Key words: *coronavirus MHV/mRNA 4/translation product*

Identification of the Coronavirus MHV-JHM mRNA 4 Product

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SUMMARY

A bacterial expression vector was constructed to encode a fusion protein which had, at its carboxy terminus, a polypeptide encoded within the 5′ proximal open reading frame of the coronavirus MHV-JHM mRNA 4. This polypeptide was isolated and used to produce an antiserum. The antiserum reacted specifically with a 15000 M_r polypeptide synthesized in MHV-JHM-infected cells, or *in vitro* translations of infected cell poly(A) RNA enriched for mRNA 4. These results demonstrate the translational activity of mRNA 4 during infection, identify conclusively the translation product and provide a means to investigate the synthesis and function of this protein.

INTRODUCTION

The murine hepatitis virus (MHV) belongs to the Coronaviridae, a mono-generic group of enveloped positive-stranded RNA viruses. The three major proteins of the MHV virion are the nucleocapsid (N), membrane (M) and surface (S) proteins. In MHV-infected cells the expression of the viral genome is mediated by a 3′ co-terminal set of subgenomic mRNAs. The subgenomic mRNAs 3, 6 and 7 encode the S, M and N proteins respectively (Lai *et al.*, 1981; Spaan *et al.*, 1982; Makino *et al.*, 1984; Rottier *et al.*, 1981; Leibowitz *et al.*, 1982*b*; Siddell, 1983). The data available from *in vitro* translations and sequence analysis suggest that, for the structural protein mRNAs at least, only the 5′ unique regions, i.e. those absent from the next smallest RNA, are translationally active and only single polypeptides are translated (Sturman & Holmes, 1983; Siddell, 1987).

The MHV genome also encodes an unknown number of non-structural proteins and it is assumed that at least some of these proteins are encoded in the unique regions of mRNAs 1, 4 and 5. However, the characterization of the translation products of these mRNAs is rudimentary (Leibowitz *et al.*, 1982*b*; Siddell, 1983; Denison & Perlman, 1986, 1987). The unique region of MHV-JHM mRNA 4 has been cloned and sequenced (Skinner & Siddell, 1985). This region contains a single open reading frame (ORF) which predicts a polypeptide of M_r 15000. A polypeptide of this size has been identified in MHV-JHM-infected cells (Siddell *et al.*, 1981) and in *in vitro* translation products of MHV-JHM RNA fractions enriched for mRNAs 4 and 5 (Siddell, 1983). However, the unique regions of mRNAs 4 and 5 contain ORFs of comparable size (Skinner *et al.*, 1985) and it has not been possible to assign the infected cell protein or *in vitro* translation product to one or the other mRNA. The aim of the work described here was to resolve this question; the strategy used was to construct a vector which directed the synthesis, in bacteria, of a fusion protein composed of β-galactosidase and the translation product of the carboxy-terminal region of the mRNA 4 ORF. The coronavirus portion of the fusion protein was isolated and used to produce monospecific antiserum in rabbits. This antiserum was then used in the experiments described herein.

METHODS

Virus, cells, infection and radiolabelling. To prepare virus stocks, the JHM strain of MHV was plaque-purified and grown on Sac(−) cells at low m.o.i. (Siddell *et al.*, 1980). For the purpose of radiolabelling, Sac(−) cells were

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infected with MHV-JHM at an m.o.i. of 10 and were labelled 10 h post-infection (p.i.) for 60 min with [^{35}S]methionine (SJ.204; Amersham; 100 µCi/ml). The cells were washed and cytoplasmic lysates were prepared using a non-ionic detergent buffer at 4 °C as previously described (Siddell *et al.*, 1981).

mRNA isolation, in vitro *translation.* Poly(A) RNA was isolated from the cytoplasm of MHV-JHM-infected Sac(−) cells (Siddell *et al.*, 1980) and fractions enriched for subgenomic mRNAs 4 and 5 were obtained by centrifugation in sucrose–formamide gradients (Smith *et al.*, 1976). *In vitro* translations were performed in a messenger-dependent cell-free system derived from L cells (Dahl & Dickson, 1979).

Molecular cloning. Restriction endonucleases and DNA-modifying enzymes were purchased from Boehringer and used according to the manufacturer's instructions. Cloning experiments were performed in *Escherichia coli* strains JM105 and BMH71-18. Plasmid pUC18 was used as the cloning vector and plasmids pUR291 and pUR292 for the expression of fusion proteins (Yanisch-Perron *et al.*, 1985; Rüther & Müller-Hill, 1983). cDNA fragments encoding coronavirus genes were derived from the plasmid pJMS1010 isolated and characterized by Skinner *et al.* (1985). The arrangement and location of known and putative MHV-JHM genes in pJMS1010 and their relationship to the subgenomic mRNAs is shown in Fig. 1.

