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Mouse Hepatitis Virus ORF 2a Is Expressed in the Cytosol of Infected Mouse Fibroblasts

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A 266-bp fragment of cDNA from within gene B, ORF 2a, of MHV-A59 was used to construct a vector encoding a bacterial/viral fusion protein. Antiserum raised against this fusion protein specifically immunoprecipitates a 30K protein from infected 17Cl-1 mouse fibroblasts. The protein is localized primarily in the cytosol and not in the membranes. This is consistent with its predicted sequence and potential role as an RNA binding protein. © 1990 Academic Press, Inc.

Mouse hepatitis virus contains four genes encoding nonstructural proteins (*2*). These include the approximately 23-kb putative polymerase gene A (*3*); gene B, which potentially encodes a 30K basic protein in ORF 2a and, in some MHV strains, an additional glycoprotein in ORF 2b (*1*); gene D, which encodes a 14K protein in the case of MHV-JHM (*4*) or a 10K protein in the case of MHV-A59 (Weiss *et al.*, unpublished data); and gene E, which encodes a 13K basic protein (ORF 5a) and a 9.6K hydrophobic protein (ORF 5b) (*5, 6*). It is possible that at least some of the nonstructural proteins encoded in genes B, D, and E may be involved in RNA synthesis as there are at least six complementation groups of temperature-sensitive mutants of MHV with RNA-negative phenotypes (*7*). The predicted amino acid sequences of ORFs 2a and 5a suggest that these ORFs may encode nucleic acid binding proteins.

Of these predicted small, nonstructural proteins the gene E, ORF 5b product (*8*) and gene D product (*9*) have been detected in infected cells by the use of specific antisera directed against these proteins. A 35K protein, the possible product of gene B, ORF 2a, was observed in infected cells and in the products of cell-free translation of mRNA 2 by the use of serum derived from an infected mouse (*10*). We report here the raising of antiserum directed specifically against the polypeptide encoded in ORF 2a of MHV-A59 and the use of this antiserum to identify the 30K protein in the cytosol of infected cells.

An antiserum was raised against the protein encoded in ORF 2a. The immunogen used to raise this antiserum was a recombinant protein encoded by a bacterial/viral fusion vector containing gene B cDNA. The vector construction is similar to those we have used previously for other viral genes (*8, 11*). A 266-bp internal fragment from within ORF 2a was excised from a 2-kb *Pst*I/*Pst* fragment of MHV-A59 cDNA clone 1033 (*3*) by digestion with *Sac*I and *Nde*I and tailed with oligo(dC) as described previously (*11*). This fragment was inserted into *Sma*I-cleaved, oligo(dG)-tailed bacterial plasmid pGE374, between the "out-of-frame" truncated recA and lacZ genes. These recombinant plasmids were used to transform MC1061 all as previously described (*8, 11*). Clones in which the reading frame of lacZ was restored and hence presumably contained all three proteins fused in-frame were selected on the basis of β-galactosidase activity. This RecA/viral/LacZ tripartite fusion protein was identified from such a clone, purified on the basis of its insolubility after lysis of cells with Triton and sonication, and used to raise antiserum in rabbits all as described previously (*11*). Both native and denatured proteins were used as immunogens to ensure a broad range of reactivity. This anti-ORF 2a serum was shown to react against β-galactosidase in a dot radioimmunoassay (*8*) and then reacted with proteins from infected cells.

Figure 1 shows the detection of the ORF 2a product using this antiserum. Mouse fibroblast 17Cl-1 cells were infected with MHV-A59 or mock-infected and cell lysates were derived at various times postinfection. A 30K protein was observed in infected cells following immunoprecipitation with anti-ORF 2a serum (Panel B) but not with serum directed against other viral proteins (for example MHV nucleocapsid protein, Panel C) or preimmune serum (Panel A). This 30K protein is the correct size for the protein predicted from the sequence of ORF 2a. (There is also nucleocapsid protein observed in the products of immunoprecipitation with anti-ORF 2a serum as well as with preimmune serum (compare Panels A, B, and C). This is due to nonspecific binding of nucleocapsid protein to the staphyloc-

