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Protein Synthesis in Cells Infected by Murine Hepatitis Viruses JHM and A59: Tryptic Peptide Analysis

By

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With 8 Figures

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Summary

The structural and intracellular proteins of the murine hepatitis viruses MHV-JHM and MHV-A59 were studied by tryptic peptide mapping. The results demonstrated that the virions contained three distinct proteins: the two related chains of the E2 complex, the nucleocapsid protein and the heterogeneous E1 complex. Five distinct virus-specific proteins were synthesized by infected cells. Three of the five intracellular proteins contained tryptic-peptides with properties similar to the three structural proteins. Models describing the evolution of the proteins are proposed. Although the pathogenic properties of MHV-JHM and MHV-A59 differ greatly, the tryptic peptide maps of the corresponding proteins of these MHV strains were remarkably similar.

Introduction

The *Coronaviridae* is a family of RNA viruses which cause a wide variety of diseases in many species of animals, including man. The murine hepatitis virus (MHV) group includes viruses which cause diseases in mice, including hepatitis, chronic and acute neurological diseases, enteritis and nephritis (15). The JHM strain of MHV is a strongly neurotropic agent which produces an acute encephalomyelitis in mice a few days after intracerebral or intranasal inoculation (11, 15, 17, 29). The A59 strain of MHV is strongly hepatotropic, but weakly neurotropic (15, 17).

MHV particles contain a large (6.1×10^6 daltons) single-stranded RNA genome with positive polarity (12, 13, 15). Cells infected by MHV contain

at least seven virus-specific RNA species that function as mRNAs (12, 13, 20, 22). Five of the seven mRNAs have been translated *in vitro* and coding assignments have been made (13, 20).

Five to nine virus-specific proteins have been identified in cells infected by MHV-JHM, MHV-A59 or MHV-3 (1, 3, 5, 18, 21). The virus-specific proteins were identified by comparison of immunoprecipitates of infected and mock-infected cell lysates with purified virions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Sturman (26) proposed a model for the structure of MHV-A59. Three structural proteins were described; the nucleocapsid phosphoprotein (N), the transmembrane glycoprotein (E1) and the peplomeric glycoprotein complex (E2), which consists of two related proteins.

Siddell (19) analyzed the structural and intracellular proteins of MHV-JHM by tryptic peptide mapping of ^{35}S-methionine-labeled proteins. The results indicated that six distinct virus-specific proteins were synthesized by infected cells during the acute infection. The virus particles contained four of these proteins.

Weiss and Leibowitz (31) demonstrated by molecular hybridization that the genomes of MHV strains JHM and A59 are closely related in sequence. The molecular weights of the MHV-JHM and MHV-A59 proteins are similar by SDS-PAGE (2, 3), yet the pathogenic properties of these two viruses are strikingly different (15, 17). In this communication we present tryptic peptide maps of MHV-JHM and MHV-A59 proteins.

Materials and Methods

Cells and Virus

The sources and conditions for growing the murine cell lines 17Cl-1 and DBT have been described (3, 7, 16, 28). The sources and preparation of the stocks of MHV-JHM and MHV-A59 have also been described (3). Cells were infected in suspension at a multiplicity of infection (MOI) of 0.3 plaque-forming units (PFU) per cell, and virus was adsorbed at 37° C for 30 minutes at a concentration of 10^7 cells per ml. Infected cells were centrifuged, resuspended in prewarmed Dulbecco's modified Eagle's medium supplemented with 2 percent fetal bovine serum (DME 2) at a concentration of 2×10^6 cells per ml, plated into plastic culture dishes at a concentration of 3×10^5 cells per cm^2, and incubated at 37° C.

Preparation of Radiolabeled Virion Proteins

Cells (17Cl-1) were infected with MHV-JHM or MHV-A59 as described above and incubated at 33° C. At 6 hours postinfection (PI), the infected cells were washed twice with DME 2 containing 1/5 the normal amount of methionine and labeled with ^{35}S-methionine (50 μCi/ml) in the same medium. The cell culture fluid was harvested at 16—18 hours p.i. and cell-associated virus was released by 2 cycles of freeze-thawing. The virus particles were purified (12), disrupted and prepared for SDS-PAGE.

Radiolabeling of Intracellular Proteins

The infected monolayers of DBT cells or 17Cl-1 cells were washed twice with methionine-free DME 2, and radiolabeled with ^{35}S-methionine (200 μCi/ml) in methionine-

free DME 2 from 6 to 8 hours p.i. Then, the cell layers were washed twice with serum-free complete medium (DME 0) and lysed *in situ* at 0° C with buffer B 10 [10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 percent (vol/vol) NP 40, 0.1 percent (wt/vol) sodium dodecyl sulfate (SDS), 1 percent (vol/vol) Aprotinin, 50 μg/ml ribonuclease, 50 μg/ml deoxyribonuclease]. The cytoplasmic lysates were prepared for SDS-PAGE.