Construction of fusion protein expression vectors. The vector that was used to express a fusion protein composed of β-galactosidase and the carboxy-terminal half (approx.) of the mRNA 4 unique region ORF (ORF 4) product was constructed as follows. The plasmid pJMS1010 was digested with *Pst*I and the viral cDNA insert was isolated and digested with *Taq*I. The resulting fragments were ligated with *Acc*I-cut, phosphatase-treated pUC18 and were used to transform JM105. Transformants were selected by colony hybridization using the 3′ *Hinf*I fragment of the pJMS1010 insert, which had been labelled with ^{32}P by nick translation. Plasmid DNA from positive clones was digested with *Bam*HI/*Hin*dIII and inserts of approximately 250 bp were subcloned into *Bam*HI/*Hin*dIII-cut, M13mp18 replicative form (RF) DNA. The inserts were then sequenced and their orientation was determined. A *Bam*HI/*Hin*dIII fragment corresponding to the 3′ half of ORF 4, i.e. essentially the 255 bp *Taq*I fragment of the pJMS1010 insert, was then ligated with *Bam*HI/*Hin*dIII-cut pUR291 and used to transform BMH71-18 cells. This forced cloning resulted in a 'gene' encoding a fusion protein (F4, 1100 amino acids, M_r 124800) composed of β-galactosidase fused to six amino acids resulting from cloning procedures and 71 amino acids preceding the ORF 4 termination codon. Finally, the plasmid DNA in one transformant (pDEF4) was purified, the insert excised with *Eco*RI, subcloned into *Eco*RI-cut, phosphatase-treated M13mp18 RF DNA and the construction checked by sequence analysis.

A second vector that was used to express a fusion protein composed of β-galactosidase and the carboxy-terminal third (approx.) of the mRNA 3 unique region ORF (ORF 3) product (i.e. the virion S protein) was also constructed. The plasmid pJMS1010 was digested with *Pst*I and the viral cDNA insert was isolated and digested with *Hpa*II. These fragments were ligated with *Pst*I-cut, phosphatase-treated pUR292. The ligation products were treated with T4 DNA polymerase to produce flush ends and were then religated. This material was used to transform BMH71-18 cells and transformants were screened directly, by gel electrophoresis of bacterial lysates, for the expression of fusion proteins. The desired construct, pDEF3, was identified by its ability to produce a fusion protein (F3, 1409 amino acids, M_r 158800) composed of β-galactosidase, five amino acids resulting from cloning procedures and the 382 amino acids preceding the ORF 3 termination codon.

Induction and purification of fusion proteins. Cultures of BMH71-18 cells containing either pDEF4 or pDEF3 were grown to mid-logarithmic phase in the presence of 0·25 mM-isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested, washed, treated with lysozyme (1 mg/ml) for 15 min at 4 °C and sonicated in 60 mM-Tris–HCl pH 6·8 containing 50 mM-2-mercaptoethanol, 100 units/ml aprotinin and 0·1 mM-EDTA. The cell membranes were pelleted and dissolved in the same buffer containing 2% SDS. The lysates were heated to 100 °C for 5 min before electrophoresis.

The coronavirus-encoded portion of fusion protein F4 was obtained by digestion of 10 mg of purified F4 protein with 1 mg of *Staphylococcus aureus* V8 protease at 37 °C for 15 h in 50 mM-NH_4HCO_3. The digestion products were separated by preparative electrophoresis and the coronavirus encoded-peptide, P4, was isolated.

Immunological methods. Immunizations were performed by subcutaneous injections of New Zealand White rabbits using standard procedures and the antisera were used without further purification. Indirect immunofluorescence was performed on MHV-JHM-infected Sac(−) cells fixed with 3% formaldehyde and permeabilized with Triton X-100 (Massa *et al.*, 1986). Rhodamine-labelled swine anti-rabbit IgG (Dakopatts) was used as the second antibody. Immunoprecipitations of cytoplasmic lysates or *in vitro* translation products were performed as described by Siddell *et al.* (1981), except that immune complexes were bound to Protein A–Sepharose (Pharmacia). The preparation of anti-JHM virion serum has been described previously (Siddell *et al.*, 1980). Western blots were performed using a semi-dry blotting system (Kyhse-Andersen, 1984). Bacterial lysates were separated on a 12% SDS–polyacrylamide gel. First antibodies were used at dilutions of 1:100. The second antibody, peroxidase-linked swine anti-rabbit IgG (Dakopatts), was used at a dilution of 1:200.

