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Detection of a Murine Coronavirus Nonstructural Protein Encoded in a Downstream Open Reading Frame

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Mouse hepatitis virus (MHV) gene 5 contains two open reading frames. We have expressed the second open reading frame of this gene (gene 5 ORF 2) in an *Escherichia coli* expression system. This system utilized a plasmid which contained the promoter and the first 36 codons of the *recA* gene fused in frame with the MHV gene 5 ORF 2, which is fused in turn to the β-galactosidase gene. The protein product of this gene fusion was used to raise antibody to gene 5 ORF 2. The specificity of the antibody was verified by immunoprecipitation of the *in vitro* transcribed and translated protein product of gene 5 ORF 2. The second reading frame of MHV gene 5 was shown to be expressed during the course of infection by immunocytochemistry and radioimmunoprecipitation using the antibody raised against the *E. coli* fusion protein and by two-dimensional gel electrophoresis. © 1988 Academic Press, Inc.

INTRODUCTION

The coronaviruses are a group of RNA viruses with genomes of positive polarity which are about 27 kb in length (Boursnell *et al.*, 1987; Lai and Stohlman, 1978; Leibowitz *et al.*, 1981). Infected cells contain five or six subgenomic mRNAs, depending upon the virus studied, and an RNA species which is indistinguishable from genome RNA in structure (Leibowitz *et al.*, 1981; Stern and Kennedy, 1980a, b; Lai *et al.*, 1981). Structural studies have established that the subgenomic mRNAs make up a "nested set" with coterminal 3′ ends (Leibowitz *et al.*, 1981; Stern and Kennedy, 1980a, b; Lai *et al.*, 1981; Cheley *et al.*, 1981; Weiss and Leibowitz, 1983; Spaan *et al.*, 1982) and contain a leader sequence of approximately 72 bases at their 5′ termini (Spaan *et al.*, 1983; Lai *et al.*, 1983, 1984). This is shown schematically in Fig. 1. The leader sequence is identical to the sequence present at the 5′ terminus of the virion RNA and contains a short sequence homologous to sequences in the corresponding regions preceding each gene in the genomic RNA.

In vitro translation studies of purified populations of mouse hepatitis virus mRNAs have demonstrated that the MHV nucleocapsid (N), transmembrane (E1), and peplomer (E2) proteins are encoded by RNA 7, RNA 6, and RNA 3, respectively (Leibowitz *et al.*, 1982; Rottier *et al.*, 1981; Siddell, 1983). RNA 2 encodes a 30- to 35-kDa nonstructural protein (Leibowitz *et al.*, 1982; Siddell, 1983). The coding assignments for RNAs 4 and 5 have been harder to determine, in part because of difficulties in resolving these two species. In cell-free translation studies, a 14-kDa nonstructural protein was synthesized in response to a mixture of these two mRNAs (Leibowitz *et al.*, 1982; Siddell, 1983). These functional studies of MHV mRNA have led to the hypothesis that the most 5′ sequences of each mRNA, not present in smaller mRNA species, contain the coding sequences utilized during infection. This hypothesis has allowed the construction of a tentative genetic map of MHV, shown in Fig. 1.

Sequencing studies of molecular cDNA clones derived from either MHV mRNA or virion RNA have, in general, confirmed the assignment of the MHV structural genes suggested by *in vitro* translation studies (Armstrong *et al.*, 1983, 1984; Skinner and Siddell, 1983, 1985). Sequence analyses of mRNA cDNA clones corresponding to the 5′ portions of RNA 4 of MHV, strain JHM, demonstrate that the unique 5′ terminus of RNA 4 contains an open reading frame encoding a 15.4-kDa protein (Skinner and Siddell, 1985). This is similar in size to the 14-kDa nonvirion protein observed in MHV-infected cells (Rottier *et al.*, 1981). Thus it is likely that RNA 4 encodes a nonstructural protein of about 15 kDa.