SDS-PAGE and Tryptic Peptide Mapping

SDS-PAGE was done as described by LAEMMLI and FAVRE (9). The analytical gels were processed for fluorography using EN^{3}HANCE (New England Nuclear) and exposed to preflashed Kodak XAR-2 X-ray film (10). Preparative gels of infected 17 Cl-1 cell lysates for tryptic peptide mapping were dried without fixation and exposed directly to Kodak XAR-2 X-ray film to locate the bands of interest. The bands were excised from the dried gel, rehydrated and electroeluted (32). A portion of each eluted protein was reanalyzed by SDS-PAGE to ensure that the band was free of cross-contamination from adjacent bands. The purified proteins were digested with trypsin and peptide maps were prepared (6, 13).

Results

MHV Structural Proteins

Virus particles radiolabeled with ^{35}S-methionine were purified from the medium of 17 Cl-1 cells infected by MHV-A 59. The virus was disrupted and analyzed by SDS-PAGE (Fig. 1). Three proteins of 180, 90 and 60

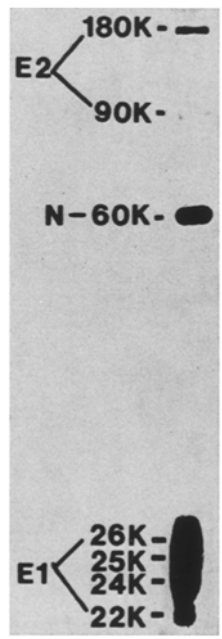

Fig. 1. Autoradiogram of the structural proteins of MHV-A 59. The autoradiogram was overexposed to detect the E 2-90 K species and to demonstrate that no other methionine containing proteins were evident

kilodaltons and a heterogeneous protein of 22 to 26 kilodaltons were resolved. The gel was overexposed to demonstrate that no other major methionine-containing proteins were detectable in the virus particles. On the basis of molecular weight, the 180 and 90 kilodalton proteins were equivalent to the E2 peplomeric complex described by Sturman and Holmes (25, 26, 27). The 22 to 26 kilodalton complex was equivalent to the E1 envelope protein complex (25, 26, 27). In repeated experiments, the 22 kilodalton protein was resolvable from the 24 to 26 kilodalton complex. However, the latter complex could not be resolved into discrete bands. The 60 kilodalton protein was equivalent to the nucleocapsid protein, N (25, 26, 27). Virus particles were purified from the culture fluids of 17Cl-1 cells infected by MHV-JHM and analyzed in the same manner (data not shown). Similar results were obtained, except the N protein of MHV-JHM was 63 kilodaltons instead of 60 kilodaltons.

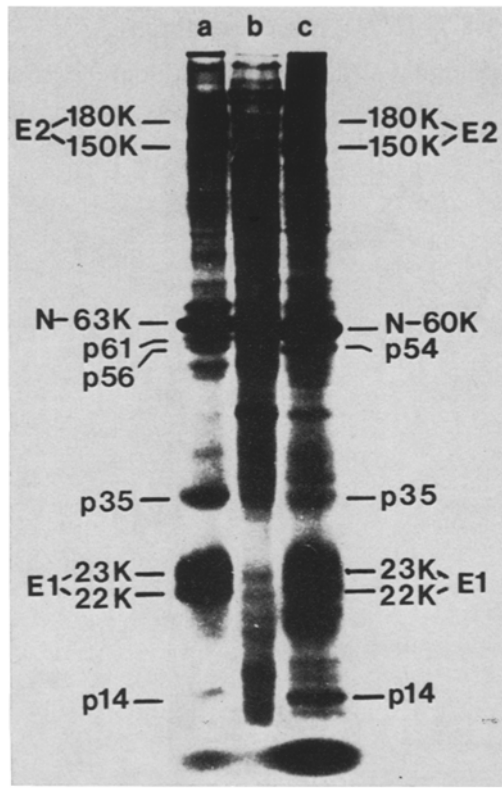

Fig. 2. Autoradigram of the MHV-specific proteins synthesized by DBT cells infected by MHV-JHM or MHV-A59. Cells (DBT) were infected by MHV-JHM (lane *a*) or MHV-A59 (lane *c*) or mock-infected (lane *b*), radiolabeled with ^{35}S-methionine, and lysates were analyzed by SDS-PAGE on a 7.5 to 15 percent gradient gel