Neutralization assay. Antibodies, serum or ascites fluid, which had been inactivated at 56 °C for 30 min, were incubated in tenfold dilutions with 50 p.f.u. of MHV-JHM virus for 1 h at 4 °C. The infectious virus was then

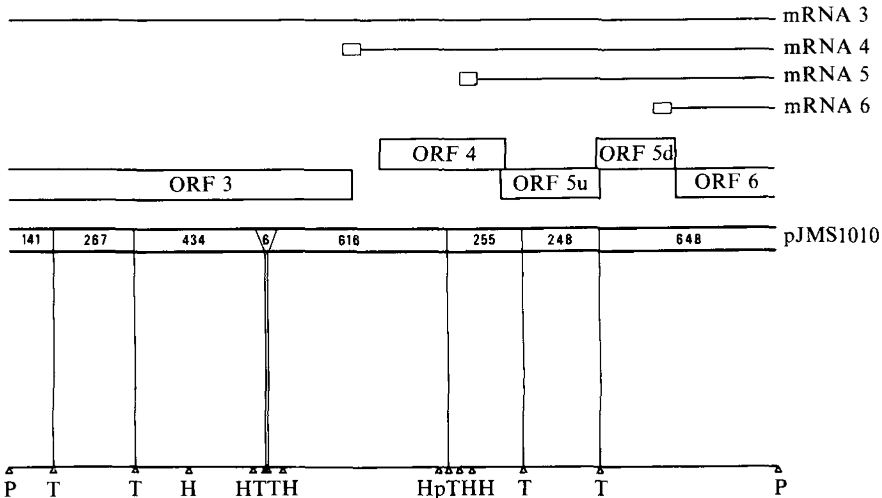

Fig. 1. Location of open reading frames and restriction sites (P, *Pst*I; T, *Taq*I, H, *Hinf*III; Hp, *Hpa*II) in pJMS1010 and their relationship to MHV-JHM subgenomic mRNAs.

measured by titration on monolayers of L929 cells. The monolayers were stained 16 h p.i. with May-Grünwald stain and the number of plaques was determined.

General methods. SDS–polyacrylamide gels were prepared as described by Laemmli (1970). Proteins were recovered from preparative gels by electroelution, using a commercial system (Renner, Dannstadt, F.R.G.). Plasmid DNA purification, agarose gel electrophoresis, electroelution of DNA fragments, colony hybridizations and nick translations were all performed according to standard procedures (Maniatis *et al.*, 1982). Transformations were carried out using the procedures described by Hanahan (1983). M13 dideoxynucleotide sequencing was carried out with [α-^{35}S]dATP and the products were analysed on buffer gradient gels (Biggin *et al.*, 1983).

RESULTS

Expression of fusion proteins

Fig. 2(*a*) shows a Coomassie Brilliant Blue-stained gel of lysates from uninduced and induced BMH71-18 cells containing pDEF4 (lanes 5 and 6) or pDEF3 (lanes 7 and 8). By comparison with BMH71-18 cells alone (lanes 1 and 2) or cells containing pUR291 (lanes 3 and 4), both recombinant plasmids gave rise to large amounts of fusion proteins when induced. The fusion proteins which were expressed were of the expected size and were synthesized in sufficient amounts to allow preparative isolation. In the case of fusion protein F3, the gel-purified material was used directly for immunization. In the case of fusion protein F4, the gel-purified material was digested with *S. aureus* V8 protease and the coronavirus-encoded portion of the protein, peptide 4 (P4; M_r 10650) was isolated by preparative gel electrophoresis. Under the conditions used V8 protease cleaved specifically at glutamate residues, an amino acid that was absent from P4. A comparison of V8 protease-digested β-galactosidase and fusion protein F4 (Fig. 2*b*) clearly demonstrated the specificity of the protease and the generation of P4 in the digested material. P4 had 15 amino acids at its amino terminus that are encoded at the carboxy terminus of the β-galactosidase gene.

