IDENTIFICATION OF THE CORONAVIRUS MHV-JHM mRNA 4 GENE PRODUCT USING FUSION PROTEIN ANTISERA

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INTRODUCTION

The murine hepatitis virus (MHV) genome is a large single-stranded RNA which encodes the major structural proteins of the virion, the nucleocapsid (N), the membrane (M) and the spike (S) proteins. Additionally, an unknown number of proteins which are either minor virion components, or non-structural proteins, are expressed during infection (Siddell, 1987). The expression of the MHV genome is mediated in the infected cell via a 3' co-terminal nested set of subgenomic mRNAs, which are numbered 1 to 7, in order of decreasing size. The available data (reviewed in Siddell, 1987) suggests that, at least for the structural protein mRNAs (i.e. mRNAs 3, 6 and 7) only the 5' unique regions i.e. those absent from the next smallest mRNA, are translationally active and only single polypeptides are translated from these regions. In contrast, the unique region of mRNA 5 has two potential open reading frames (ORFs, Skinner et al., 1985) and although the translation products have not yet been identified, this unusual organization suggests that this mRNA may be functionally bicistronic.

The MHV mRNA 4 unique region has also been cloned and sequenced (Skinner and Siddell, 1985). This region contains a single ORF which predicts a basic polypeptide having the size and charge characteristic of a 14-15,000 molecular weight (mol.wt) polypeptide previously detected in infected cells (Siddell et al., 1981) or in vitro translations (Siddell, 1983). The aim of the work described here was to unambiguously assign the 14-15,000 mol.wt. polypeptide from MHV-JHM infected cells to the MHV mRNA 4. The strategy used was to construct a vector which allowed for the expression in bacteria of a fusion protein, composed of ß-galactosidase and the hydrophilic C terminal region of the mRNA 4 ORF "product". The coronavirus encoded portion of the fusion protein was isolated and used to produce monospecific antiserum in rabbits. This antiserum was then used in the immunofluorescence and immunoprecipitation experiments described here.

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METHODS

The construction of the two bacterial expression vectors used will be described in detail elsewhere. Both are based upon the pUR expression vectors of Rüther and MÜller-Hill (1983). Essentially, p417 contains a Taq 1 fragment of clone pJMS1010 extending from the position 272 (Skinner and Siddell, 1985, fig. 1) to position 138 (Skinner et al., 1985, fig. 3) cloned, in the correct orientation, between the Bam HI and Hind III sites of pUR 291. Upon expression this results in a fusion protein comprised of almost the entire ß-galactosidase protein together with the C-terminal half (70 amino acids) from the predicted mRNA 4 product. This region of the mRNA 4 product was chosen because of its hydrophilic nature and an absence of glutamic acid residues, which facilitated purification of the virally encoded portion of the molecule.

p300 is comprised of a Pst I-Hpa II fragment from clone pJMS1010 extending from the position 2585 (Schmidt et al., 1987, fig. 2) to position 254 (Skinner et al., 1985; fig. 1) cloned, in the correct orientation, between the Pst I site of pUR292. Upon expression this results in the ß-galactosidase protein fused to approximately the C terminal third (383 amino acids) of the spike protein. Both fusion proteins, FUS4 and FUS3C, were expressed in E. coli BMH 71-18 and isolated by preparative PAGE. The virally encoded portion of FUS4, (PEP4), was further purified by preparative PAGE following S. aureus V8 protease digestion. FUS3C was also mildly digested with S. aureus protease before immunization, but specific peptides were not purified. Immunization of NZ White rabbits was performed using standard procedures and the antisera were used without further purification.

MHV JHM infected Sac(-) cells were labelled with ^{35}S-methionine and cytoplasmic lysates were prepared as previously described (Siddell et al., 1981). In vitro translations in a cell free L-cell system were performed as previously described using poly A containing RNA (poly A + RNA) isolated from MHV--infected cells (Siddell, 1983). Prior to translation the poly A + RNA was fractionated in aqueous sucrose gradients and pooled fractions enriched for MHV mRNAs 4 and 5 or 6 and 7 were obtained. Immunoprecipitations of lysates or in vitro translation products were performed as described (Siddell et al., 1981; Siddell, 1983). Indirect immunofluorescence was performed on MHV-JHM infected cells fixed with 3 % formaldehyde and permeabilized with Triton X100 (Massa et al., 1986). Rhodamine-labelled swine anti rabbit IgG was used as the second antibody.

