CHARACTERIZATION OF THE MHV-JHM NON-STRUCTURAL PROTEIN ENCODED BY mRNA 2

Birgit Schwarz, Edward Routledge and Stuart Siddell

Institute of Virology
Versbacher Straße 7
D - 8700 Würzburg

INTRODUCTION

The genome of the murine coronavirus MHV-JHM is a positive-stranded RNA of approximately 30 Kb, which encodes 4 structural proteins (N, M, S and HE) and at least 4 non-structural proteins. The location of the major open reading frames (ORFs) in the genome is shown in Fig. 1. In the infected cell, expression of the viral proteins is mediated by a set of subgenomic mRNAs. In relation to the genome the mRNAs are 3' coterminal and extend to different positions in a 5' direction. The mRNAs 7, 6, 3 and 2-1 have been shown to encode the N, M, S and HE proteins respectively (Siddell, 1983; Pfleiderer et al., this volume). mRNA 4 encodes a 15,000 molecular weight (mol.wt.) non-structural protein (Ebner et al, 1988) and mRNA 5 has the potential to encode two non-structural proteins of 12,400 and 10,200 mol.wt. (Skinner et al., 1985). Earlier *in vitro* translation studies (Siddell, 1983), indicated that the MHV-JHM subgenomic mRNA 2 encodes a 30,000 mol.wt. non-structural protein, the expression of which was translationally regulated. Recent sequence analysis of the 5' proximal region of the closely related MHV-A59 mRNA 2 confirms the presence of a 261 aminoacid ORF at this position. Analysis of the predicted polypeptide sequence indicates a non-membrane protein which may possibly have nucleotide binding and phosphorylating properties (Luytjes et al., 1988).

In the experiments described here our aims were:

1. to construct and express a fusion protein containing sequences derived from the 5' proximal ORF of the MHV-JHM mRNA 2

2. to isolate monoclonal antibodies (MoABs) specific for the MHV-JHM mRNA 2 gene product

3. to identify the MHV-JHM mRNA 2 gene product *in vitro* and *in vivo*.

Coronaviruses and Their Diseases
Edited by D. Cavanagh and T.D.K. Brown
Plenum Press, New York, 1990