Sequencing studies of cDNA clones representing the 5′ portion of MHV-A59 RNA 5 have shown that this mRNA contains two open reading frames which if expressed in MHV-infected cells would encode proteins

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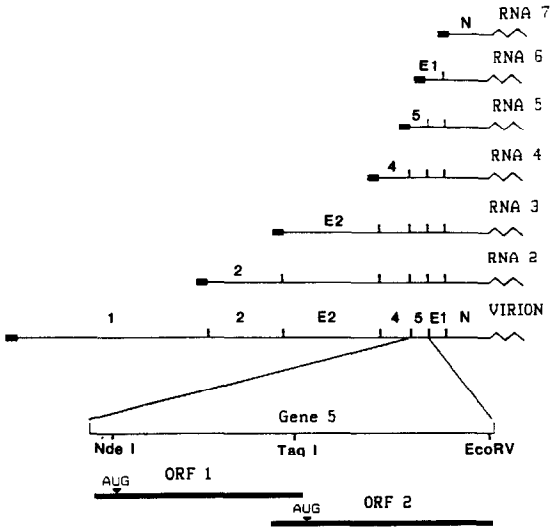

FIG. 1. Schematic drawing of MHV gene 5. The approximately 72-base leader sequence at the 5′ terminus of each RNA is depicted by the dark rectangles. The gene order was deduced from the results of *in vitro* translation (Leibowitz *et al.*, 1982; Rottier *et al.*, 1982; Siddell, 1983) and sequencing experiments (Skinner and Siddell, 1985; Skinner *et al.*, 1985; Budzilowicz and Weiss, 1987), assuming that each mRNA is translated from its 5′ unique sequences. A map of the portion of clone g344 which contains gene 5 is shown at the bottom of the figure. ORF 1 and ORF 2 are depicted as solid dark lines. The positions of their initiating AUG codons and the restriction sites relevant to this work are indicated.

of 13 and 9.6 kDa (Budzilowicz and Weiss, 1987). Similar results have been obtained in sequencing studies of cDNA clones representing the genome of MHV, strain JHM (Skinner *et al.*, 1985). The question of which of the two open reading frames present in MHV RNA 5 is expressed in infected cells is not addressed by the above data. Recently Budzilowicz and Weiss (1987) have demonstrated that RNA synthesized *in vitro* from pGEM recombinant vectors containing these open reading frames can be translated *in vitro* into polypeptides corresponding in size to both reading frames, although the downstream open reading frame is translated preferentially. In this work, we show that the downstream open reading frame within RNA 5 is expressed in MHV-infected cells.

MATERIALS AND METHODS

Cells and virus

The origin and growth of the 17Cl-1 and L-2 cell lines have been described (Sturman and Takemoto, 1972; Rothfels *et al.*, 1959; Leibowitz *et al.*, 1981). The origin and growth of MHV-A59 has been described (Sturman and Takemoto, 1972; Leibowitz *et al.*, 1981).

Plasmids

The isolation and characterization of the plasmid g344 has been described previously (Budzilowicz *et al.*, 1985). This plasmid carries an 1.8-kbp cDNA clone of MHV-A59 spanning the 3′ portion of gene 4, all of gene 5, gene 6 (encoding the E1 protein), and the 5′ portion of gene 7 (encoding the nucleocapsid protein).

The plasmids pGE372 (6.58 kbp) and its derivatives are shown in Fig. 2. Plasmid pGE372 is a derivative of the plasmid pBR322 in which the *tet* gene (between the *Eco*RI and *Ava*I sites in pBR322) has been replaced by a fusion of the *E. coli recA* and *lacZ* genes. The fusion contains the regulatory region from *recA* as well as the first 36 codons of the structural gene. The *recA* sequence is followed by *lacZ*, encoding β-galactosidase. The *lacZ* sequence is missing its promoter, translation start site, and first eight codons. However, the *lacZ* sequence is fused in frame to *recA*, such that a RecA–β-galactosidase hybrid protein is produced. A *Bam*HI site is located between *recA* and *lacZ* in pGE372. Plasmid pGE374 is analogous to pGE372 except that the spacer sequence between *recA* and *lacZ* differs such that the *lacZ* reading frame is +2 with respect to the *recA* reading frame. Consequently a hybrid protein is not produced.

