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Murine Coronavirus Nonstructural Protein ns2 Is Not Essential for Virus Replication in Transformed Cells

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Two isolates of the murine hepatitis virus (MHV) strain JHM, which differed in their ability to express the nonstructural gene product ns2, were characterized. The MHV Wb3 isolate encodes a 30,000-molecular-weight ns2 protein that can be readily detected in infected cells by using a specific monoclonal antibody, MAb 2A. The MHV Wb1 isolate is a deletion mutant that lacks a functional ns2 gene and the transcriptional signals required for the synthesis of an ns2 mRNA. However, there are no obviously significant differences in the growth of the MHV Wb1 and MHV Wb3 isolates in continuous cell lines or in the synthesis of viral mRNAs or proteins in infected cells. These results demonstrate that the ns2 gene product is not essential for MHV replication in transformed murine cells and suggests that the function of the ns2 gene may only be manifest in vivo.

The coronaviruses are enveloped, positive-strand RNA viruses associated with diseases of economic importance in both animals and humans (41). In the last decade our understanding of coronavirus molecular biology has increased considerably; in this respect, the murine hepatitis virus (MHV) and the prototype coronavirus, infectious bronchitis virus, have been widely studied (for a recent review, see reference 34). The MHV virion has a genomic RNA of approximately 30 kilobases (21), a lipid envelope, and three or four major proteins: the nucleocapsid protein N (50 to 60 kilodaltons [kDa]), the membrane glycoprotein M (23 to 26 kDa), the spike or surface glycoprotein S (180 kDa), and, in some MHV strains, the hemagglutinin-esterase glycoprotein HE (65 kDa) (27). Within the genome, the structural protein genes are arranged in the order 5′ HE-S-M-N 3′ (34).

The expression of viral proteins in MHV-infected cells is mediated by a 3′-coterminal set of subgenomic mRNAs. The synthesis of these mRNAs involves a process of discontinuous transcription (1, 33), but is is not yet clear whether genomic- or subgenomic-length templates are used (14, 25). In general, each mRNA is translated into a single polypeptide that is encoded in the 5′ sequences absent in the smaller mRNAs (34). In addition to the structural genes, the MHV genome contains a number of open reading frames (ORFs) that encode proteins not found in the virion. The largest of these nonstructural genes is located at the 5′ end of the genome and is assumed to encode components of the viral RNA-dependent RNA polymerase (21, 32). Between the S and M genes there are 3 ORFs, ORF4, ORF5a, and ORF5b, which encode small (10- to 15-kDa) proteins, two of which, ns4 and ns5b, have been identified in infected cells (8, 15). Finally, proximal to the 5′ end of the HE gene, is a third ORF, ORF2, which encodes a polypeptide of 30 kDa. The predicted polypeptide has no potential *N*-glycosylation sites and lacks obvious membrane protein characteristics (17, 26).

The gene product of ORF2, now designated as ns2 (4a), was first detected by in vitro translation of mRNA 2 (16, 28). A protein of the same size was also detected in MHV-infected cells, but its identity remained unconfirmed (30). Recently, Zoltick et al. (44) and Bredenbeek et al. (4) used purified fusion proteins to obtain ns2-specific polyvalent antisera, with which they were then able to confirm the identity of the ns2 gene product and demonstrate that the ns2 protein is localized to the cytosol. Although synthesis of the ns2 protein continues throughout the infection, accumulation is limited because of a relatively short half-life. In common with most of the nonstructural gene products of MHV, the function of the ns2 protein is unknown. It has been noted that the predicted amino acid sequence reveals elements that resemble those proposed to be involved in nucleotide binding, and consequently it has been proposed that the protein may have a role in replication, for example, as an RNA-binding protein (17). In the experiments reported here, we were able to characterize an MHV isolate that did not express the ns2 protein, and our results clearly demonstrate that this gene product is not essential for virus replication in transformed murine cells in culture.

MATERIALS AND METHODS

Cells and virus. DBT cells are a continuous cell line derived from a Schmidt-Ruppin Rous sarcoma virus-induced mouse tumor (11). Sac(−) cells are a continuous cell line of Moloney sarcoma virus-transformed murine fibroblasts (42). Both lines were grown in monolayers in minimal essential medium (041 01095; GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal calf serum. Sac(−) cells were also grown in suspension in modified minimal essential medium (041 01385; GIBCO) containing 5% fetal calf serum.