Analysis of anti-P4 serum specificity

The antiserum produced by immunization with P4 was tested for its specificity by Western blotting. Fig. 2(*c*) (lanes 1, 2 and 3) shows that β-galactosidase, fusion protein F4 and peptide P4 were all recognized by the serum. The inclusion of soluble β-galactosidase in the first antibody reaction buffer essentially blocked the reaction with filter-bound β-galactosidase but did not significantly reduce the reactions with F4 or P4 (lanes 4, 5 and 6). This demonstrates that the

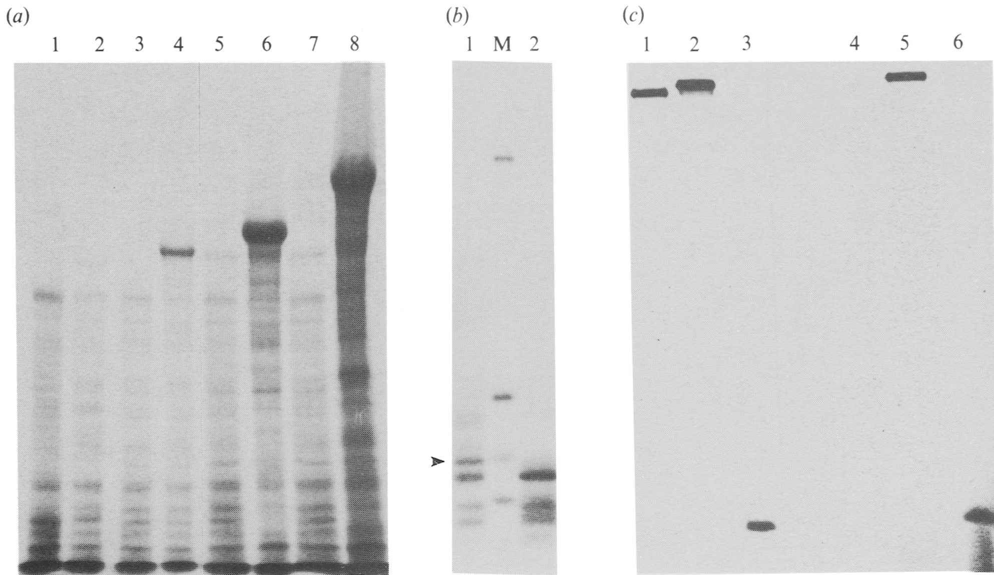

Fig. 2. (a) Induction of fusion protein expression in bacteria, induced by growth in 0·25 mM-IPTG. Cell lysates were prepared, electrophoresed on a 7·5% SDS–polyacrylamide gel and then stained with Coomassie Brilliant Blue R250. Lane 1, BMH71-18/–, uninduced; lane 2, BMH71-18/–, induced; lane 3, BMH71-18/pUR291, uninduced; lane 4, BMH71-18/pUR291, induced; lane 5, BMH71-18/pDEF4, uninduced; lane 6, BMH71-18/pDEF4, induced; lane 7, BMH71-18/pDEF3, uninduced; lane 8, BMH71-18/pDEF3, induced. (b) Digestion of fusion protein F4 (lane 1) and β-galactosidase (lane 2) with *S. aureus* V8 protease at an enzyme:protein ratio of 1:10. The digestion products were analysed on a 15% SDS–polyacrylamide gel. M, molecular weight markers, albumin (69000), trypsin inhibitor (21500), cytochrome *c* (12500), aprotinin (6500). The arrow shows the location of the peptide P4. (c) The specificity of the anti-P4 serum was tested by Western blotting using β-galactosidase (lanes 1 and 4), fusion protein F4 (lanes 2 and 5) or peptide P4 (lanes 3 to 6) in the absence (lanes 1, 2 and 3) or presence (lanes 4, 5 and 6) of soluble β-galactosidase (100 μg/ml) in the first antibody buffer.

serum contained antibodies specific for both P4 and the carboxy-terminal region of β-galactosidase. No immune reactions were observed with preimmune serum (data not shown).

Analysis of anti-F3 serum specificity

The specificity of the anti-F3 serum was demonstrated by the immunoprecipitation of a 150000 M_r polypeptide from MHV-JHM-infected Sac(−) cells (Fig. 3, lane 4). This polypeptide has previously been shown by tryptic peptide fingerprinting to be the co-translationally glycosylated intracellular precursor to the virion S protein (Siddell, 1982). Furthermore, it was possible to demonstrate that the anti-F3 serum did not contain detectable amounts of virus-neutralizing activity Fig. 4 shows that a previously characterized neutralizing monoclonal antibody, A1 (E2-Aa) (Wege *et al.*, 1984), at a dilution of 1:10000 was able to neutralize 50 p.f.u. of JHM virus fully (Fig. 4*a*). The anti-F3 serum, or preimmune serum at dilutions ranging from 1:10000 to 1:10, did not display any detectable neutralizing activity (Fig. 4*b*, *c*).