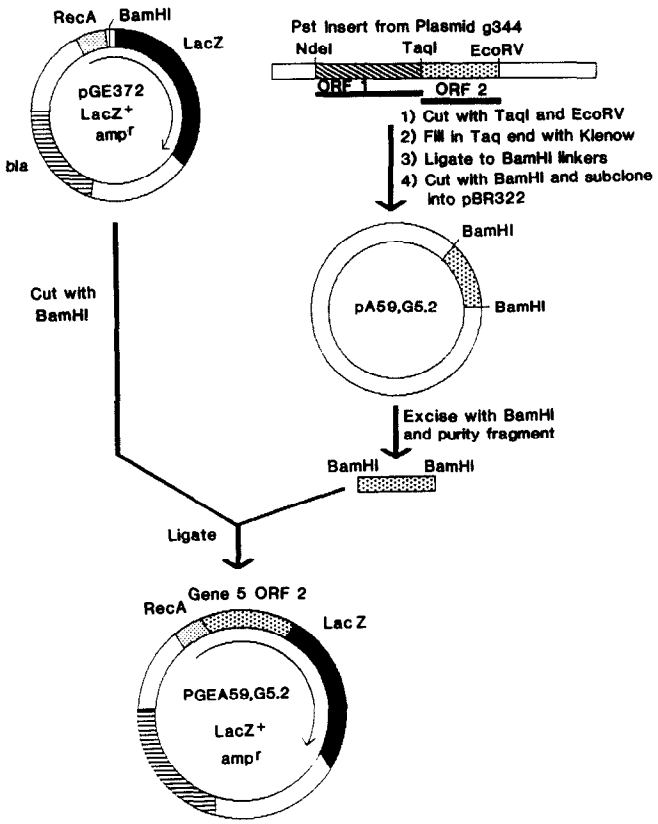

FIG. 2. Construction of pGEA59.G5.2. The β-lactamase gene of pGE372 and its derivative, pGEA59.G5.2, are indicated by ▤. The *recA* and *lacZ* coding sequence are indicated by ▨ and ■, respectively. Gene 5 ORF 1 is represented by ▧; gene 5 ORF 2 is represented by ▩. The details of the construction are presented under Materials and Methods.

Construction of tribrid plasmids

The entire 1.8-kb MHV-specific insert was excised from the plasmid g344 by digestion with *Pst*I and purified by gel electrophoresis. This fragment was digested with *Taq*I and *Eco*RV and the resulting *Taq*I–*Eco*RV fragment of MHV gene 5 containing ORF 2 (254 bases) was purified by agarose gel electrophoresis. The *Taq*I end was repaired using the Klenow fragment of DNA polymerase. Octameric *Bam*HI linkers were then ligated to this fragment, and after digestion with *Bam*HI, it was subcloned into the *Bam*HI site of pBR322. This subcloned fragment was then excised from pBR322 with *Bam*HI, purified by gel electrophoresis, and ligated into the *Bam*HI site of pGE372. This yields a construct in which the *recA* promoter and the first 108 bases of the coding sequence of the *recA* gene are fused in frame to MHV gene 5 ORF 2, which is, in turn, fused in frame with the *lacZ* gene. This construction is shown schematically in Fig. 2.

Recombinant DNA techniques

Large-scale growth and purification of plasmids was performed as described by Clewell and Helinski (1972). Small-scale preparations of plasmid (<20 ml) were made using the procedure of Birboim and Doty (1979). Transformation of HB101 was by the method of Hanahan (1983); transformation of MC1061 was by Dagert and Ehrlich's (1979) modification of the calcium chloride procedure. Ligations and other manipulations of plasmid DNA were essentially as described by Maniatis *et al.* (1982). Computer analyses were performed using the programs provided by Dr. Charles Lawrence, Baylor College of Medicine.

Preparation of bacterial extracts

Bacterial cultures were harvested by centrifugation and resuspended in a small volume of 10 m*M* Tris, pH 7.4, 10 m*M* NaCl, and 1.5 m*M* $MgCl_2$. The bacterial suspension was sonicated for three 20-sec bursts, mixed with an equal volume of 2× SDS–PAGE sample buffer (Maizel, 1971), and immersed in a boiling water bath for 5 min. The extract was then clarified in either a microfuge or a table top centrifuge.

Western blots

Bacterial extracts were prepared in sample buffer and electrophoresed on 8% polyacrylamide gels as described by Maizel (1971). The resolved proteins were transferred to nitrocellulose at 250 mA for 16 hr as described by Towbin *et al.* (1979). The transferred proteins were either stained with 0.1% amido black or tested for the presence of RecA sequences with a rabbit anti-RecA antibody. Blots were blocked overnight at 4° with 3% nonfat dry milk in Tris saline, 1 m*M* PMSF, washed with Tris saline, reacted for 2 hr with 1:1000 dilution of anti-RecA antibody in Tris saline, washed three times with Tris saline containing 0.05% Tween 20, reacted with a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit Ig (Cappel) for 2 hr, washed three times with Tris saline, 0.05% Tween 20, and the bound antibody was visualized by incubation for no more than 10 min with 0.06% 4-chloronaphthol plus 0.02% hydrogen peroxide.