The MHV isolates described in this study were derived from material originally supplied by L. P. Wiener. The original suckling mouse brain homogenate (MHV JHM SMB 7) was propagated twice in suckling mouse brain, plaque purified three times on L_{929} cells, and then grown for approximately 20 passages at low multiplicities of infection (MOI) in Sac(−) cells. This material, which was referred to as MHV JHM in publications before 1986, was used to isolate MHV Wb1 and MHV Wb3. Both isolates were plaque purified twice on DBT cells and amplified by minimal low-MOI passage to stocks of approximately 2×10^7 50% tissue culture infectious doses ($TCID_{50}$) per ml. The virus referred to as MHV JHM in our publications after 1986 is the MHV Wb1 isolate. The MHV A59 isolate was obtained from P.

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Carthew, plaque purified twice in DBT cells, and amplified as described above to a stock of 2×10^7 $TCID_{50}$/ml. Infectious virus titrations were performed by end point dilution and calculated as described by Reed and Muench (23).

cDNA cloning and sequence analysis. Poly(A)-containing RNA was obtained from MHV-infected Sac(−) cells as described previously (29). cDNA synthesis was essentially by the method of Gubler and Hofmann (9) with MHV-specific oligonucleotides as first-strand primers. The cDNAs 2/C3 and 2/G1 were derived from MHW Wb1 poly(A) RNA by using the primers 5′ TTA GAT TAT GCC TCA TGC 3′ (corresponding to positions 1307 to 1319+5 in the HE gene [26]) and 5′ ACG CTT CCT CAT CAT CTG 3′ (corresponding to positions 671 to 688 in the ns2 gene; see Fig. 1), respectively. The cDNA 2/A2 was derived from MHV Wb3 poly(A) RNA by using the primer 5′ ACC AGC TGA CAG CCA ATC 3′ (corresponding to positions 48 to 65 in the HE gene [26]). The synthesized double-stranded cDNAs were treated with T4 DNA polymerase, ligated into *Sma*I-linearized Bluescript pKSII$^+$ DNA (Stratagene, Heidelberg, Federal Republic of Germany), and used to transform competent *Escherichia coli* TG1 cells.

Recombinant clones were identified by colony hybridization with MHV-specific oligonucleotides, and the cDNA inserts were characterized by restriction endonuclease and sequence analyses. Sequencing was performed on single- and double-stranded DNA templates by the chain termination method. The sequences presented herein were determined completely on both cDNA strands. Sequence data were assembled with the programs of Staden (35) and analyzed with the programs of the University of Wisconsin Computer Genetics Group (7). MHV-specific oligonucleotides were synthesized by phosphoramidite chemistry on a Cyclone DNA synthesizer and purified by gel electrophoresis. Plasmid purification, agarose gel electrophoresis, and standard recombinant DNA procedures were performed as described by Maniatis et al. (19). Colony hybridizations were performed as described by Woods (43).

Construction and expression of fusion protein vectors. The 486-base-pair 3′ end of the ns2 gene was isolated as a 597-base-pair *Eco*RV-*Pvu*II fragment from the 2/C3 cDNA clone. This fragment was then cloned into the *E. coli* expression vector pEX2 (36), which had been digested with *Eco*RV and *Eco*RI to remove 1,890 base pairs from the 3′ end of the *cro-lacZ* gene. The fusion protein predicted from this construct consists of the amino-terminal 407 amino acids of the Cro–β-galactosidase fusion protein, two artifactual amino acids and the carboxy-terminal 161 amino acids of the ns2 gene product (63 kDa). This construct, ΔpEX2-ns2, along with a self-ligated *Eco*RV-*Eco*RI pEX2 DNA, designated as ΔpEX2, and the original pEX2 DNA were used to transform competent *E. coli* RR1 cells, which carried the pRK248 *c*Its plasmid (2). Thus, expression of the fusion protein was under the control of the temperature-sensitive λ *c*Its repressor.

Expression was induced by raising the temperature of the bacterial culture from 30 to 42°C during exponential growth. Analytical-scale (2- to 5-ml) lysates were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described by Crowl et al. (5). On a preparative scale, induced bacteria were pelleted, suspended in 1 mM Tris hydrochloride (pH 7.2)–15 mM NaCl, disrupted in a glass-bead milling machine (IMA, Dreieich, Federal Republic of Germany), and solubilized by boiling in reducing sample buffer (12) without bromophenol blue. Insoluble material was removed by centrifugation, and the lysates were dialyzed against 1 mM Tris hydrochloride (pH 7.2)–15 mM NaCl to remove SDS and β-mercaptoethanol. The fusion protein used for mouse inoculation was, in addition, partially purified by SDS-PAGE and electroelution before dialysis.