Identification of the mRNA 4 product in vitro

Fig. 5 shows the results of *in vitro* translation in an L cell system of poly(A) RNA from MHV-JHM-infected cells and fractions enriched for mRNAs 4 and 5. As previously described (Siddell, 1983), the total poly(A) RNA directed the synthesis of two major products, identified as the intracellular M (M_r 23000) and N (M_r 60000) polypeptides (lane 3). Both polypeptides were specifically immunoprecipitated by the anti-JHM virion serum (lane 9) compared to preimmune

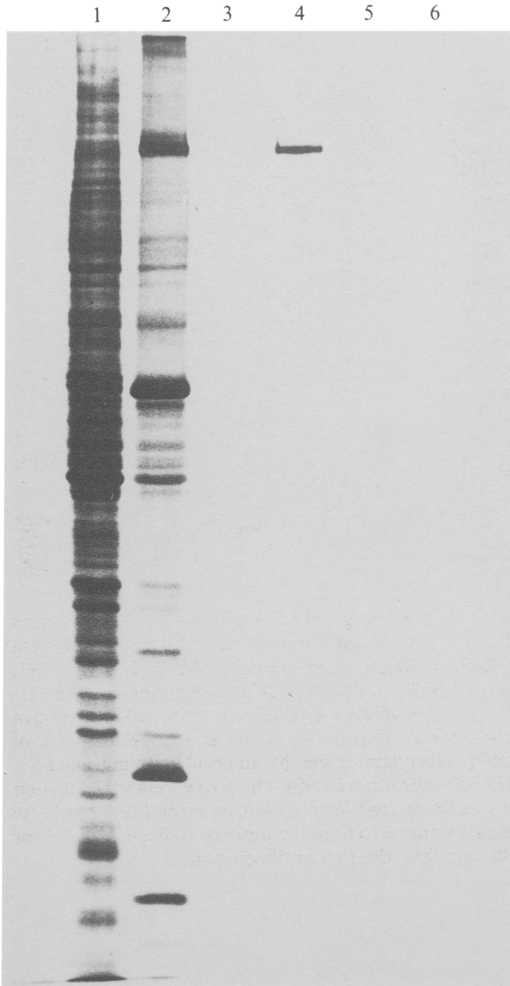

Fig. 3

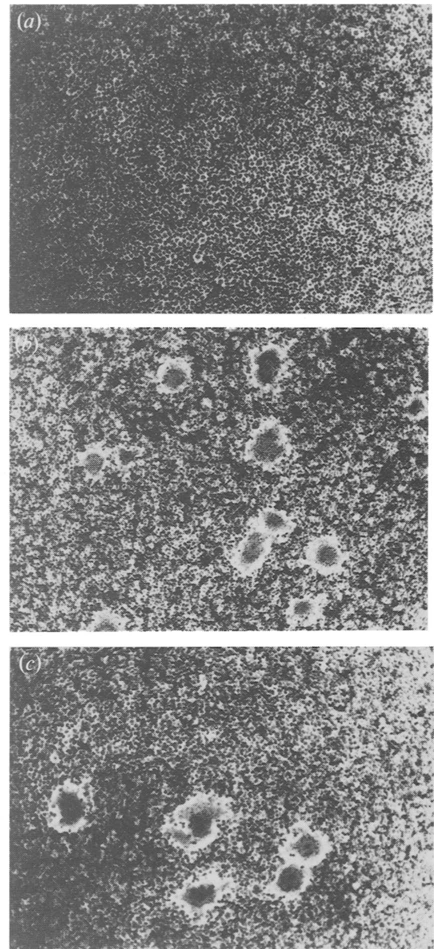

Fig. 4

Fig. 3. Immunoprecipitation of *in vivo* labelled cytoplasmic lysates with anti-F3 serum. [^{35}S]Methionine-labelled cytoplasmic lysates were analysed on a 12% SDS–polyacrylamide gel, directly or following immunoprecipitation. Lane 1, mock-infected cell lysate; lane 2, MHV-JHM-infected cell lysate; lane 3, anti-F3 serum, mock-infected; lane 4, anti-F3 serum, MHV-JHM-infected; lane 5, preimmune serum, mock-infected; lane 6, preimmune serum, MHV-JHM-infected.