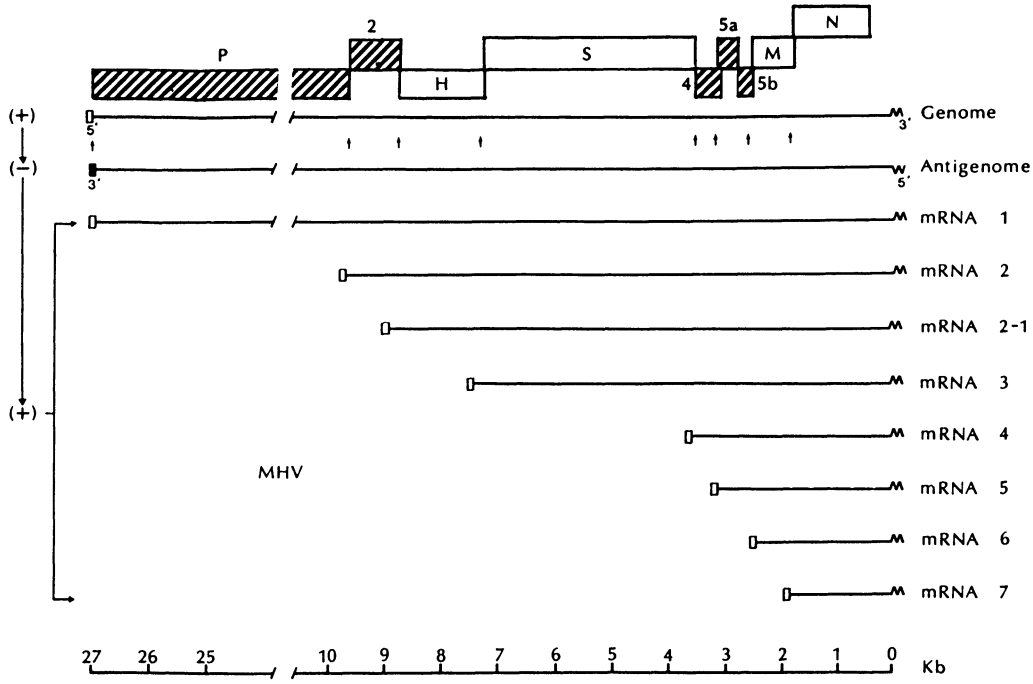

Fig. 1. A summary of the replication strategy of murine hepatitis virus, MHV-JHM. The organization of the major open reading frames in the genome and their relationship to the subgenomic mRNAs is shown. The figure includes data submitted for publication.

RESULTS

A cDNA clone encompassing the 5' proximal ORF of the MHV-JHM mRNA 2 has been isolated and sequenced (Schwarz et al, in preparation). This analysis reveals an ORF of 265 aminoacids and the predicted polypeptide has 97 % similarity to the corresponding MHV-A59 protein. A fusion protein vector, based upon the pEX system (Stanley and Luzio, 1984) was constructed to express sequences derived from the 5' proximal ORF of MHV-JHM mRNA 2. Details of this construction will be given elsewhere. Essentially, the fusion protein consisted of a tripartite construct with amino acids 105 to 265 of the mRNA 2 ORF. The construct was introduced into E coli strain RR1 (pRK248cIts) where expression of the fusion protein is regulated by the temperature-sensitive λ phage repressor. Fig. 2 shows the proteins synthesized in induced (lanes 1 and 3) or non-induced (lanes 2 and 4) bacteria which carry the ΔpEX vector (lanes 1 and 2) (a modified pEX vector which expresses a carboxyterminal truncated approx. 50,000 mol.wt. cro-ß-galactosidase) or the fusion protein construct, ΔpEX-2, described above (lanes 3 and 4). These results show that the fusion protein (approx. 67,000 mol.wt) is expressed in both induced and non-induced cells indicating the loss of the control, pRK248cIts, plasmid.

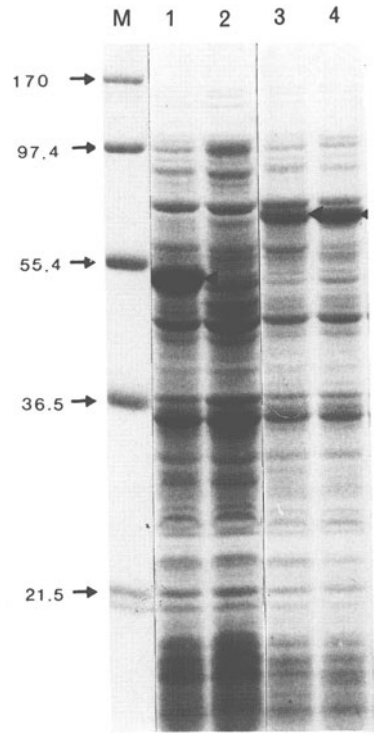

Fig. 2. Expression of the MHV-JHM mRNA 2 ORF fusion protein in bacteria.

The fusion protein was purified by preparative SDS-polyacrylamide gel electrophoresis and electroelution and used to immunize three months old Balb/c mice by a variety of routes over a 14 week period. Three days after the final inoculation spleen cells were fused to NSI plasmocytoma cells using polethylene glycol. Hybridomas selected in HAT medium were screened for fusion protein specific antibody production by ELISA using lysates from induced bacteria carrying the ΔpEX or the ΔpEX-2 as capture antigen. Ten specific cell lines could be cloned and these were tested for their ability to recognize MHV specific polypeptides *in vitro* and *in vivo*.

PolyA+ RNA from MHV-JHM and MHV-A59 infected DBT cells was isolated and translated in a rabbit reticulocyte lysate as previously described (Siddell, 1983) (Fig. 3, lane 2 and 3). The *in vitro* translation products were immunoprecipitated using a mixture of the fusion protein specific MoAbs (Fig. 3, lanes 5 and 6). The results clearly show that the fusion protein specific antibodies immunoprecipitate an approximately 30,000 mol.wt. polypeptide from both the MHV-JHM and MHV-A59 translates. These polypeptides were not immunoprecipitated using MoAbs directed against the MHV-N protein (data not shown).