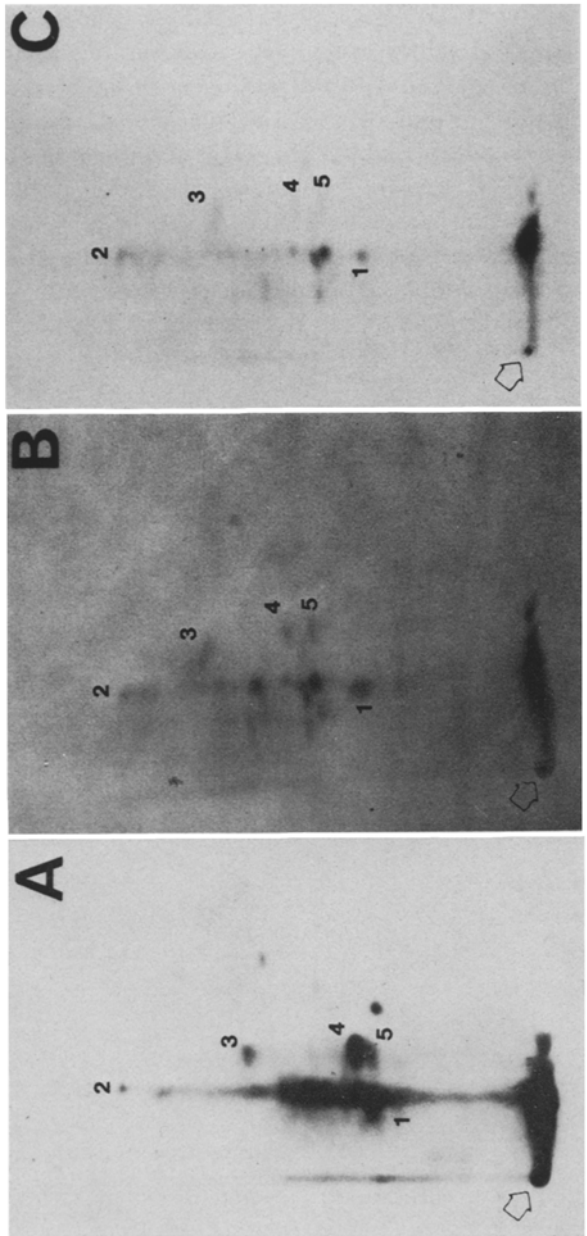

Fig. 3. Tryptic peptide maps of E2 proteins of MHV-A59. The intracellular E2-150K species (panel *A*) was purified by SDS-PAGE from a lysate of 17Cl-1 cells infected by MHV-A59, eluted, digested with trypsin and mapped. Maps of the E2-180K species (panel *B*) and E2-90K species (panel *C*) from purified MHV-A59 particles were prepared by the same procedure. Origins are indicated by arrows and positions of common peptides are numbered

Intracellular MHV-Specific Proteins

DBT cells infected with MHV-JHM or MHV-A59 or mock-infected were radiolabeled with ^{35}S-methionine. The cell lysates were analyzed by gradient SDS-PAGE (Fig. 2). MHV-specific proteins were readily observable in the lanes of infected cell lysates due to the significant decrease in cellular protein synthesis compared to the lane of mock-infected cell lysate. Eight MHV-A59-specific proteins of 180, 150, 60, 57, 54, 35, 23, 22 and 14 kilodaltons were detectable. Eight MHV-JHM-specific proteins of 180, 150, 63, 61, 56, 35, 23, 22 and 14 kilodaltons were detectable. Similar results were obtained in our previous studies when lysates or immunoprecipitates of lysates of infected 17Cl-1 cells were analyzed by SDS-PAGE (2, 3). However, the 180 kilodalton protein was not detectable in 17Cl-1 cells infected by either MHV-A59 or MHV-JHM.