MAbs. Three-month-old female BALB/c mice were inoculated four times with the partially purified truncated Cro–β-galactosidase–ns2 fusion protein (approximately 60 to 260 μg of protein per inoculation) by a variety of routes over a 14-week period. Three days after the final inoculation, the spleen cells of one mouse were fused to NS1 plasmacytoma cells by using polyethylene glycol. Hybridomas, selected in RPMI 1640 medium (041-2400; GIBCO) containing 10% fetal calf serum and 1× HAT supplement (043-1060; GIBCO), were screened for ns2 specific activity by an enzyme-linked immunosorbent assay (40) with the truncated Cro–β-galactosidase or truncated Cro–β-galactosidase–ns2 fusion proteins as capture antigens. Hybridomas secreting antibodies specific for the ns2 polypeptide were cloned twice by limiting dilution. Two control antibodies used in this study were an MHV JHM nucleocapsid protein-specific monoclonal antibody (MAb), 556 (H. Wege, unpublished), and an MHV surface protein-specific MAb, 30B (E. Routledge, submitted for publication), both of which react in immunoprecipitation and Western immunoblotting with their respective antigens (data not shown).

SDS-PAGE and immunoblotting. SDS-PAGE was performed under reducing conditions on 15% polyacrylamide gels by the method of Laemmli (12). Western blotting was carried out as described by Samson et al. (24). Briefly, blots were stained with undiluted hybridoma tissue culture supernatant, followed by peroxidase-linked rabbit anti-mouse immunoglobulin and the substrate 4-chloronaphthol.

Molecular weight markers were visualized by Coomassie blue or India ink staining and by autoradiography. The markers used were ^{14}C-labeled methylated myosin (200 kDa), α-2-macroglobulin (170 kDa), ^{14}C-labeled methylated phosphorylase *b* and phosphorylase *b* (97.4 kDa), ^{14}C-labeled methylated bovine serum albumin (69 kDa), glutamate dehydrogenase (55.4 kDa), ^{14}C-labeled methylated ovalbumin (46 kDa), lactate dehydrogenase (36.5 kDa), ^{14}C-labeled methylated carbonic anhydrase (30 kDa), trypsin inhibitor (soybean, 20 kDa), and ^{14}C-labeled methylated lysozyme (14.3 kDa).

Virus infection, labeling, and cell lysates. DBT cell monolayers were infected with MHV at an MOI of 6 $TCID_{50}$ per cell as previously described (29). To label intracellular polypeptides, the medium was replaced for 1 h with methionine-free medium containing 2% dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine (SJ 204, 800 Ci/mmol; Amersham Buchler) per ml. To prepare cell lysates, labeled or unlabeled cell monolayers were washed twice with ice-cold phosphate-buffered saline, scraped into phosphate-buffered saline, and pelleted at $800 \times g$ for 2 min. The cells were then lysed at 4°C in 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–0.2% Nonidet P-40–500 U of aprotinin per ml (TNPA buffer). The cytoplasmic lysates were centrifuged at $10,000 \times g$ for 2 min, and the supernatants were stored at −70°C.

Cell-free protein synthesis. Poly(A) RNA was obtained from MHV-infected Sac(−) cells as described previously (29). For cell-free protein synthesis, 1 μg of RNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine as previously described (28).

Immunoprecipitation. Cytoplasmic lysates or in vitro

translation products were incubated with 2 volumes of TNPA buffer containing 1 mg of bovine serum albumin per ml and 1 volume of hybridoma tissue culture supernatant for 3 h at 4°C. The immunocomplexes were adsorbed to a suspension of protein A-Sepharose, washed three times with 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–5 mM EDTA–0.05% Nonidet P-40, and heated in reducing sample buffer for 5 min at 55°C. After removal of the Sepharose, the immunocomplexes were incubated at 100°C for 2 min and analyzed by SDS-PAGE.

Northern blots. Poly(A) RNA from MHV-infected Sac(−) cells was electrophoresed in 1% agarose gels containing formaldehyde, subjected to mild alkaline hydrolysis in situ, and transferred to nitrocellulose filters (19). After baking at 80°C for 2 h, the filters were prehybridized at 42°C for 4 h in 50% formamide–1× Denhardt solution–5× SSC (1× SSC is 150 mM NaCl–10 mM sodium acetate [pH 7.0])–250 μg of denatured herring sperm DNA per ml. Hybridizations were performed under the same conditions for 16 h with a nick-translated DNA probe, pSS 38, which is a cDNA copy of the MHV JHM nucleocapsid gene (31), or a ^{32}P-labeled oligonucleotide corresponding to positions 97 through 114 in the MHV Wb3 ns2 gene (Fig. 1). The filters were washed twice for 30 min in 0.2× SSC–0.1% SDS at 55°C (cDNA) or 45°C (oligonucleotide) and autoradiographed.

Nucleotide sequence accession number. The sequence data presented in this article appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession number M34035.

