingerprint of the wt JHM virus used in these studies is very similar to that obtained in other laboratories, and we therefore numbered the oligonucleotides according to previous publications (19, 29). When the wt and cl-2 virus genomic RNAs are compared, there are only two differences in the fingerprinting pattern. Oligonucleotide 7 (shown by an arrow in Fig. 4A) is missing in the genome RNA of cl-2 (shown by a circle in Fig. 4) and, instead, a new oligonucleotide designated 7a (shown by an arrow in Fig. 4B) was found. The similar electrophoretic mobilities of oligonucleotides 7 and 7a suggests that they are closely related in base composition. A similar comparative analysis of the oligonucleotide fingerprints of the intracellular mRNA 3 of wt and cl-2 viruses is shown in Fig. 5. In this case, there is no detectable difference in the oligonucleotide fingerprints, although mRNA 3 of cl-2 virus is ca. 500 nucleotides larger than that of wt virus. We have not been able to incorporate sufficient radioactivity into the mRNA 2 produced in cl-2 virus-infected cells to perform a similar analysis for this mRNA species.

Comparison of proteins synthesized in wt and cl-2 virus-in-

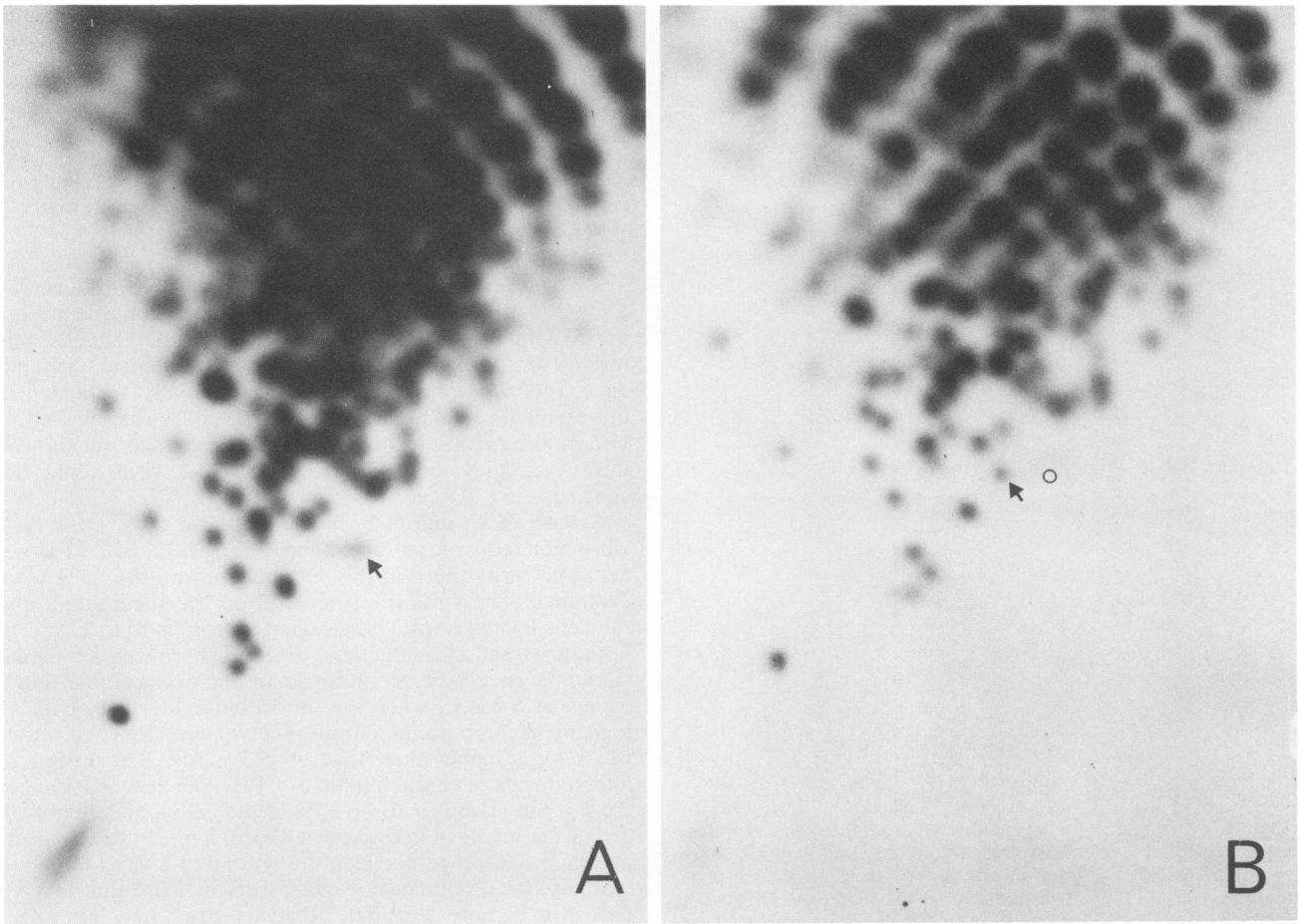

FIG. 4. T_1-resistant oligonucleotide fingerprints of genomic RNA. Sac(−) cells were infected (MOI, 1) and labeled with $^{32}P_i$, and RNA was isolated from virions purified from the tissue culture supernatant. Genome-sized RNA was fractionated in agarose-urea gels and digested with RNase T_1, and oligonucleotides were subjected to two-dimensional electrophoresis as described in the text. (A) Wild-type virus genome RNA; (B) cl-2 virus genome RNA. The oligonucleotides referred to in the text are indicated.