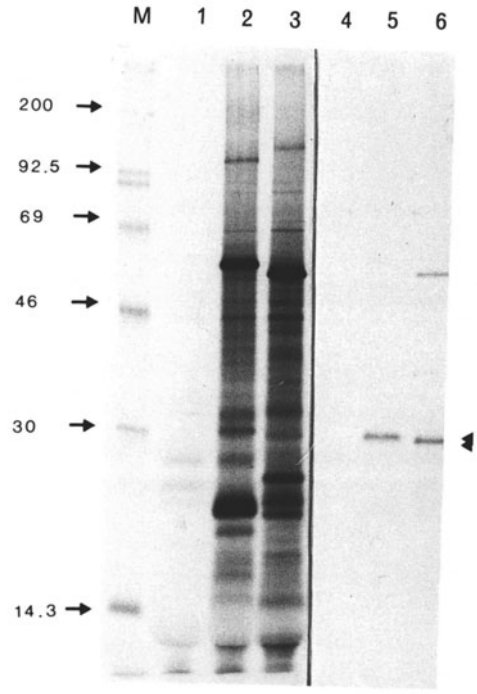

Fig. 3. Analysis of MHV <u>in vitro</u> translation products with MoAbs specific for the MHV-JHM mRNA 2 ORF fusion protein. The lanes 1 and 4 are H_2O controls.

To identify the MHV-JHM mRNA 2 gene product <u>in vivo</u> we performed immunofluorescence, immunoprecipitation and Western blotting. Indirect immunofluorescence was performed on acetone fixed MHV-JHM infected and mock-infected DBT cells. Fig. 4 shows the staining of infected cells with the mixture of fusion protein MoAbs (A) or an anti-spike protein MoAb (kindly provided by H. Wege) (C). In both cases a clear positive immune reaction was evident, albeit less intensely for the fusion protein MoAb. The immunostaining was restricted to the cytoplasm of syncytia, the characteristic cytopathic effect of MHV-JHM infection. There was no staining in the nuclei of infected cells nor in mock-infected cells (B).

The immunoprecipitation of ^{35}S methionine cell lysates (Siddell et al., 1981) derived from MHV-JHM or MHV-A59 infected DBT cells using either the fusion protein specific MoAb mixture, an anti-S MoAb, or an anti ß-galactosidase MoAb is shown in Fig. 5. The results show that, as expected, the anti S MoAb immunoprecipitates a polypeptide of approximately 150-160,000 mol.wt, the intracellular precursor of the spike