RESULTS

Sequence analysis of the MHV Wb1 and MHV Wb3 ns2 genes. Sequence analysis of the cDNA clones encompassing the ns2 gene regions of the MHV Wb1 and MHV Wb3 isolates is shown in Fig. 1. These data indicate that the MHV Wb3 genome contains a 265-amino-acid ORF that is identical to the MHV ORF2a reported by Shieh et al. (26). In contrast, the MHV Wb1 genome contains a 318-base deletion that extends from a position 19 bases upstream of the ns2 gene to a position 299 bases within the ns2 gene. This deletion encompasses the consensus region of homology, which is upstream of the ns2 gene and is believed to have an essential role in the synthesis of MHV mRNAs (13). Thus, the MHV Wb1 isolate does not have a complete ns2 gene or the transcriptional signals required for the synthesis of an mRNA that is able to express the gene product. Also shown for comparison in Fig. 1 is the MHV A59 ns2 gene sequence (17), which indicates that, compared with MHV Wb3, the MHV A59 ns2 ORF is truncated by 4 amino acids at the amino terminus.

Isolation of ns2 protein-specific MAbs. The expression of fusion proteins from the ΔpEX2 (truncated Cro–β-galactosidase), pEX2 (Cro–β-galactosidase), and ΔpEX2-ns2 (truncated Cro–β-galactosidase–ns2) constructs was analyzed by SDS-PAGE of bacterial lysates. In the case of ΔpEX2 and pEX2 induction resulted, as expected (36), in the expression of proteins of 53 and 117 kDa respectively. In *E. coli* cells that carry the ΔpEX2-ns2 construct, a protein of 63 kDa is constitutively expressed. The identity of all fusion proteins was confirmed by Western blotting and immunodetection with a hyperimmune rabbit antiserum with a high titer of anti β-galactosidase antibodies (8; data not shown). With the truncated Cro–β-galactosidase–ns2 fusion protein as an antigen, 10 hybridoma cell lines secreting ns2-specific MAbs were obtained by standard procedures. The isotypes of the MAbs were determined, and their reactivity in Western blots and immunoprecipitation was established (data not shown). One MAb, designated 2A, was used in the experiments described below.

Identification of the ns2 gene product in vitro and in vivo. Poly(A) RNA from MHV Wb1-, Wb3-, and A59-infected Sac(−) cells was translated in a reticulocyte lysate, and the products were analyzed by SDS-PAGE. The poly(A) RNA from Wb3- and A59-infected cells directed the synthesis of a polypeptide of approximately 30 kDa (Fig. 2A, lanes 2 and 3), which was immunoprecipitated by the ns2-specific MAb 2A (lanes 6 and 7). This protein was not found among the translation products directed by MHV Wb1 poly(A) RNA (lanes 4 and 8). Analysis of the polypeptides synthesized in MHV Wb1-, MHV Wb3-, and MHV A59-infected cells also led to a similar conclusion (Fig. 2B). In MHV Wb3- and MHV A59-infected cells a 30 kDa polypeptide was synthesized (lanes 1 and 2), and this protein could be specifically immunoprecipitated with the ns2-specific MAb (lanes 5 and 6).

The specificity of the immunoprecipitations shown in Fig. 2 was confirmed by using tissue culture supernatants from NS1 cells and an MHV JHM N protein-specific hybridoma cell line (data not shown). These results confirmed the identity of the MHV ns2 gene product and showed that the MHV Wb1 isolate was deficient in ns2 protein synthesis. It is also evident from Fig. 2 that, compared with MHV Wb3, the MHV A59 ns2 protein has a slightly faster electrophoretic mobility. This would be consistent with the differences in the lengths of the predicted ns2 proteins (Fig. 1) and suggests that synthesis of the proteins is initiated at the 5′-proximal AUG of mRNA2 in both cases.

Immunoblotting of cytoplasmic lysates from MHV Wb1- and MHV Wb3-infected cells. To demonstrate that the MHV Wb1 isolate is deficient in ns2 protein expression throughout the replication cycle, we performed a Western blot analysis of cytoplasmic lysates from infected cells. The ns2 protein was first detected in MHV Wb3-infected cells approximately 6 h postinfection (Fig. 3B). A steady-state level was achieved by 8 h postinfection, and the protein did not accumulate further. No ns2-related polypeptides could be detected in MHV Wb1-infected cells, irrespective of the time of infection (Fig. 3D). This contrasted with the accumulation of S protein-related polypeptides (pre-S, S, and S_1), which was essentially identical in both infections (Fig. 3A and C). These results confirm again that the MHV Wb1 isolate is not able to express the ns2 protein.