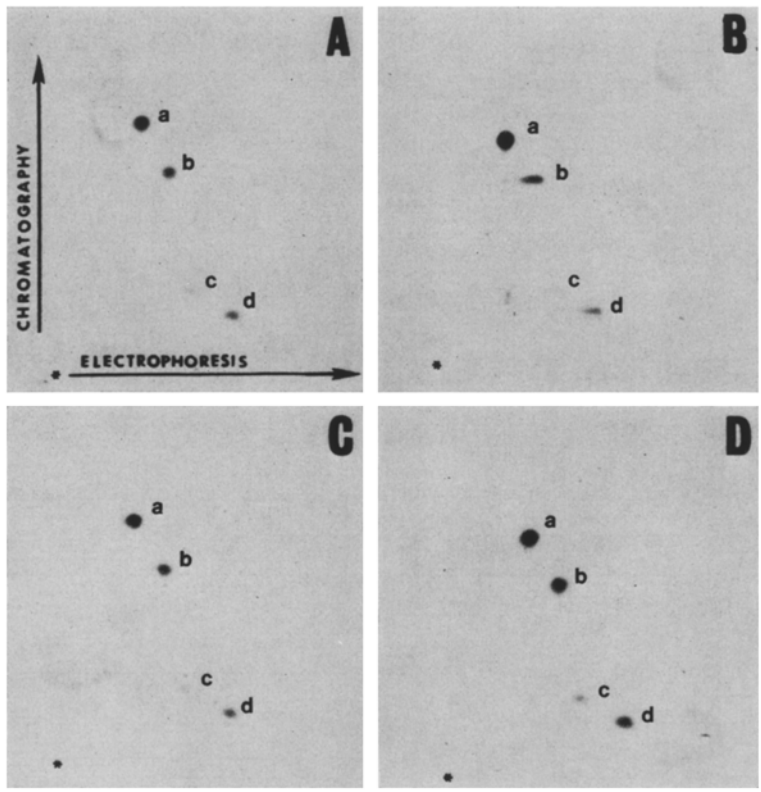

Fig. 4. Tryptic peptide maps of nucleocapsid (N) proteins of MHV-A59. The intracellular nucleocapsid-related proteins (N-60K, panel *A*; p54, panel *C*; p57, panel *D*) were purified by SDS-PAGE from a lysate of 17Cl-1 cells infected by MHV-A59. The map of the nucleocapsid protein N-60K (panel *B*) from purified virus particles was prepared the same way. Origins are indicated by asterisks and positions of common peptides are lettered

Tryptic Peptide Maps of MHV-Specific Proteins

MHV-specific proteins radiolabeled with ^{35}S-methionine were excised and eluted from SDS-polyacrylamide gels of purified virus particles or of lysates of 17Cl-1 cells infected by either MHV-A59 or MHV-JHM. The eluted proteins, which were homogeneous by SDS-PAGE, were digested with trypsin and tryptic peptide maps were prepared.

The structure of E2-150K from MHV-A59 infected cells was compared to E2-180K and E2-90K from purified MHV-A59 particles (Fig. 3). The tryptic maps of the E2-180K (panel B) and E2-90K (panel C) species were essentially the same. The tryptic map of the E2-150K (panel A) species was similar to the former species, but appeared to be more complex.

The nucleocapsid protein of MHV-A59 (N-60K) was smaller than the nucleocapsid protein of MHV-JHM (N-63K) (Fig. 2, ref. 2, 3). Two proteins

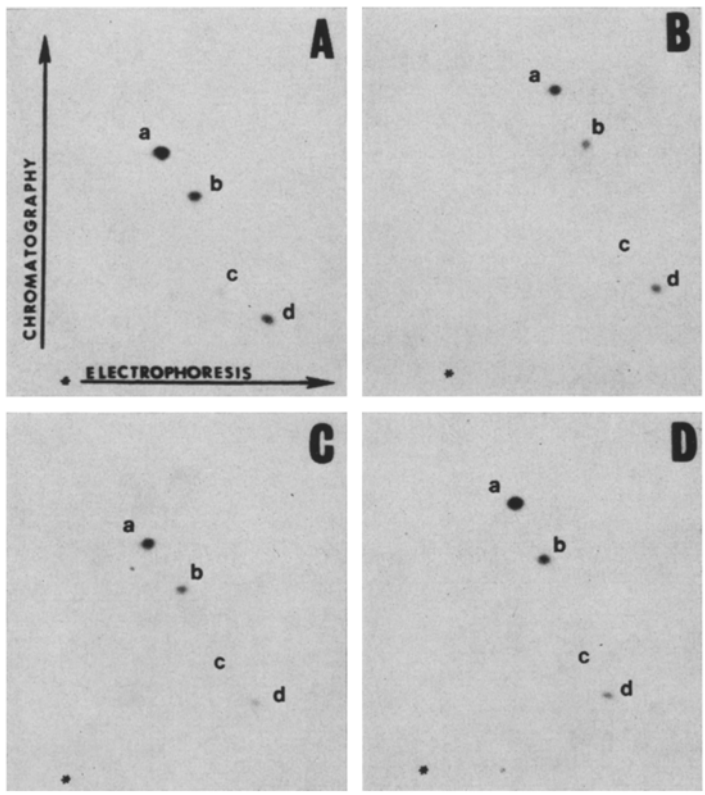

Fig. 5. Tryptic peptide map of nucleocapsid (N) proteins of MHV-JHM. The intracellular nucleocapsid-related proteins (N-63K, panel *A*; p61, panel *C*; p56, panel *D*) were purified by SDS-PAGE from a lysate of 17Cl-1 cells infected by MHV-JHM. The map of the N-63K nucleocapsid protein (panel *B*) from purified virus particles was prepared by the same procedure. Origins are indicated by asterisks and positions of common peptides are lettered