Preparation of anti-tribrid protein antibodies

A 20-ml culture was inoculated with a single colony carrying the desired plasmid and grown to an OD_{600} of 0.2. These cells were then inoculated into a mass culture (400–1000 ml) which was incubated until an OD_{600} of 0.2 was reached. Mitomycin C was added to the cells at a concentration of 1 μg/ml to induce the *recA* promoter and the cultures were incubated for an additional 3 hr. Induction with mitomycin C was necessary since the presence of MHV inserts dramatically decreased the amount of the tribrid protein synthesized relative to the parental RecA–LacZ fusions (data not shown). The cells were harvested and protein extracts were prepared as described above. The tribrid proteins were resolved by electrophoresis on 8% polyacrylamide gels, located by Coomassie blue staining of strips cut from the ends of the preparative gel, electroeluted as described previously (Welch *et al.*, 1981), and quantitated. Between 50 and 100 μg of protein was homogenized with complete Freund's adjuvant and injected subcutaneously in two NZW rabbits. Rabbits were subsequently boosted at approximately 4-week intervals by injection of antigen in incomplete Freund's adjuvant.

Antisera were initially evaluated by immunoblotting against bacterial extracts which had been resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. These extracts were prepared from bacteria carrying the plasmids pGEA59.G5.2, pGE372, and pGE374. At least three immunizations were required before activity against the appropriate bands on the Western blots was observed. This assay merely measured the reactivity of the sera with the tribrid protein and does not distinguish between reactivity to RecA, LacZ, and MHV determinants.

In vitro transcription

The MHV-A59 specific insert present in plasmid g344 was excised with *Pst*I and inserted into the *Pst*I site of pGEM-1 as described previously (Budzilowicz and Weiss, 1987). For the synthesis of RNA representing ORF 2, the MHV-specific insert was excised from

plasmid g344 and digested with *Taq*I, and the 1103-nucleotide *Taq*I/*Pst*I fragment was subcloned into pGEM-2 which had been digested with *Acc*I and *Pst*I. The resulting plasmid was digested with *Hin*dIII and RNA was synthesized using the SP6 polymerase. All transcription reactions were carried out as described by Krieg and Melton (1984).

In vitro translation

Approximately 1 μg of *in vitro* transcribed RNA was translated in a wheat germ cell-free extract (Amersham) as described previously (Budzilowicz and Weiss, 1987). Translation products were analyzed on 8–16% gradient gels (Maizel, 1971) and processed for fluorography with En^{3}Hance (New England Nuclear).

Immunocytochemistry

Cells infected with MHV-A59 or mock-infected were stained at 24 hr postinfection for ORF 2 antigens by the immunoperoxidase method using an ABC kit (Vector Labs). Cells were counter-stained with hematoxylin and photographed.

Intracellular labeling with radioactive amino acids, immunoprecipitation, and two-dimensional gel electrophoresis

L-2 cells were infected with MHV-A59 (multiplicity of infection, 10) and labeled at the times indicated in the figure captions with either [^{35}S]methionine or [^{35}S]cysteine in methionine- or cysteine-depleted medium, respectively.

For radioimmunoprecipitation, labeled cells were washed with phosphate-buffered saline and lysed in NET (0.1 *M* NaCl, 0.01 *M* Tris, pH 7.4, 0.001 *M* EDTA) with 0.5% NP-40. Approximately 10^6 cpm was reacted with either preimmune or immune antibody overnight at 4°. Antigen–antibody complexes were bound to protein A–Sepharose (Pharmacia) and purified away from unbound, labeled protein by extensive washing with PBS, pH 8.6, containing 0.1% bovine serum albumin, 0.1% SDS, 0.1% NP-40, and 0.1% sodium azide. Complexes were dissociated by boiling in Laemmli buffer (Laemmli, 1970) and the labeled products were analyzed by electrophoresis through polyacrylamide gels (Maizel, 1971). Gels were prepared for autoradiography as described previously (Denison and Perlman, 1986). *In vitro* translation products were diluted into 10 m*M* phosphate, 0.4 *M* NaCl, 1% aprotinin (Sigma), 0.5% NP-40, pH 7.4, and bound for 2 hr at 4° to preformed antibody–protein A–Sepharose complexes. The resulting antigen–antibody–protein A–Sepharose complexes were then washed as described above for cell lysates.