Comparison of MHV Wb1 and MHV Wb3 replication. Having established that the MHV Wb1 isolate is deficient in ns2 protein expression, we decided to analyze in detail the replication of this virus and compare it with that of the MHV Wb3 isolate, which has a functional ns2 gene. We made a one-step growth curve for both viruses and analyzed viral RNA and protein synthesis in infected cells. In DBT cells infected at an MOI of 6 $TCID_{50}$ per cell, the kinetics of replication for both viruses were very similar (Fig. 4). The development of syncytia, the characteristic cytopathic effect of MHV, was slightly accelerated in MHV Wb3-infected cells, and the final titer of infectious virus released to the medium was reproducibly higher. However, these effects were not dramatic.

Analysis of the viral RNA synthesized in MHV Wb1- or MHV Wb3-infected cells also did not reveal any obviously significant differences. The well-characterized MHV subgenomic mRNAs (mRNA1 to mRNA7 [34]) were detected in approximately equivalent amounts in both infections (Fig. 5,

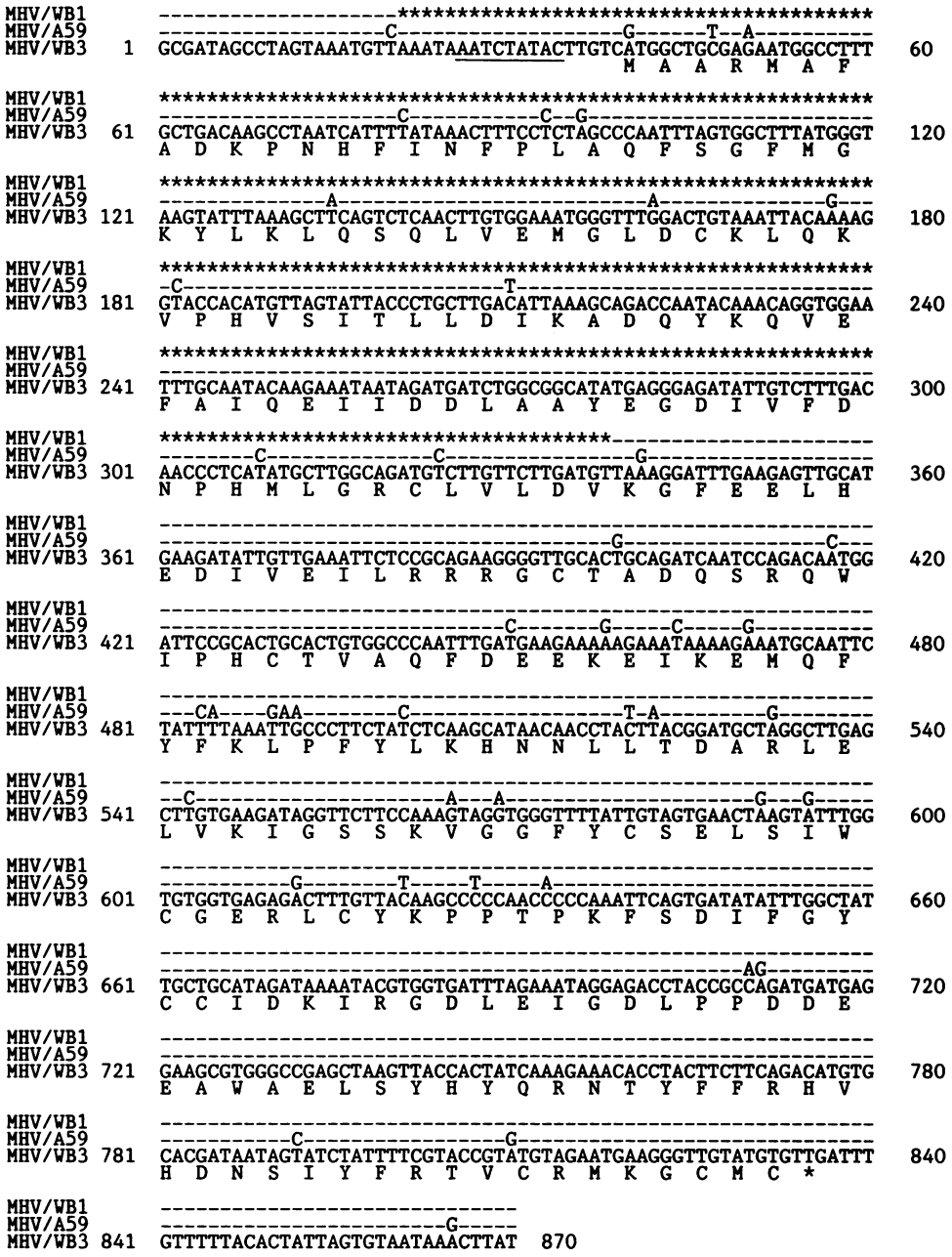

FIG. 1. Nucleotide sequence of the cDNA clones encompassing the ns2 gene regions of MHV Wb1 and MHV Wb3. The nucleotide sequence of the flanking regions and ns2 gene of MHV Wb3 is shown, together with a translation of the ns2 ORF. Differences (A, C, G, T) and identities (— —) with the MHV A59 ns2 gene sequence (17) and the MHV Wb1 sequence are indicated above the MHV Wb3 sequence. Deleted nucleotides are indicated by an asterisk (*). The region of homology preceding the ns2 gene (26) is underlined. The nucleotide numbering is arbitrary.