that migrated slightly faster than the nucleocapsid proteins (MHV-A59: p57, p54; MHV-JHM: p61, p56) were regularly detected in infected cell lysates (Fig. 2, ref. 2, 3). The structures of the three intracellular proteins N-60K, p57, p54 and the virion nucleocapsid protein N-60K were compared by tryptic peptide mapping (Fig. 4) as were the corresponding proteins of MHV-JHM (Fig. 5). The tryptic peptide maps of the virion and intracellular nucleocapsid proteins were nearly identical. The same conclusion was drawn from the tryptic peptide maps of the corresponding MHV-JHM proteins. Although the molecular weights of the MHV-nucleocapsid proteins differed by a significant amount, the positions of the peptides in the maps were remarkably similar.

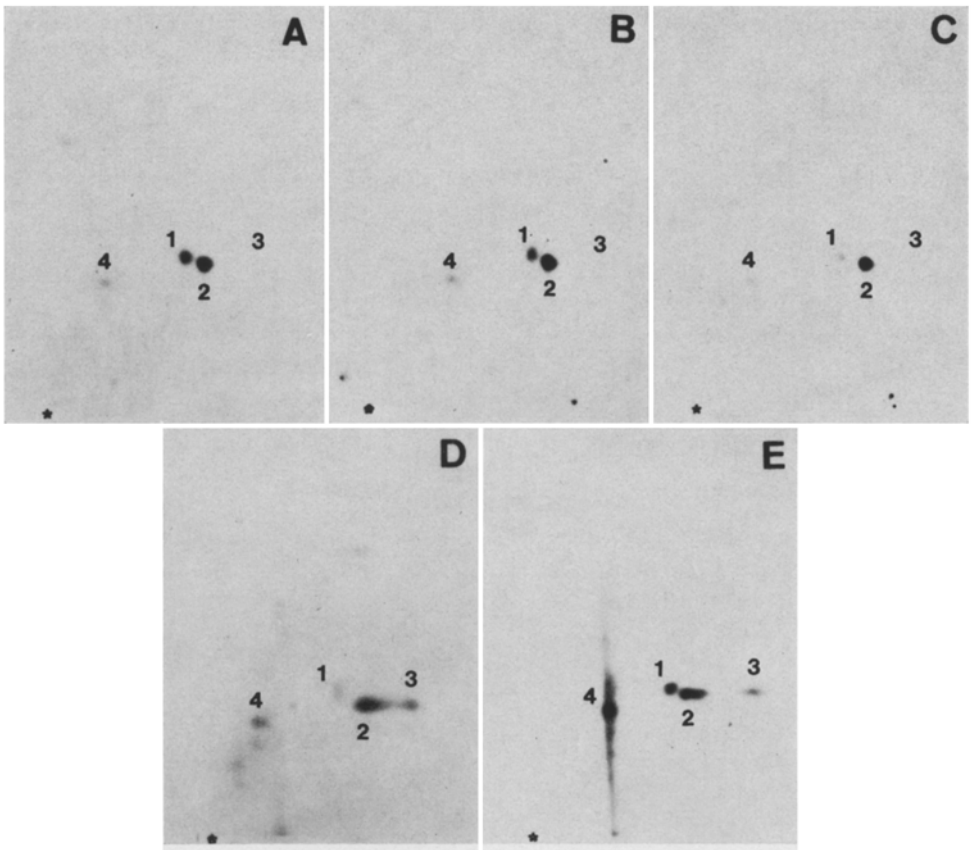

Fig. 6. Tryptic peptide maps of E1 of MHV-A59. Intracellular E1-22K protein (panel *E*) was purified by SDS-PAGE from a lysate of 17Cl-1 cells infected by MHV-A59. The heterogeneous E1 protein was purified from virus particles by SDS-PAGE. The four fractions of the heterogeneous E1 protein indicated in Fig. 1 were independently eluted and mapped (E1-22K, panel *A*; E1-24K, panel *B*; E1-25K, panel *C*; E1-26K, panel *D*). Origins are indicated by asterisks and the positions of common peptides are numbered