Fig. 4. Neutralization of MHV-JHM. Fifty p.f.u. of MHV-JHM was incubated with (*a*) monoclonal antibody A1 (E2-Aa), 1:10000 dilution, (*b*) anti-F3 serum, 1:100 dilution and (*c*) pre-immune serum, 1:100 dilution. The remaining virus was titrated in L929 cells. A representative area of the cell monolayer is shown.

serum (lane 8). Immunoprecipitation of the total poly(A) RNA translation products with the anti-P4 serum (lane 10) did not reveal the immunoprecipitation of any specific polypeptide. The observed precipitation of the M protein appeared to be non-specific as the preimmune serum (lane 8) also produced a similar result. Additionally, we observed a non-specific binding of the M protein to Protein A–Sepharose alone, which may be due to the hydrophobic properties of the integral membrane protein (Rottier *et al.*, 1984).

When the translation products of the poly(A) RNA fraction enriched for mRNAs 4 and 5 were reacted with the anti-P4 serum (lane 7) a clear and specific immunoprecipitation of a 15000 M_r

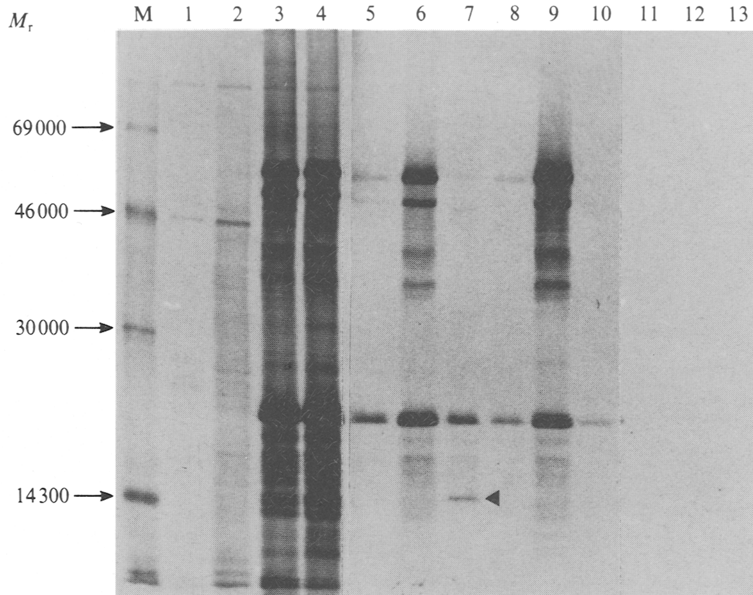

Fig. 5. Immunoprecipitation of *in vitro* translation products with anti-P4 serum. *In vitro* translations were performed with equivalent amounts (0·5 µg) of poly(A) RNA. The products were analysed on a 15% SDS–polyacrylamide gel directly or following immunoprecipitation. Autoradiography of lanes 5 to 13 was three times longer than for lanes 1 to 4. Lane M, M_r markers, albumin (69000), ovalbumin (46000), carbonic anhydrase (30000), lysozyme (14300); lane 1, no RNA; lane 2, poly(A) RNA, mock-infected cells; lane 3, poly(A) RNA, MHV-JHM-infected cells; lane 4, RNA pool 4 + 5, MHV-JHM-infected cells; lane 5, preimmune serum, RNA pool 4 + 5; lane 6, anti-JHM virion serum, RNA pool 4 + 5; lane 7, anti-P4 serum, RNA pool 4 + 5; lane 8, preimmune serum, poly(A) RNA, MHV-JHM-infected; lane 9, anti-JHM virion serum, poly(A) RNA, MHV-JHM-infected; lane 10, anti-P4 serum, poly(A) RNA, MHV-JHM-infected; lane 11, preimmune serum, poly(A) RNA, mock-infected; lane 12, anti-JHM virion serum, poly(A) RNA, mock-infected; lane 13, anti-P4 serum, poly(A) RNA, mock-infected.

polypeptide was observed. The polypeptide was not detected with preimmune serum (lane 5), anti-JHM virion serum (lane 6) or in the products of translation of uninfected cell RNA (lanes 2, 11, 12 and 13).

Identification of the mRNA 4 product in vivo

Fig. 6 shows the immunofluorescence appearance of MHV-JHM-infected Sac(−) cells using the anti-F3, anti-P4 or preimmune serum. By comparison with the preimmune serum (*c*), the anti-F3 serum (*a*) produced a clear staining of syncytia, the characteristic cytopathic effect of MHV-JHM infection. The immune staining was diffuse and restricted to the cytoplasm. The anti-P4 serum (*b*) also produced a clear, albeit less intense, staining of the syncytial cytoplasm. Mock-infected cells did not show any specific immunofluorescence with anti-P4 serum (*d*).