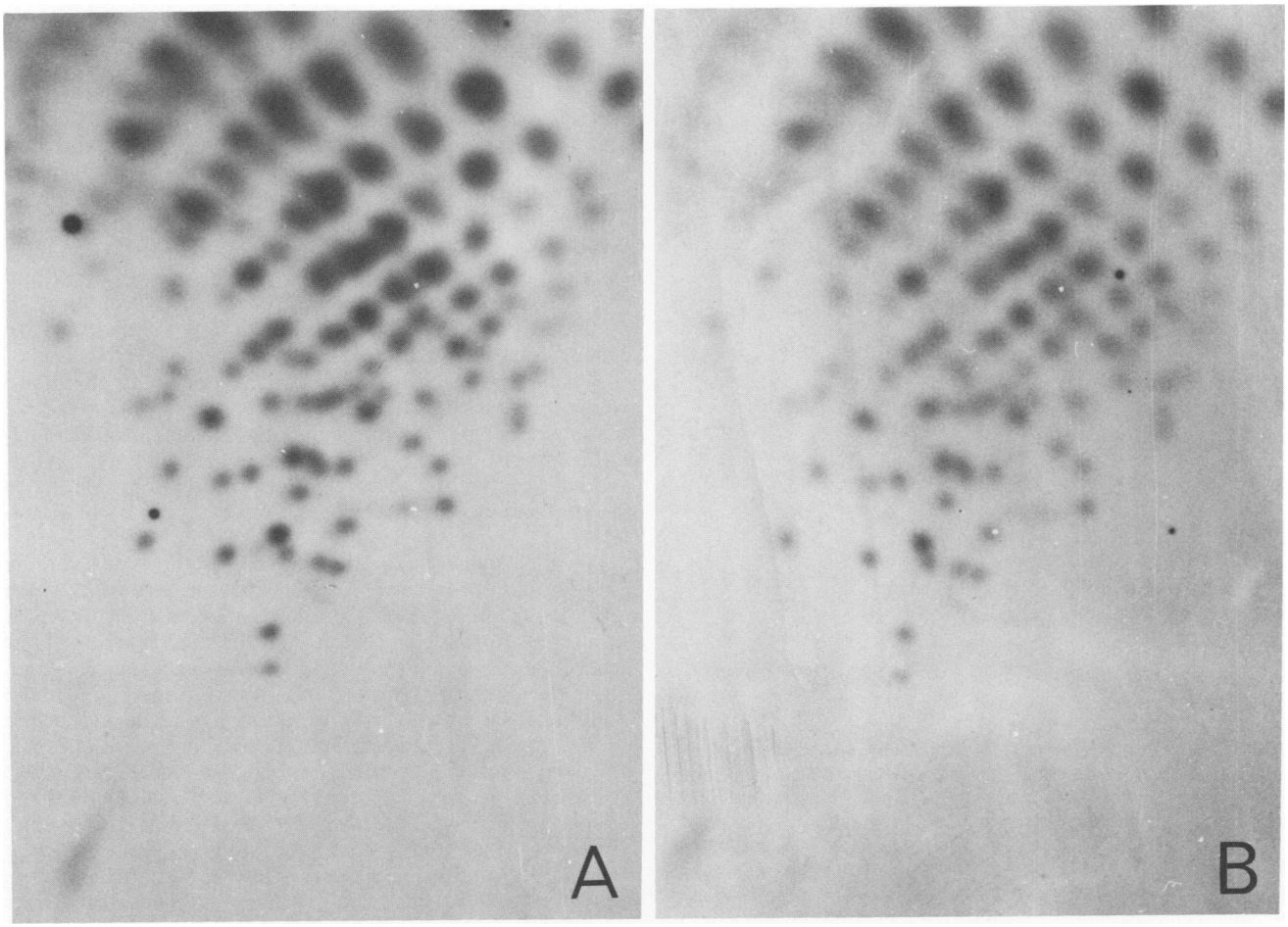

FIG. 5. T_1-resistant oligonucleotide fingerprints of intracellular mRNA 3. Sac(−) cells were infected (MOI, 1), and intracellular viral mRNA was labeled with $^{32}P_i$ in the presence of actinomycin D. The mRNA 3 was isolated and analyzed as described in the text. (A) Wild-type virus mRNA 3; (B) cl-2 virus mRNA 3.

fected cells. The mRNA 3 produced by cl-2 virus is significantly larger than that of wt virus, so we examined whether the protein encoded by mRNA 3, the E2 glycoprotein, was also larger in cl-2-infected cells. DBT cells were infected and labeled with [^{35}S]methionine, and cell lysates were immunoprecipitated with an antiserum raised against purified wt virus. The intracellular precursor to the E2 protein produced in cl-2 virus-infected cells is ca. 15,000 MW larger than the wt virus intracellular E2 precursor (Fig. 6). As this apparent MW difference could be due to differences in glycosylation, we compared the sizes of the E2 polypeptides synthesized in the presence of tunicamycin. The E2 polypeptide in cl-2 virus-infected cells is 15,000 MW larger than that found in wt virus-infected cells (Fig. 6).

DISCUSSION

The infection of rodents with MHV-JHM is an important animal model for the study of virus-related central nervous system disorders (6, 10–12, 21, 35). However, to our knowledge, it has not previously been noted that the mouse hepatitis virus population which preferentially replicates in the animal brain can differ systematically from the virus used for infection. Our studies have also demonstrated that although overt biological differences between different virus populations may be absent, significant biochemical differences exist which can be detected by using techniques such as Northern blot analysis. This finding emphasizes that animal experiments dealing with, for example, the role of virus-specified factors in determining disease patterns need to be carefully controlled.

The virus population that predominates in the brains of Lewis rats 5 to 7 days p.i. has a different mRNA profile compared with the virus used for infection. We have never detected the mRNA profile characteristic of wt virus in material derived from brain tissue, nor have we been able to reisolate from animal brains a virus that produced the wt virus mRNA profile in tissue culture. We conclude, therefore, that acute encephalitis (12, 21, 37), the disease pattern characteristic of acute infection, is associated with the propagation of a variant virus population and not of wt virus. The development of acute encephalitis is apparently dependent upon mutation within the wt virus population and selection within the animal of a specific population of variant viruses.

This conclusion raises two questions. First, is the variant a minority component of the "wt" virus population used for infection, or do (phenotypically) similar variants arise de novo during each infection? The wt virus stock used in these experiments was repeatedly cloned, and the mRNA profile of the population presents no evidence of a cl-2-like component. However, it is technically difficult to rigorously exclude the "minority component" argument (without analyz-