RESULTS

Fig. 1 shows the immunofluorescent appearance of MHV-JHM infected Sac(-) cells using the antisera raised against FUS3C, PEP4 or a preimmune serum. Compared to the preimmune serum, the anti-FUS3C serum produces a clear staining of syncytia, the characteristic cytopathic effect of MHV-JHM infection. The immune reaction is diffuse, is restricted to the cytoplasm and is not seen in the nuclei of the syncytium. The anti PEP4 serum also produces a clear, albeit less intense, staining of the syncytial cytoplasm.

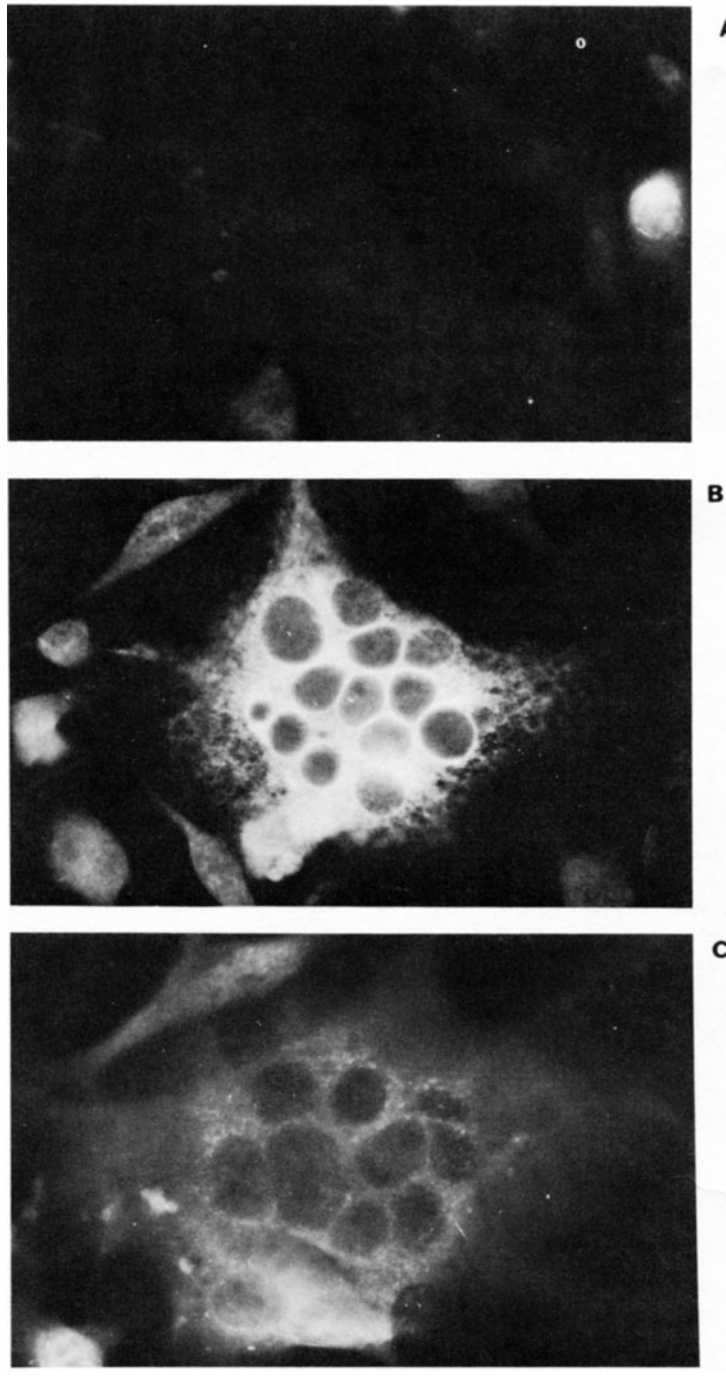

Figure 1

Indirect immunofluorescence of MHV infected cells using a) pre-immune serum, b) anti FUS3C serum, c) anti PEP4 serum.