lanes 1 and 2). One expected difference was the absence of mRNA2 in the MHV Wb1 poly(A) RNA population (lane 2), but otherwise the major mRNAs (species 3, 6, and 7) as well as the minor mRNAs (species 1, 4, and 5) were equally represented. Also shown in Fig. 5 is a Northern blot of MHV A59 poly(A) RNA (lane 3). The pattern of subgenomic mRNAs was very similar to that of MHV JHM, and an mRNA2 capable of encoding the ns2 protein was detected (lane 3). The same RNAs were also probed with an oligonucleotide corresponding to the region deleted in the MHV Wb1 ns2 gene. As expected, this probe detected mRNAs 1 and 2 for MHV Wb3 (lane 4) but failed to hybridize to MHV Wb1 mRNA (lane 5).

Finally, we analyzed the synthesis of viral polypeptides in MHV Wb1- and MHV Wb3-infected cells (Fig. 6). Again, we were unable to detect any obviously significant differences. In both infections the synthesis of viral polypeptides followed approximately the same time course, and the relative rates of synthesis of the polypeptides that could be readily detected (N, M, S, and ns4) were similar. Also, the MHV-

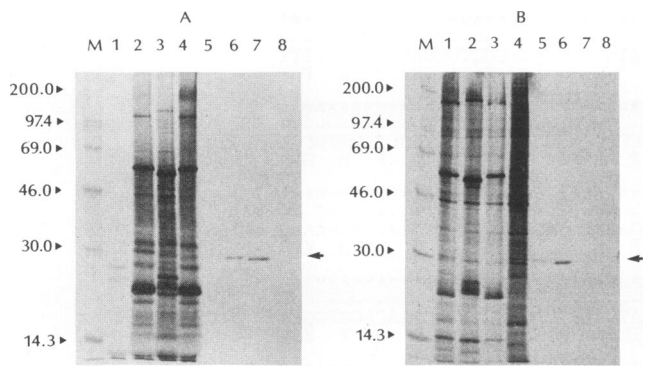

FIG. 2. Identification of the MHV ns2 protein in vitro and in vivo. (A) The ^{35}S-labeled in vitro translation products directed by poly(A) RNA isolated from Sac(−) cells infected with MHV Wb3 (lanes 2 and 6), MHV A59 (lanes 3 and 7), or MHV Wb1 (lanes 4 and 8) were analyzed by SDS-PAGE directly (lanes 2, 3 and 4) or after immunoprecipitation with the ns2-specific MAb 2A (lanes 6, 7, and 8). A control translation with H_2O (lanes 1 and 5) and molecular weight markers (lane M) are also shown. The position of the ns2 (30 K) protein is indicated (◄). (B) [^{35}S]Methionine-labeled cytoplasmic lysates from DBT cells infected with MHV Wb3 (lanes 1 and 5), MHV A59 (lanes 2 and 6), or MHV Wb1 (lanes 3 and 7) were analyzed by SDS-PAGE directly (lanes 1, 2, and 3) or after immunoprecipitation with the ns2-specific MAb 2A (lanes 5, 6, and 7). Control lysates from mock-infected cells (lanes 4 and 8) and molecular weight markers (lane M) are also shown. The position of the ns2 (30-kDa) protein is indicated (◄).

mediated shutoff of host-cell protein synthesis (29) was equally evident in the later stages of both infections.

DISCUSSION

On the basis of the results presented in this article, three important conclusions can be reached. First, we determined the sequence of the MHV JHM ns2 gene and identified and characterized the ns2 gene product in infected cells. These data confirm and extend our own earlier work (28, 30) and the more recent studies of Shieh et al. (26), Zoltick et al. (44), and Bredenbeek et al. (4).