Fig. 7 shows the immunoprecipitation of [^{35}S]methionine-labelled cytoplasmic lysates from MHV-JHM-infected, or mock-infected, cells using preimmune or anti-P4 serum. In the non-immunoprecipitated lysates (lanes 1 and 2) infection-specific polypeptides of 150000, 60000 and 23000 M_r, previously identified as the intracellular S, M and N proteins (Siddell, 1983), are seen. In comparison to the preimmune serum, the anti-P4 serum specifically immunoprecipitated only a 15000 M_r polypeptide from infected cell lysates (lanes 3, 4, 5 and 6). Again, a small amount of non-specific M protein precipitation was seen using each serum and the preimmune serum appeared to contain two activities (cross-)reacting with cellular polypeptides.

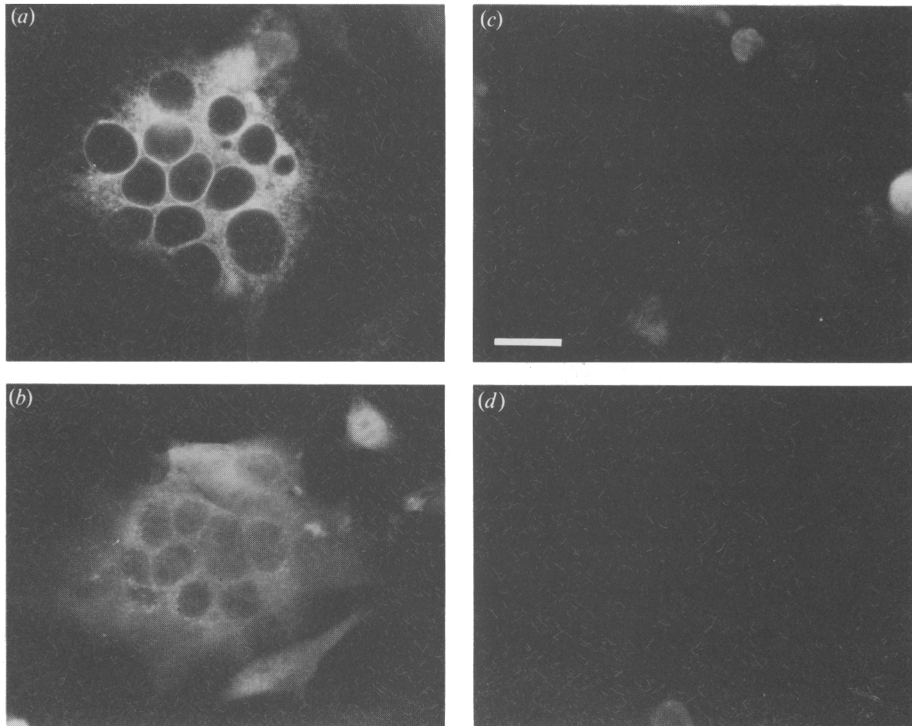

Fig. 6. Indirect immunofluorescence of MHV-JHM-infected cells. Infected cells were fixed, permeabilized, reacted with first antibody and stained with rhodamine-labelled second antibody. Photographs were taken with a Leitz Dialux 22EB fluorescence microscope. Bar marker represents 20 μm. (*a*) Anti-F3 serum, MHV-JHM-infected cells; (*b*) anti-P4 serum, MHV-JHM-infected cells; (*c*) preimmune serum, MHV-JHM-infected cells; (*d*) anti-P4 serum, mock-infected cells.

DISCUSSION

The data presented in this paper show conclusively that the translation product of the MHV-JHM mRNA 4 is a 15000 M_r polypeptide encoded in a single ORF within the 5′ unique region of the mRNA. Sequence analysis of this gene (Skinner & Siddell, 1985) has shown that the initiation codon for ORF 4 lies in a favoured context, GUUAUGG (Kozak, 1983), and is not preceded by any potential upstream initiation codons. The translation product predicted from the nucleotide sequence is a threonine-rich polypeptide with a hydrophobic amino terminus and a basic carboxy terminus. The inability of anti-JHM virion serum to precipitate this polypeptide supports the proposal that mRNA 4 product is a non-structural protein (Siddell, 1983). However, the possibility that it is a minor virion component has not been entirely excluded. At the present time, nothing is known about the function of the 15000 M_r protein; however, the anti-P4 serum described here will be useful for defining its cellular location, the kinetics of its synthesis and possibly its association with either viral or host cell proteins.