For two-dimensional gel electrophoresis, cells were directly lysed in lysis buffer as described previously (O'Farrell, 1975; Denison and Perlman, 1987). Samples were analyzed by two-dimensional gel electrophoresis as described previously, except that the first dimension was an isoelectric focusing gel containing ampholines 3.5–10 and 5–7 at final concentrations of 0.4 and 1.6%, respectively, and the second dimension was a 15% SDS–polyacrylamide gel.

RESULTS

Construction of a tribrid plasmid

Sequence analysis of the plasmid g344 revealed a *Taq*I site (position 412 using the coordinate system of Budzilowicz and Weiss, 1987) at the junction of the two open reading frames in MHV-A59 gene 5. Cleavage of g344 at this site and at the *Nde*I and *Eco*RV sites at positions 88 and 664, respectively, allows the convenient separation and subsequent separate expression of the two open reading frames.

The *Taq*I–*Eco*RV fragment of MHV gene 5 containing ORF 2 was inserted into the *Bam*HI site of pGE372 as illustrated in Fig. 2. The sequences of the vector and the MHV insert predict that those inserts which are ligated in the correct orientation will be in frame with the N-terminal portion of the *recA* gene and also preserve the reading frame of the *lacZ* gene. Therefore bacteria containing this construct will synthesize a tribrid protein consisting of the first 36 amino acids of the RecA protein, the amino acids encoded by the second open reading frame of MHV gene 5, and the 1015 C-terminal amino acids of β-galactosidase. Bacteria containing this plasmid will form blue colonies in the presence of an appropriate chromagen such as X-Gal. Bacteria containing plasmids with the MHV insert in the opposite orientation will not express β-galactosidase activity due to encountering a termination codon within the MHV insert. pGE372 is an in frame *recA–lacZ* fusion and thus transformants containing this plasmid will also be β-galactosidase positive. Therefore we selected colonies containing the correct construction by the presence of β-galactosidase activity and the presence of the MHV insert as detected by colony hybridization.

Several of these colonies were analyzed further. Small-scale plasmid preps were digested with *Bam*HI, electrophoresed on an agarose gel, and blotted to nitrocellulose. These blots were then probed for the presence of MHV sequences using a random primed cDNA probe prepared from MHV-A59 genomic RNA. The results of this analysis for the clone used in all subsequent experiments, designated pGEA59.G5.2,

are shown in Fig. 3. This clone carries a plasmid containing an insert of the predicted size which strongly hybridized with the MHV probe. Protein extracts were prepared from this bacteria carrying this plasmid, unmodified pGE372, or pGE374 as described under Materials and Methods. These extracts were then electrophoresed on 8% polyacrylamide gels and the resolved proteins were visualized by Coomassie blue staining. A band representing the putative RecA–MHV–LacZ tribrid protein was tentatively identified by comparison with pGE372, which directed the synthesis of the RecA–LacZ fusion protein, and with pGE374, which did not since the RecA–LacZ fusion in this vector was out of frame. No difference in electrophoretic mobility could be demonstrated between the putative tribrid protein and the RecA–LacZ fusion protein synthesized in bacteria bearing the plasmid pGE372 (data not shown). To demonstrate that synthesis of the putative RecA–ORF 2–LacZ fusion protein was initiating at the desired site, we performed a Western blot analysis of protein extracts of pGE372, pGE374, and clone pGEA59.G5.2 (Fig. 3B). Inspection of the amido black stained filter allows the identification of a protein present in extracts of pGE372 and pGEA59.G5.2 and missing from pGE374. The apparent molecular weight of this protein was about 120 kDa, the predicted molecular weight of the RecA–LacZ fusion present in pGE372. An identical filter was probed for the presence of RecA antigen using a rabbit anti-RecA antibody. As shown in Fig. 3B, the 120-kDa protein synthesized by pGE372 and pGEA59.G5.2, but absent in extracts of bacteria containing pGE374, reacted with the anti-RecA antibody. Thus we concluded that the plasmid pGEA59.G5.2 contained the desired MHV sequences inserted into the *Bam*HI site between the *recA* and *lacZ* sequences, conferred β-galactosidase activity on bacteria containing it, and directed the synthesis of a protein which reacts with anti-RecA antisera and therefore presumably contains RecA sequences at its N-terminus. This led us to conclude that the putative tribrid protein did in fact express the second open reading frame of MHV-A59 gene 5.