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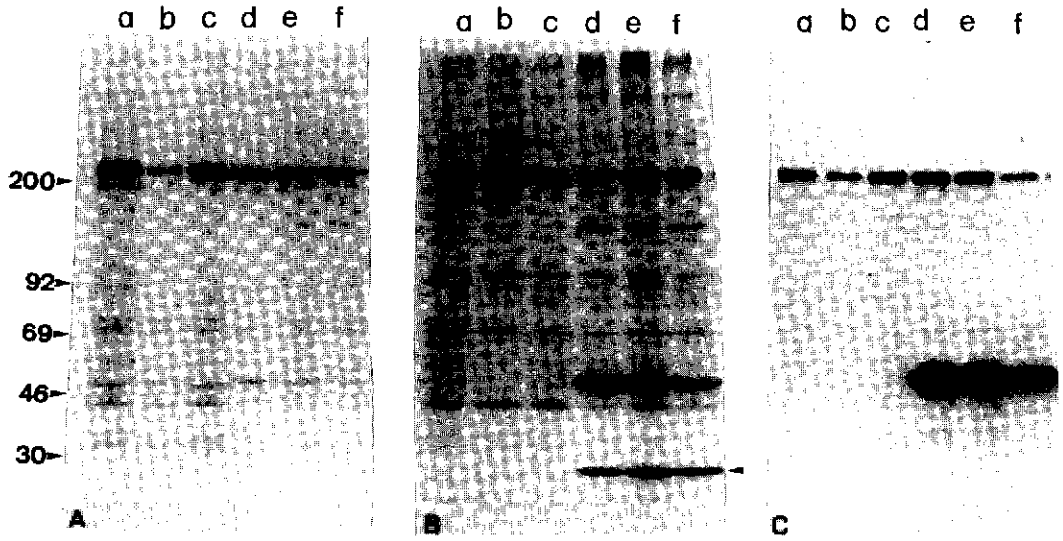

FIG. 1. Kinetics of synthesis of the 30K protein in 17Cl-1 infected fibroblasts. 17Cl-1 cells were infected with MHV-A59 (5–10 PFU/cell) or mock-infected. At various times postinfection as listed below, cells were starved for 1 hr in methionine- and cysteine-free medium and then labeled for 1 hr with 100 μCi/ml each of [^{35}S]methionine and -cysteine. Cells were then lysed in 0.15 *M* NaCl, 0.01 *M* Tris, pH 7.4, 1% NP-40, 1% DOC, 0.1% SDS, 20 μg/ml aprotinin, and 20 μg/ml PMSF; the nuclei were removed by centrifugation, lysates immunoprecipitated, and the proteins resolved on 4–15% polyacrylamide gradient gels (*14*) all as previously described (*8*). (A) Preimmune rabbit serum. (B) Anti-ORF 2a serum. The 30K protein is indicated by the arrowhead. (C) Anti-nucleocapsid monoclonal antibody (1.16.1). Lane a, mock-infected; lane b, 3 hr postinfection; lane c, 7 hr postinfection; lane d, 11 hr postinfection; lane e, 18 hr postinfection; lane f, 19 hr postinfection.

cal protein A used in the immunoprecipitations (J. Leibowitz, unpublished observation).)

The sequence of ORF 2a led to the suggestion that this protein is probably not membrane associated and might be a nucleic acid binding protein (*1*, our unpublished results). Thus we used the antiserum to examine the cellular location of the 30K protein. Figure 2 shows the immunoprecipitation of proteins from cytosol and membrane fractions of 17Cl-1 cells using antiserum directed against the 30K protein. This protein is found mostly in the cytosol and not in the membranes (Panel B). This is consistent with the fact that sequence analysis of ORF 2a does not reveal any hydrophobic, potential membrane-spanning regions. As a control, samples were also reacted with a monoclonal antibody directed against E1, the coronavirus glycoprotein that is located primarily in intracellular membranes (Panel A). Furthermore, the nucleocapsid protein which is present due to its nonspecific binding during immunoprecipitation (Panel B) is present only in the cytosol as expected. It is difficult to determine if there is any 30K protein in the nucleus as we were unable to recover nuclei completely free of membranes, cytoplasm, and unlysed cells. However, it is unlikely that the 30K protein is located in the nucleus as MHV replicates in enucleated cells (*12*).

Immunofluorescent staining was also used to localize the 30K protein. As shown in Fig. 3, the staining is specific for infected cells (Panel A) and the pattern is