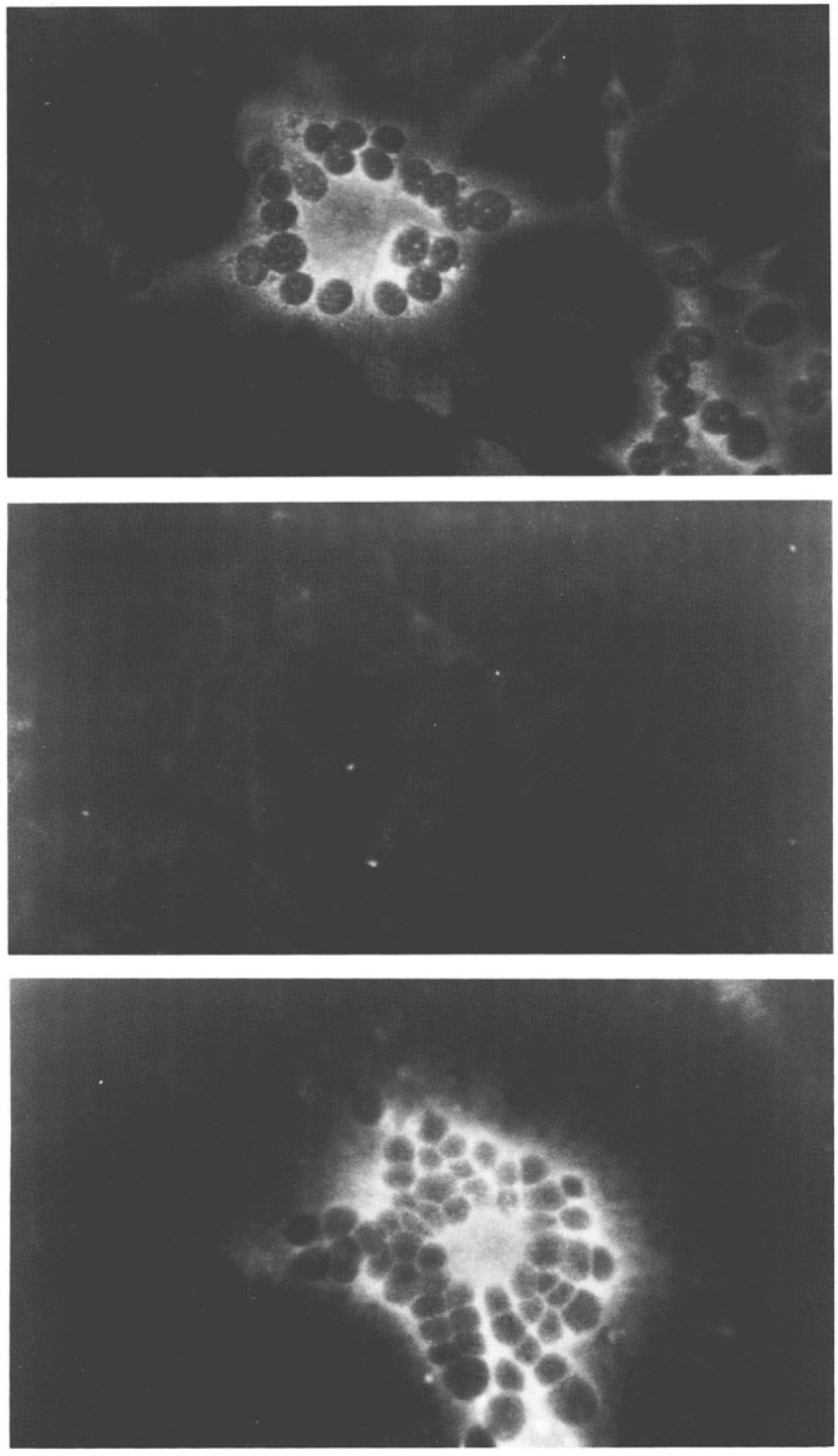

Fig. 4. Immunofluorescence of MHV-JHM infected cells using MoAbs specific for the mRNA 2 ORF fusion protein.

protein (Fig. 5, lanes 5 and 6). The fusion protein specific MoAbs again specifically immunoprecipitate an approximately 30,000 mol.wt. polypeptide from both JHM and A59 infected cells (Fig. 5 lanes 3 and 4). The anti ß-galactosidase MoAb did not show any immunoprecipitation (Fig. 5, lanes 7 and 8).

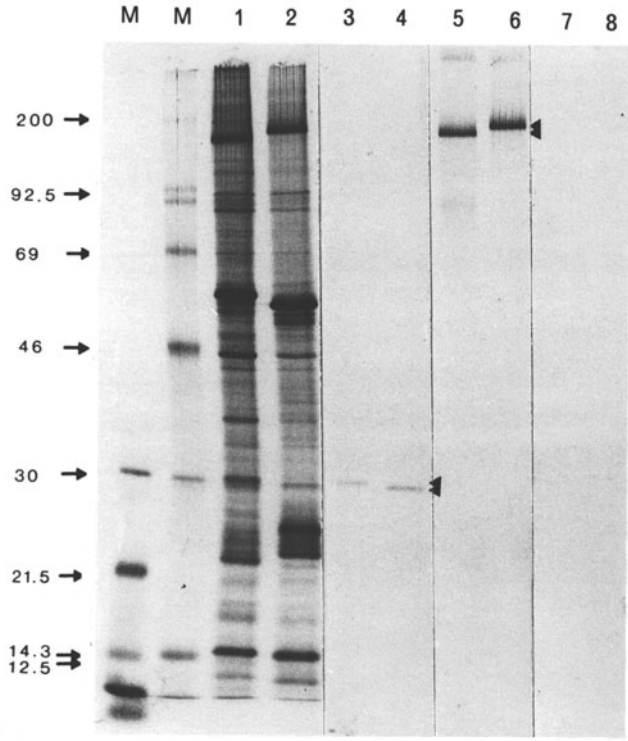

Fig. 5. Immunoprecipitation of polypeptides from MHV infected cells using MoAbs specific for the mRNA 2 ORF fusion protein.