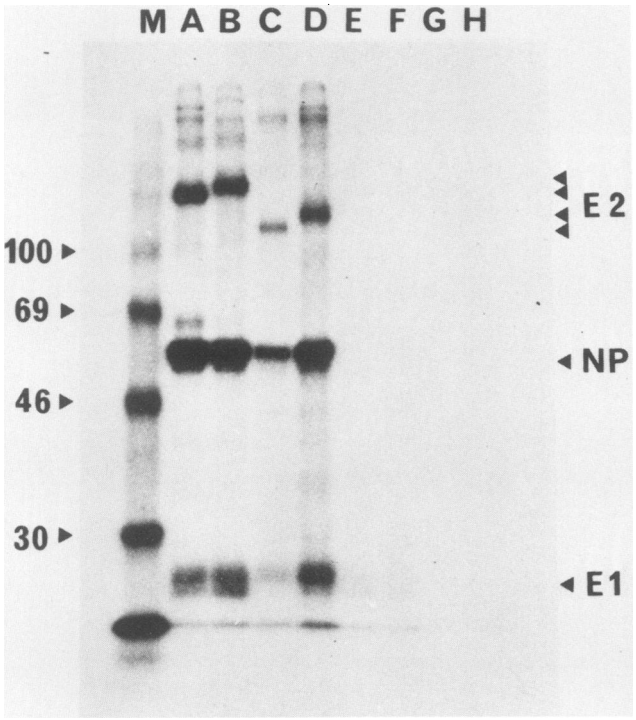

FIG. 6. Electrophoresis of intracellular virus-specific proteins. DBT cells were infected (MOI, 3), treated with tunicamycin, and labeled with [^{35}S]methionine, and cytoplasmic lysates were prepared and electrophoresed after immunoprecipitation as described in the text. Lanes A, C, E, and G, lysates from wild-type-infected cells; lanes B, D, F, and H, lysates from cl-2-infected cells; lanes C, D, G, and H, lysates from tunicamycin-treated cells; lanes A, B, C, and D, immunoprecipitated with anti-JHM rabbit serum; lanes E, F, G, and H, immunoprecipitated with normal rabbit serum. M, Molecular weight markers (Amersham, CFA 626); NP, nucleocapsid protein.

ing innumerable isolates from the wt virus population), and therefore we cannot at the moment provide a definitive answer to this question. However, it should be noted that RNA viruses are believed to have relatively high mutation rates (7) and that MHV has an RNA genome that is among the largest known.

The second and more important question is the nature of the selective advantage for viruses with the cl-2-like phenotype which results in preferential propagation in the brains of Lewis rats. There are a number of possibilities. First, the cl-2 like virus may be able to replicate in brain cells that the wt virus cannot infect. This hypothesis could be tested directly in primary cultures of defined brain cell types. It would be reasonable to think that such selectivity could be mediated by differences in the cl-2 virus E2 surface glycoprotein, since this protein is responsible for virus adsorption onto susceptible cells (4, 8, 9). The E2 protein is known to play a central role in determining the diversity of MHV strains as well as their virulence and tropisms (2, 34, 36), and recent reports indicate that variant MHV viruses derived from persistently infected cells have antigenically distinguishable E2 proteins (1). The demonstration that the sizes of the E2 proteins of cl-2 and wt virus differ is at least compatible with this idea.

Alternatively, the selective replication of cl-2 virus could be explained by other mechanisms. One could be simply that in animals the growth of cl-2-like virus is accelerated compared with that of wt virus. It has to be remembered that we have also observed a change in the size of mRNA 2 produced by cl-2-like virus infection. This mRNA encodes a 30,000-MW nonstructural protein of unknown function (26), and this gene product could be important in this respect.

Yet again, it could be that cl-2-like virus is more resistant than wt virus to host defense mechanisms, especially those generated in an early phase of infection. For example, we have tested the ability of macrophages, which are believed to play an important protective role during MHV-JHM infection of mice (30, 33), to support the growth of wt and cl-2 virus. However, neither virus was able to multiply or produce CPEs in rat macrophages (data not shown), so we cannot invoke this particular mechanism for the selection of cl-2-like virus in the brain. In conclusion, although we are not at present able to define the nature of the selective advantage, we believe that a detailed molecular and biological comparison of the genes and gene products of cl-2 virus and wt virus will provide important information relating to the viral parameters governing the "neurotropism" of MHV-JHM.

The final question to be addressed is the nature of the genetic differences between cl-2 virus and wt virus (which result in the altered mRNA profile and, at least for mRNA 3, an altered translation product) and the mechanism of their generation. Essentially, two possibilities seem plausible. First, the alterations may result from the insertion in the genomic RNA of cl-2 virus of additional sequences in the coding region of the E2 protein. If this were correct, however, it would be predicted that the genomic RNA of cl-2 virus should be larger than that of wt virus, which does not seem to be the case. Alternatively, the differences in the cl-2 virus mRNAs 2 and 3 compared with wt virus could be generated during the synthesis of these mRNAs, which is known to involve the fusion of noncontiguous transcripts (13, 28). We believe it is significant that the only difference detected in the oligonucleotide fingerprints of the cl-2 and wt virus genomes was an alteration of oligonucleotide 7, which has been previously mapped to a region at or near the 5′ end of MHV-JHM mRNA 3 (19, 29). There is also recent evidence suggesting that alternative combinations of noncontiguous transcripts can be fused during the generation of MHV mRNAs (19). Finally, the mRNA 3 oligonucleotide fingerprints of both viruses, which are identical, indicate either that the additional sequences found in cl-2 virus mRNA 3 contain no "characteristic" oligonucleotides or, for example, that they may be a repetition of sequences normally present in wt mRNA 3. These questions and their relevance to the transcription of MHV mRNAs can best be resolved by sequence analysis. These experiments are currently in progress.

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