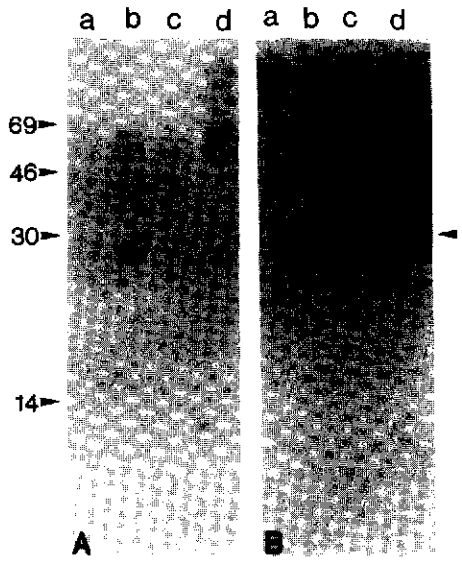

FIG. 2. Subcellular fractionation of the 30K protein. MHV-A59-infected 17Cl-1 cells were labeled with [^{35}S]methionine and -cysteine as in Fig. 1 and at 14 hr postinfection fractionated into nuclear, cytosol, and membrane fractions (*15*). Samples from the membrane and cytosol fractions were immunoprecipitated with antiviral antibodies or control sera. (A) Lane a, membranes precipitated with an anti-reo virus σ-1 protein monoclonal antibody (9BG5); lane b, membranes immunoprecipitated with an anti-E1 (JA1.8) monoclonal antibody; lane c, cytosol precipitated with anti-reovirus σ-1 protein monoclonal antibody; lane d, cytosol precipitated with anti-E1 monoclonal antibody. (B) Lane a, membranes precipitated with preimmune rabbit serum; lane b, membranes precipitated with anti-ORF 2a serum; lane c, cytosol precipitated with preimmune rabbit serum; lane d, cytosol precipitated with anti-ORF 2a serum.

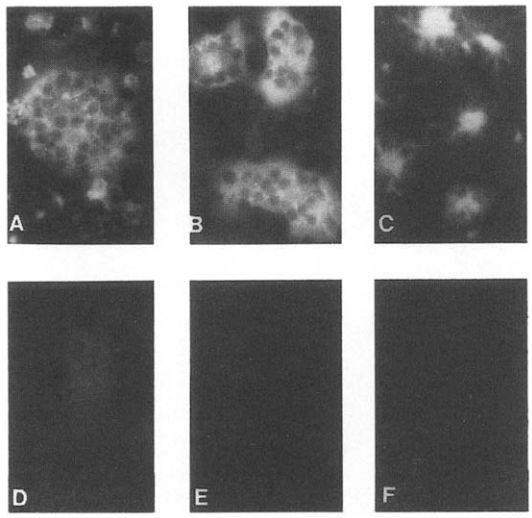

FIG. 3. Indirect immunofluorescent staining of MHV-infected cells. L-2 cells were mock-infected or infected with MHV-JHM (1 PFU/cell). At 9 hr postinfection cells were either fixed for 5 min with 2% buffered paraformaldehyde or left unfixed and then stained by indirect immunofluorescent microscopy using FITC goat anti-rabbit IgG or FITC goat anti-mouse IgG as secondary antibody. (A) Infected fixed cells stained with anti-30K serum at a dilution of 1:400. (B) Infected fixed cells stained with anti-nucleocapsid monoclonal antibody. (C) Infected fixed cells stained with anti-E1 (J2.7) monoclonal antibody. (D) Infected fixed cells, stained with preimmune serum at a dilution of 1:400. (E) Mock-infected fixed cells stained with anti-30K serum at a dilution of 1:400. (F) Infected unfixed cells, stained with anti-30K serum at a dilution of 1:50.

similar to that obtained by staining with the anti-nucleocapsid monoclonal antibody (Panel B). It is diffusely cytoplasmic staining and not observed when unfixed cells are stained with anti-30K serum (Panel F). This is in contrast to the more localized, perinuclear staining of syncytia observed after staining with anti-E1 monoclonal antibody (Panel C). This confirms the subcellular localization of the 30K protein.

In summary, the antiserum generated against a fusion protein containing an internal portion of the protein encoded in ORF 2a detects a 30K protein in cells infected with MHV. This protein is the same size as that predicted from the sequence of the ORF suggesting it is the product of this ORF. This is further supported by the fact that the 30K intracellular protein is the same size as a protein synthesized by *in vitro* translation of an mRNA generated from a pGEM construct containing ORF 2a DNA (data not shown).

The anti-30K serum was generated against both native and denatured fusion proteins. It is useful in detection of the 30K protein by immunoprecipitation, Western blot (data not shown), and immunofluorescence. Therefore, it must contain antibodies to epitopes that are sequential as well as those that are conformational.

The 30K protein is found during both lytic infections in 17Cl-1 cells (Fig. 1) and persistent infections in glial cells (data not shown) in proportions similar to those of other viral proteins. We are presently determining whether it is expressed during infection in animals.

The cytosolic localization of the 30K protein as well as the predicted amino acid sequence of the protein encoded in ORF 2a is consistent with this protein having a role as an RNA-binding protein. Thus it may be part of the very complex polymerase functions of MHV-A59. We are presently attempting to determine the function of the 30K protein by expressing gene B in eukaryotic cells to be used to complement temperature-sensitive RNA-negative mutants and examining the possible RNA binding activity of the protein with virus-specific RNA sequences.

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