Finally, the identity of the MHV-JHM mRNA 2 gene products was confirmed in vivo by immunoblotting. Cytoplasmic lysates were prepared from MHV-JHM and MHV-A59 infected DBT cells, electrophoresed on a 15 % SDS polyacrylamide gel and transferred to nitrocellulose. The 30,000 mol.wt. mRNA 2 gene product was then detected in both JHM and A59 infected cell lysates using the fusion protein specific MoAbs as shown in Fig. 6, lanes 1 and 2.

DISCUSSION

In this paper we have clearly demonstrated that the MHV-JHM mRNA 2 product is a 30,000 mol.wt. protein which has been previously identified as an *in vitro* translation product (Siddell, 1983) and has been detected in small amounts in MHV-JHM infected cells (Siddell et al., 1981). The results also show that a homologous protein is synthesized in MHV-A59 infected cells. Furthermore, as predicted from the sequence analysis, the JHM and A59 proteins differ slightly in size (265 and 261 amino-acids, respectively) (this paper, Luytjes et al., 1988). The experiments we have reported also clearly demonstrate the

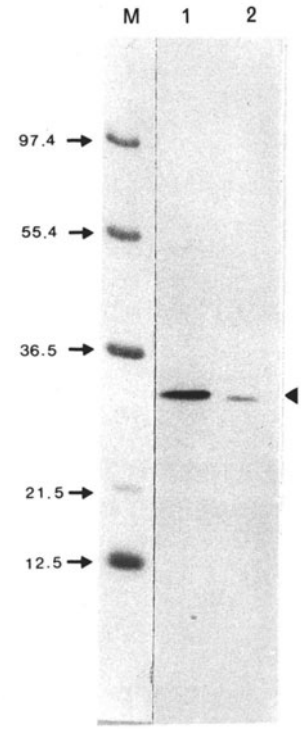

Fig. 6. Western blotting of cytoplasmic lysates from MHV infected cells using MoAbs specific for the mRNA 2 ORF fusion protein.

usefulness of the "fusion protein" strategy and the advantages of isolating fusion protein specific MoAbs, as compared to polyvalent sera. It should be noted that the mixture of fusion protein specific MoAbs is able to function in immunofluorescence, immunoprecipitation and immunoblotting. We believe that the MoAbs we have isolated may also be useful in elucidating the function of this protein, which at the moment is unknown.

ACKNOWLEDGMENT

We would like to acknowledge the technical help of Frau B. Schelle-Prinz. We thank Helga Kriesinger for typing the manuscript. This work was supported by the SFB 165 (B1).

REFERENCES

Ebner, D., Raabe, T., and Siddell, S.G., 1988, Identification of the coronavirus MHV-JHM mRNA4 product. J. gen. Virol. 69: 1041.

Luytjes, W., Bredenbeek, P.J., Noten, A.F.H., Horzinek, M.C., and Spaan, W.J.M., 1988, Virology 166:415.

Siddell, S.G., Wege, H., Barthel, A., and ter Meulen, V., 1981, Coronavirus JHM. Intracellular protein synthesis. J. gen. Virol. 53:145.

Siddell, S.G., 1983, Coronavirus JHM. Coding assignment of subgenomic mRNAs. J. gen. Virol. 64:113.

Skinner, M.A., Ebner, D., and Siddell, S.G., 1985, Coronavirus MHV-JHM mRNA5 has a sequence arrangement which potentially allows translation of a second, downstream open reading frame. J. gen. Virol. 66:581.

Stanley, K.K., and Luzio J.P., 1984, Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins, EMBO J. 3:1429.