The structure of the E1-22 kilodalton protein from MHV-A59-infected cells was compared to the structure of the E1 proteins of the MHV-A59 virus particle (Fig. 6). The virion-associated E1 protein was heterogeneous by SDS-PAGE (Fig. 1). Four fractions of the E1 complex from virus particles corresponding to 22, 24, 25 and 26 kilodaltons were analyzed (Fig. 6, panels A, B, C, D respectively). The peptide map of the intracellular E1-22K protein (panel E) was similar to the peptide map of the structural E1-22K protein (panel A) suggesting that the peptide structure of the proteins were the same. The E1 proteins of MHV-JHM were analyzed in the same manner (Fig. 7). The E1-23K and E1-22K intracellular proteins were both analyzed (panels E, F). The peptide maps of these two proteins were similar. The maps of the 4 size classes of structural E1

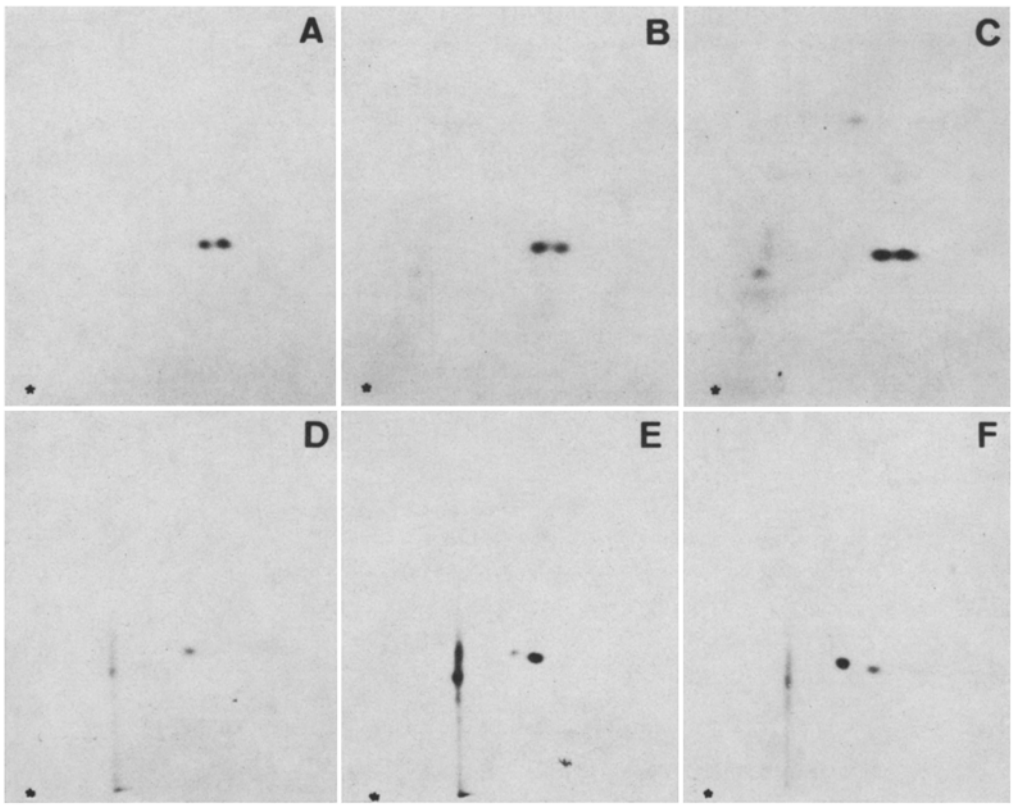

Fig. 7. Tryptic peptide maps of E1 proteins of MHV-JHM. Intracellular E1-23K (panel *E*) and E1-22K (panel *F*) protein were purified from a lysate of 17Cl-1 cells infected by MHV-JHM. The heterogeneous E1 protein was purified from virus particles by SDS-PAGE. The four fractions of the heterogeneous E1 protein as shown for MHV-A59 in Fig. 1 were independently eluted and mapped (E1-22K, panel *A*; E1-24K, panel *B*; E1-25K, panel *C*; E1-26K, panel *D*). Origins are indicated by asterisks and positions of common peptides are numbered

proteins (panels A, B, C, D) were similar to each other and to the 2 intracellular species. The peptide maps suggest that the intracellular E1 protein(s) was the precursor to the structural E1 proteins in the MHV virus particles. The differences in size of the E1-22K protein compared to the larger E1 species (24 to 26 kilodaltons) may be due to differential glycosylation of the E1-22K protein. The positions of the peptides in the maps of the MHV-A59 E1 proteins (Fig. 6) were similar to those of the MHV-JHM E1 proteins (Fig. 7).