Second, our data indicate that the MHV genome can be subject to an unusual degree of deletion mutation. In the MHV Wb1 isolate, we identified for the first time a deletion in a nonstructural MHV gene. However, Taguchi et al. (37, 38) and Morris et al. (20) previously demonstrated that there is considerable variability in the size of the MHV S protein gene, and recently Buchmeier and his collegues (22) identified a region in the MHV S protein gene that is heterogeneous with respect to deletions. Also, there is evidence from comparative sequence data that deletion mutation has occured in the 3′ noncoding regions of the infectious bronchitis virus M41 and human coronavirus 229E genomes (3, 20a). At the moment we do not know how these deletion mutations affect the outcome of an in vivo infection, but it is possible that factors such as tropism, virulence, and cytopathology might be altered. Also the mechanism of deletion mutation is not clear. It has been proposed that the transcription of MHV RNA is a discontinuous, nonprocessive event in which deletions might occur as a result of inaccurate reinitiation during template switching (22). Lai and his collegues have shown that during MHV replication recombination

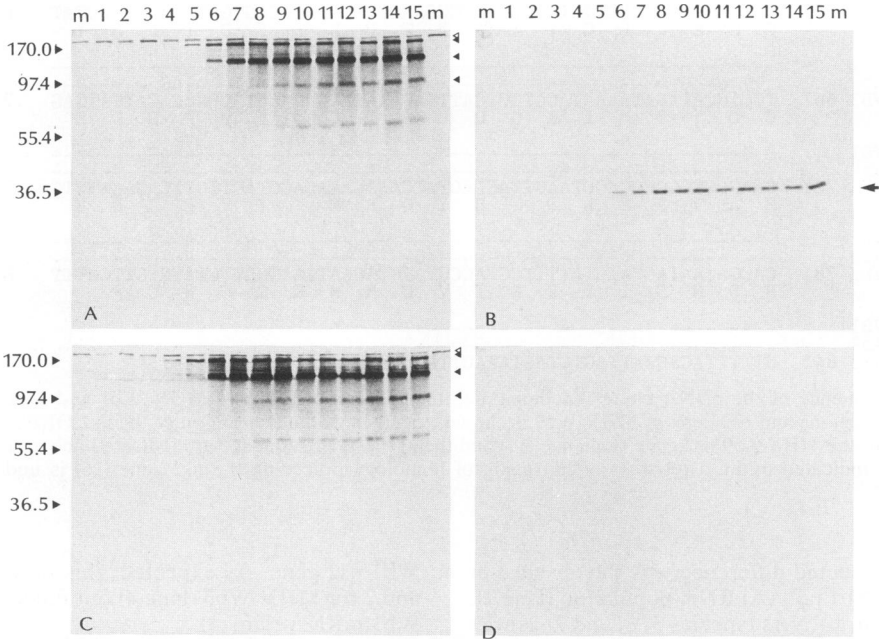

FIG. 3. Western blot analysis of cytoplasmic lysates from MHV Wb1- and MHV Wb3-infected cells. Cytoplasmic lysates were prepared from DBT cells infected with MHV Wb3 (A and B) or MHV Wb1 (C and D) at 1-h intervals (lanes 1 through 15) after infection. The lysates were analyzed by SDS-PAGE, transferred to nitrocellulose filters, and immunostained with the MHV S protein-specific MAb 30B (A and C) or the MHV ns2 protein-specific MAb 2A (B and D). Lysates from mock-infected cells (lanes m), the positions of molecular weight markers stained on the nitrocellulose filters by India ink, and the positions of S protein-related polypeptides (◄) are indicated. The S protein MAb used in this experiment also cross-reacts with a high-molecular-weight cellular polypeptide (E. Routledge, submitted), which is indicated (◁).

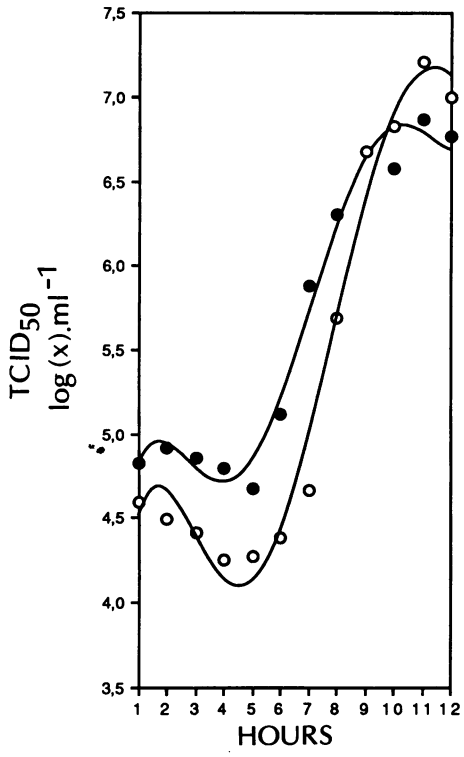

FIG. 4. One-step growth curve of MHV Wb3 and MHV Wb1 in DBT cells. DBT cells were infected at an MOI of 6 $TCID_{50}$ per cell with MHV WB1 (●) or MHV Wb3 (○), and the titer of infectious virus accumulating in the culture medium was determined at hourly intervals.