The strategy that has been used to produce the anti-P4 serum can also be applied to other coronavirus gene products. The immunofluorescence and immunoprecipitation data indicate that the anti-F3 serum recognizes the MHV-JHM S protein. The antibodies present within the serum are directed against epitopes within the carboxy-terminal region of the S2 polypeptide (Schmidt *et al.*, 1987) and we hope that such antiserum will be useful in delineating biological functions. For example, we have shown that the anti-F3 serum does not contain any detectable virus-neutralizing activity. It should be borne in mind that the anti-F3 serum was produced using an antigen synthesized in bacteria but, nevertheless, this result correlates with the

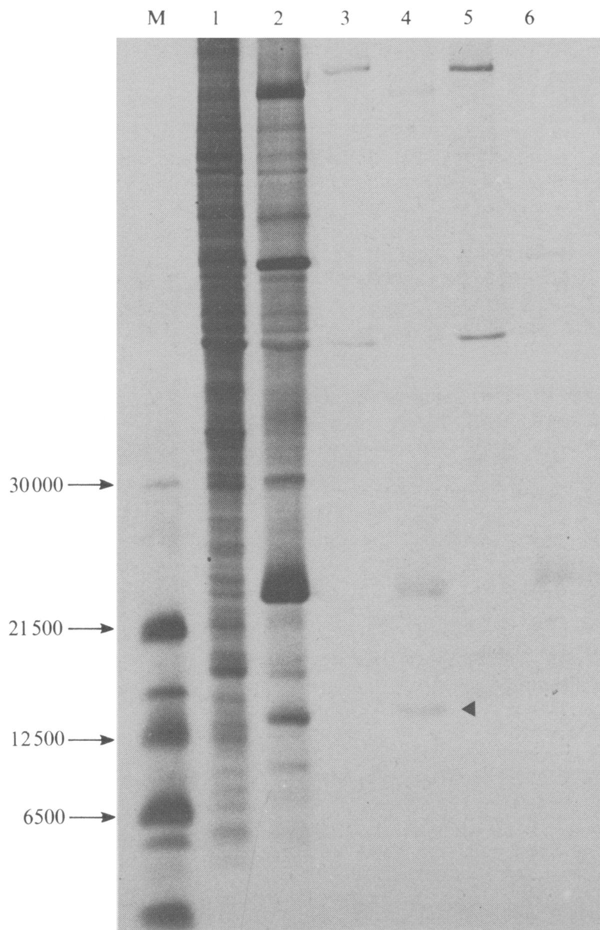

Fig. 7. Immunoprecipitation of *in vivo* labelled cytoplasmic lysates with anti-P4 serum. [^{35}S]Methionine-labelled cytoplasmic lysates were analysed on a 15% SDS–polyacrylamide gel directly or following immunoprecipitation. Autoradiography of all lanes was for equivalent times. Lane M, M_r markers, carbonic anhydrase (30000), trypsin inhibitor (21500), cytochrome *c* (12500), aprotinin (6500); lane 1, mock-infected cell lysate; lane 2, MHV-JHM-infected cell lysate; lane 3, anti-P4 serum, mock-infected; lane 4, anti-P4 serum, MHV-JHM-infected; lane 5, pre-immune serum, mock-infected; lane 6, pre-immune serum, MHV-JHM-infected.

conclusions of Cavanagh *et al.* (1986) who proposed that neutralizing epitopes on the coronavirus S protein are located within the amino-terminal S1 polypeptide.

Finally, the strategy described here may be useful in characterizing further coronavirus non-structural proteins. For example, attempts to identify the coronavirus RNA polymerase have proved to be generally unsuccessful and there is only a single report of an intracellular polypeptide which is thought to be a component of this enzyme (Denison & Perlman, 1987). The analysis of the genomic sequence of coronavirus IBV (Boursnell *et al.*, 1987), *in vitro* translations of MHV genomic RNA (Leibowitz *et al.*, 1982*b*; Denison & Perlman, 1986) and the complementation analysis of MHV temperature-sensitive mutants (Leibowitz *et al.*, 1982*a*) suggest that the virus-coded RNA polymerase is a multifunctional, high M_r polypeptide which may undergo extensive post-translational modification. Specific antiserum to the whole or parts of the RNA polymerase would be invaluable in the investigation of the synthesis, processing and function of this enzyme, which is central to the replication of coronaviruses.

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