The peptide maps of the MHV-A59 specific intracellular proteins p14 and p35 (Fig. 8, panels b, d) demonstrated that the 2 proteins were unrelated to each other or to any of the other MHV-A59 specific proteins. The peptide maps of the corresponding MHV-JHM specific proteins (Fig. 8, panels a, c) indicated that these proteins were also unrelated to each other or to any of the other MHV-JHM specific proteins. However, the positions of the peptides in the maps of p14 (Fig. 8, panels a, b) and p35 (Fig. 8,

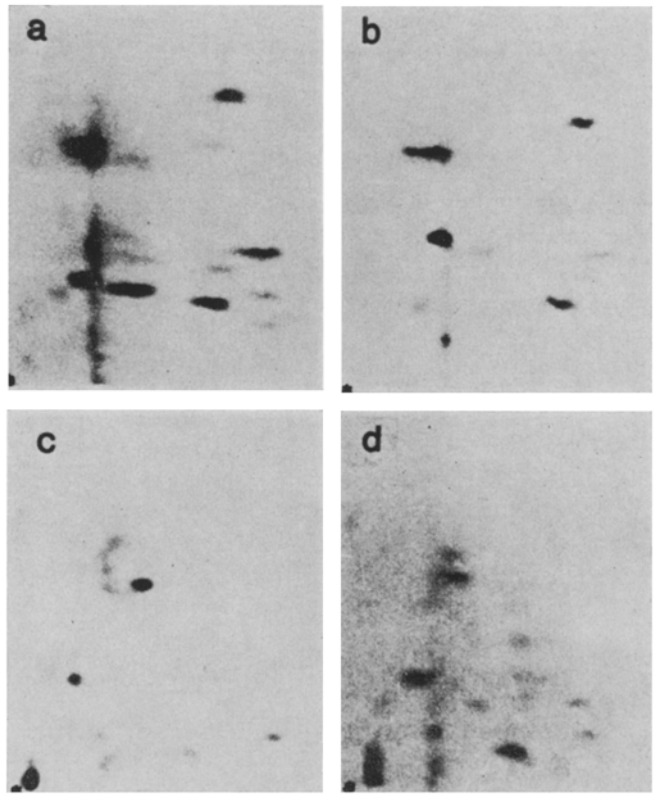

Fig. 8. Tryptic peptide maps of the p35 and p14 proteins. Intracellular p35 and p14 proteins were purified from lysates of 17Cl-1 cells infected by MHV-JHM or MHV-A59 (MHV-JHM: p14, panel *a*; p35, panel *c*; MHV-A59: p14, panel *b*; p35, panel *d*). Origins are indicated by asterisks

panels c, d) suggested that the peptide structures of the corresponding proteins of the two strains of MHV were similar.

Discussion

Cells infected by MHV-A59 or MHV-JHM have been reported to synthesize from five to nine virus-specific proteins (1, 3, 5, 18, 21). In this study, we compared the structures of the intracellular and structural proteins of MHV-JHM and MHV-A59 by tryptic peptide mapping of ^{35}S-methionine-labeled proteins purified by SDS-PAGE.

The tryptic peptide maps shown in Fig. 3 indicated that the structural proteins E2-90K and E2-180K share common peptides with the intracellular protein E2-150K. The map of E2-150K was somewhat more complex than the maps of E2-90K or E2-180K, suggesting that the protein E2-150K may be processed by cleavage during maturation. A 180 kilodalton protein was detected in MHV-infected DBT cell lysates (Fig. 2). We have not detected this species in MHV-infected 17Cl-1 cell lysates (2, 3). The reason for this observation is not known. Leibowitz *et al.* (13) demonstrated by immunoprecipitation with a monospecific antiserum that a 120 kilodalton protein synthesized in a cell-free system primed with MHV-A59 mRNA shared determinants with E2-150K. A 120 kilodalton protein has been detected in immunoprecipitates of MHV-infected cells treated with tunicamycin, an inhibitor of dolichol-mediated glycosylation (8, 18, 21). Sturman and Holmes (27) demonstrated that the MHV-A59 structural proteins E2-180K and E2-90K shared peptides on the basis of tryptic peptide maps. Furthermore, these workers have suggested that treatment of MHV-A59 particles with trypsin results in the cleavage of E2-180K to two 90 kilodalton species (25, 26, 27). This result indicates the presence of a trypsin-sensitive site near the midpoint of the molecule. The results of this study coupled with the results of others (8, 13, 18, 19, 20, 21, 25, 26, 27) suggest two possible mechanisms for the maturation of the E2 protein. The 120 kilodalton primary gene product is glycosylated to the E2-150K species, cleaved to the E2-90K species and dimerized to the E2-180K species. In the alternative mechanism, the 120 kilodalton primary gene product may be glycosylated to the E2-150K species, further glycosylated to the E2-180K species and then cleaved at the trypsin-sensitive site near the midpoint of the molecule to form two E2-90K species with different primary structures. Resolution of the mechanism will require an analysis of the primary structure of each species of protein. The extensive glycosylation of the primary gene product may prevent complete tryptic digestion of the proteins for the mapping studies. This suggestion is supported by the observation that a large proportion of the sample remained at the origin of the maps in Fig. 3, panels A, B and C.