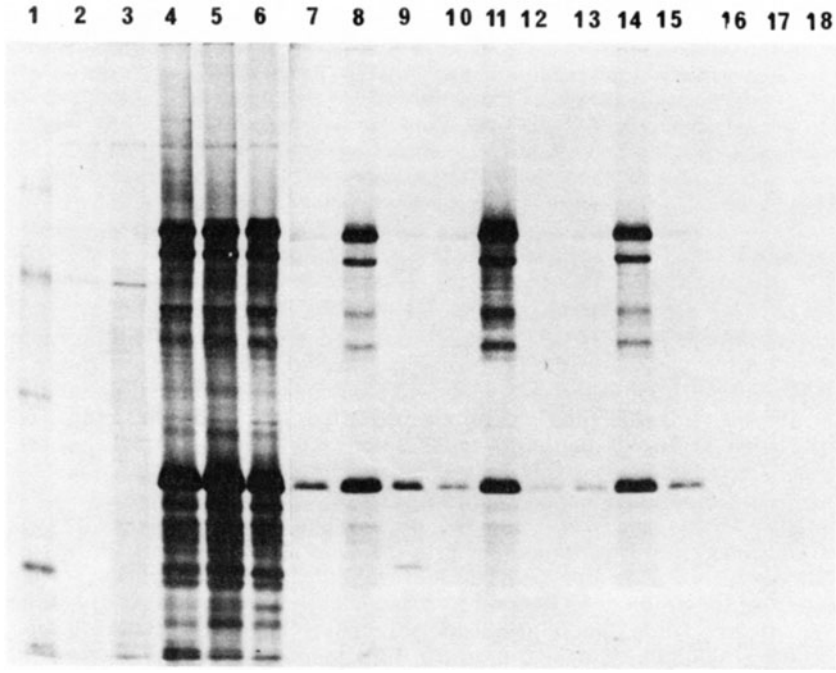

Figure 2

Immunoprecipitation of in vitro translation products directed by poly A + RNA from MHV infected cells.
1 Mol wt. markers
2 minus RNA
3 RNA from mock infected Sac(-) cells
4 RNA from MHV infected Sac(-) cells, mRNA pool 4 + 5
5 RNA from MHV infected Sac(-) cells, mRNA pool 6 + 7
6 RNA from MHV infected Sac(-) cells, total poly A + RNA
7 preimmune serum, mRNA pool 4 + 5
8 anti JHM-serum, mRNA pool 4 + 5
9 anti PEP4 serum, mRNA pool 4 + 5
10 preimmune serum, mRNA pool 6 + 7
11 anti JHM-serum, mRNA pool 6 + 7
12 anti PEP4 serum, mRNA pool 6 + 7
13 preimmune serum, total poly A + RNA
14 anti JHM-serum, total poly A + RNA
15 anti PEP4 serum, total poly A + RNA
16 preimmune serum, mock infected mRNA
17 anti JHM-serum, mock infected mRNA
18 anti PEP4 serum, mock infected mRNA

Fig. 2 shows the in vitro translation in a L cell system of poly A + RNA from MHV JHM infected cells and fractions enriched for either mRNAs 4 and 5 or 6 and 7. As has been reported (Siddell, 1983) the total poly A + RNA directs the synthesis of two major products, identified as the intracellular M (mol.wt

23,000) and N (mol.wt. 60,000) polypeptides. Both polypeptides are specifically immunoprecipitated by the anti JHM virion serum (Fig. 2, track 14). Immunoprecipitation of the total poly A + RNA directed translate with the anti PEP4 serum did not clearly reveal any specific polypeptide (Fig. 2, track 15). The observed precipitation of the M protein appears to be non-specific as the preimmune serum also produced a similar result (Fig. 2, track 13). However, when the translation products of the poly A + RNA enriched for mRNAs 4 and 5 were reacted with the anti PEP4 serum a clear and specific immunoprecipitation of a 14-15,000 mol.wt. polypeptide was observed (Fig. 2, track 9). This polypeptide was not detected with preimmune serum (Fig. 2, track 7) or translates from uninfected cell mRNA (Fig. 2, track 18).