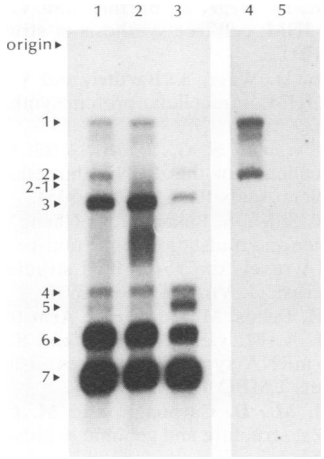

FIG. 5. Northern blot analysis of poly(A) RNA in MHV Wb1-, MHV Wb3-, and MHV A59-infected Sac(−) cells. Poly(A) RNA was isolated from Sac(−) cells that had been infected with MHV Wb3 (lanes 1 and 4), MHV Wb1 (lanes 2 and 5), or MHV A59 (lane 3) 16 h previously at an MOI of 0.5 $TCID_{50}$ per cell. The RNA was transferred to nitrocellulose filters and hybridized to ^{32}P-labeled pSS38 DNA (31) (lanes 1, 2, and 3) or a ^{32}P-labeled oligonucleotide corresponding to the region deleted in the MHV Wb1 ns2 gene (lanes 4 and 5). After washing, the filters were autoradiographed. The positions of the MHV subgenomic mRNAs (34) are indicated (▶) and numbered in order of decreasing size.

occurs in vivo and in vitro at relatively high frequencies (10, 18), and these two sources of genetic variation may be related.

The third important conclusion we have reached is that the MHV ns2 gene product is not essential for virus replication in continuous cell culture. This would suggest that either the ns2 gene function is not required or it is duplicated in transformed cells. In this context parallels can be drawn to the MHV HE protein. Luytjes et al. (17) have shown that this structural protein gene is not functional in the MHV A59 strain studied in their laboratory. However, there is ample evidence with specific inhibitors of the HE protein acetylesterase function (39) or HE-specific MAbs (6) that this gene product plays an important role in coronavirus infections. In any case, our results lead to the conclusion that the function of the ns2 gene product will not be elucidated by studies in continuous cell lines with, for example, as has been suggested, temperature-sensitive mutants and a recombinant ns2 protein (4, 44). We believe that the ns2 gene function will only be manifested in primary cell cultures or in vivo; based upon this reasoning, experiments are now in progress with

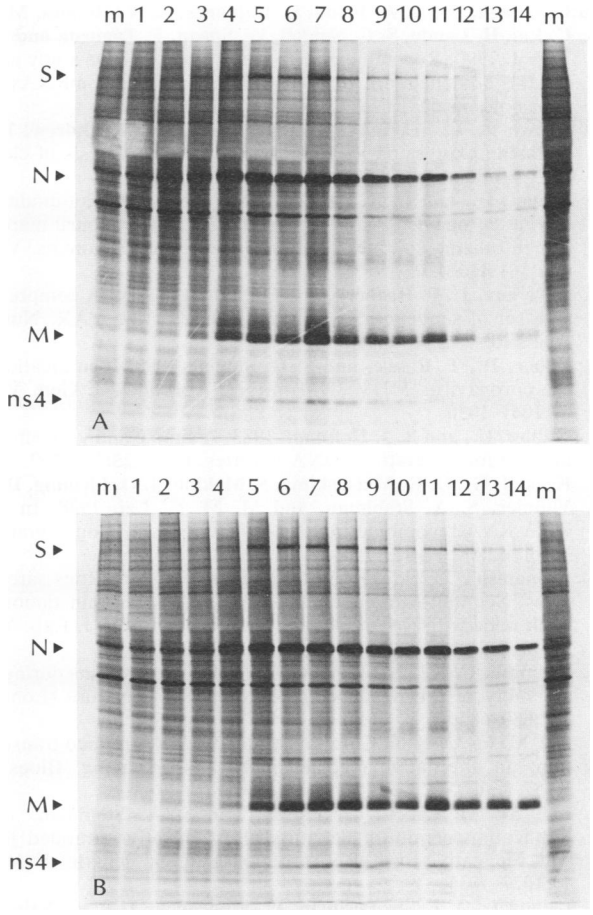

FIG. 6. Intracellular protein synthesis in MHV Wb1- and MHV Wb3-infected DBT cells. DBT cells were infected with MHV Wb1 (A) or MHV Wb3 (B) at an MOI of 6 $TCID_{50}$ per cell, and [^{35}S]methionine-labeled cytoplasmic lysates were prepared at hourly intervals (lanes 1 through 14). The lysates were analyzed directly by SDS-PAGE and autoradiography. Lysates from mock-infected cells (lanes m) and the positions of the MHV intracellular polypeptides S, N, M, and ns4 are also indicated (▶).

the isolates and ns2-specific reagents described in this report.

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