The tryptic peptide maps of the nucleocapsid proteins of MHV-A59 (N-60K) (Fig. 4) and MHV-JHM (N-63K) (Fig. 5) demonstrated that the corresponding proteins from the virus particles and from the lysates of infected cells were essentially the same. In addition, 2 more rapidly migrating species (MHV-A59: p57, p54; MHV-JHM: p61, p56), which were found only in infected cells, had tryptic peptide maps similar to the nucleocapsid proteins. There was also tryptic peptide homology between the N-60K nucleocapsid and p57 proteins synthesized by cell-free translation of MHV-A59 mRNA (13). The more rapidly migrating species may be a result of proteolysis following cell lysis (1, 21), premature termination of transcription (13) or a result of posttranslational processing of the nucleocapsid protein. The function of the more rapidly migrating species related to the nucleocapsid proteins is not known.

The virion-associated E1 proteins of MHV-A59 were heterogeneous in size (Fig. 1). The tryptic peptide maps of the E1 proteins of MHV-A59 shown in Fig. 6 demonstrated that the intracellular E1-22K was related to the four fractions of structural E1 from the virus particles. The tryptic peptide maps of the E1 proteins of MHV-JHM shown in Fig. 7 demonstrated that the intracellular E1-23K and E1-22K species were related to the four fractions of structural E1 from the virus particles of MHV-JHM. Pulse-chase studies have demonstrated that the E1-23K protein of both MHV-A59 and MHV-JHM is processed posttranslationally to the E1-22K species (2). It has been shown that the larger E1 species (E1-24K to E1-26K) are glycosylated (14, 18, 26). Since the tryptic peptide maps of these E1 species are similar and coupled with the data from our other study (2), a model for the maturation of the E1 species may be proposed. The primary gene product E1-23K is translated, cleaved to the E1-22K species and then differentially glycosylated to the heterogeneous E1 complex found in the virion. The glycosylation is resistant to tunicamycin (8, 14, 18) and has been found to be of the O-linkage type (14). The corresponding proteins in the virions of avian infectious bronchitis virus also have a common primary sequence and are differentially glycosylated (4, 23).

A glycoprotein of 65 kilodaltons has been described as a surface protein of the MHV-JHM virus particle purified from infected Sac(-) cells (19, 21, 30). There is no evidence of this protein in the virus particles of MHV-A59 (Fig. 1, ref. 2, 3, 18, 25, 26, 27) or in preparations of MHV-JHM from other laboratories (2, 3, 24). An explanation of this paradox is that the strain of MHV-JHM used by these investigators (19, 21, 30) is either capable of synthesizing an unique glycoprotein of 65 kilodaltons in Sac(-) cells or a cellular protein may be specifically assembled into the virus particle. No *in vitro* MHV-specific translation product related to this protein has been described to date (13, 20).

The tryptic maps of the p35 and p14 proteins shown in Fig. 8 demon-

strated that these proteins were not related to each other or to any of the other MHV-specific proteins. These proteins have been detected previously in cells infected by MHV-JHM (3, 19, 21) or MHV-A59 (3, 18). The proteins p35 and p14 have been shown to be primary gene products by cell-free translation of MHV-A59 or MHV-JHM specific mRNAs (13, 20). The functions of these proteins are not known at present.

There is extensive nucleic acid homology between the genomes of MHV-A59 and MHV-JHM (31). However, there is a major difference in the degree of neuropathogenicity between the 2 strains of MHV (17). The tryptic peptide maps of ^{35}S-methionine labeled nucleocapsid-related proteins (Fig. 4, 5), E1-related proteins (Fig. 6, 7) and the intracellular proteins p35 and p14 (Fig. 8) demonstrated that substantial homology existed between the structural and intracellular proteins of the two strains of MHV.

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