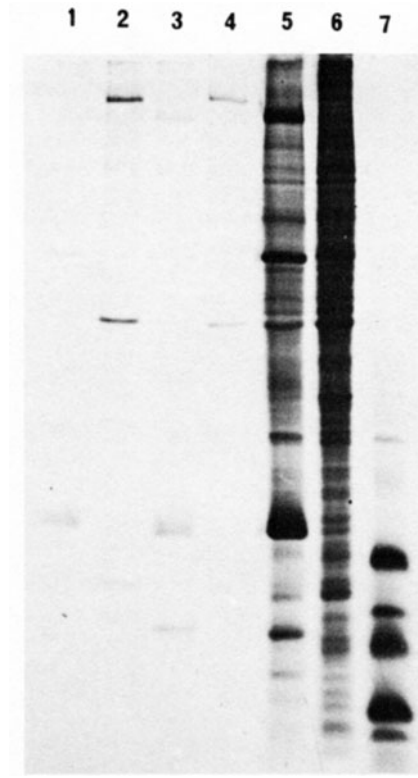

Figure 3

Immunoprecipitation of ^{35}S-methionine labelled cytoplasmic lysates from MHV infected cells.
1 infected cell lysate, preimmune serum
2 mock infected cell lysate, preimmune serum
3 infected cell lysate, anti PEP4 serum
4 mock infected cell lysate, anti PEP4 serum
5 infected cell lysate
6 mock infected cell lysate
7 molecular weight markers

Fig. 3 shows the immunoprecipitation of ^{35}S methionine labelled cytoplasmic lysates from MHV-infected, or mock infected, cells using preimmune and the anti PEP4 serum. In the non-immunoprecipitated lysates (Fig. 3, tracks 5,6) infection specific polypeptides of 150,000, 60,000 and 23,000 mol.wt. which have been previously identified as the intracellular S, M and N proteins are seen. Compared to the preimmune serum the anti PEP4 serum specifically immunoprecipitates only the 14-15,000 mol.wt. polypeptide from infected cell lysates (Fig. 3, tracks 1, 3). Again, a small amount of non-specific M protein precipitation is seen in both sera and the preimmune serum appears to contain two activities (cross-) reacting with cellular polypeptides.

DISCUSSION

The data presented here shows that the translation product of the MHV-JHM mRNA 4 is a protein which has been previously identified in infected cells and has been described as having a mol.wt. of 14-15,000. The data also suggests that mRNA 4 encodes a single polypeptide in its 5' unique region and presumably functions monocistronically. The initiation codon of the mRNA 4 ORF is in a favoured context (Kozak 1983), but the initiation codon and the region of homology sequence, which is assumed to be at or near the 5' end of the mRNA body, are about 60 nucleotides apart, compared to less than 10 nucleotides for mRNAs 3, 6 and 7 (Siddell, 1987). The effect this may have upon the translational efficiency of the MHV-JHM mRNA 4 is not known.

The major translation product of mRNA 4 is predicted to be a protein of 139 amino acids, mol.wt. 15,200. The protein is basic, rich in threonine, has a large hydrophobic aminoterminus and a basic carboxyterminus (Skinner et al., 1985). The data presented here indicate that this gene product is a non-structural protein. It is not detectable in radiolabelled, purified virions (Siddell et al., 1981) and is not immunoprecipitated from infected cell lysates by the anti-JHM virion serum. However, nothing is known about the possible functions of this protein, although the specific antiserum described here will be useful in elucidating this question. Furthermore the strategy adopted here for the mRNA 4 gene product can also be applied to the products of other MHV non-structural genes. In particular the synthesis, processing and biological activity of the virus-coded RNA polymerse, which has so far proven difficult to analyse will be ameanable to this approach.

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