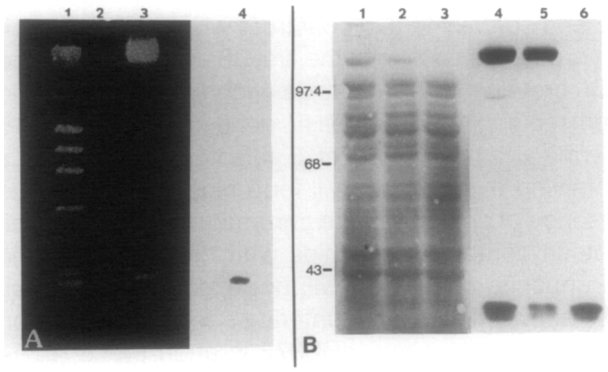

FIG. 3. (A) Southern blot analysis of pGEA59.G5.2. Plasmid pGEA59.G5.2 was digested with *Bam*HI and electrophoresed on a 2% agarose gel, and the DNA was transferred to nitrocellulose. The ethidium stained gel is shown on the left. A mixture of *Hin*dIII-cut λ and *Hae*III- cut φX174 molecular weight markers was in lane 1, lane 2 was empty, and lane 3 contained the *Bam*HI digest of pGEA59.G5.2. An autoradiograph of the nitrocellulose filter after hybridization to a random-primed cDNA probe prepared from MHV-A59 virion RNA is shown in lane 4. (B) Western blot analysis of extracts of pGEA59.G5.2 (lanes 1 and 4), pGE372 (lanes 2 and 5), and pGE374 (lanes 3 and 6). Bacterial extracts were prepared, electrophoresed on an 8% SDS–polyacrylamide gel, and transferred to nitrocellulose as described under Materials and Methods. Lanes 1–3 were stained with amido black. Lanes 4–6 were stained with rabbit anti-RecA antibody used at a dilution of 1:1000. The positions of molecular weight markers are indicated on the left.

Characterization of anti-fusion protein antibodies

Antisera to the gene 5 ORF 2 containing tribrid protein were prepared and initially characterized by immunoblotting against bacterial extracts containing the immunogen used to raise these antibodies. This test did not distinguish anti-MHV antibodies from anti-RecA or anti-β-galactosidase antibodies. Therefore when sera demonstrated reactivity in this assay they were then tested by radioimmunoprecipitation of the MHV-specific products encoded in gene 5. Transcripts of MHV-A59 gene 5 ORF 2 were produced *in vitro* from subclones of this gene in the vector pGEM-1 as described previously (Budzilowicz and Weiss, 1987) and translated in a wheat germ cell-free system. The *in vitro* translation products were immunoprecipitated with hyperimmune and preimmune sera. The resulting precipitates were then analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Fig. 4, the sera raised against the tribrid protein containing the second open reading frame react with the 9.6-kDa cell-free translation product corresponding to that reading frame.

Demonstration of gene 5 products in infected cells

The product of the second open reading frame contained in MHV gene 5 could be demonstrated by immunoperoxidase staining of infected cells, by immunoprecipitation and analysis of products by one-dimensional gel electrophoresis, and by two-dimensional gel electrophoresis. As shown in Figure 5B, MHV-infected cells were stained by the immunoperoxidase procedure with sera raised against the tribrid protein containing ORF 2. These same sera failed to react with uninfected cells (Fig. 5C). Preimmune sera from the same rabbit did not stain infected cells (A).

The ORF 2 product synthesized in the cell-free translation system migrated as a single spot when analyzed by two-dimensional gel electrophoresis (Fig. 6A). When infected cell lysates labeled at late times p.i.

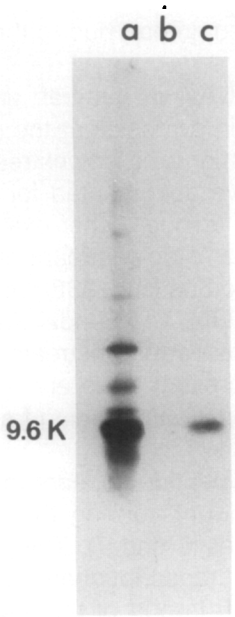

FIG. 4. Immunoprecipitation of *in vitro* synthesized ORF 2 product. ORF 2 RNA, transcribed from a recombinant pGEM vector, was translated in a wheat germ cell-free system. The [^{3}H]leucine-labeled protein products were immunoprecipitated with preimmune serum (lane b) or anti-ORF 2 serum (lane c). Lane a shows all of the translation products before immunoprecipitation. Electrophoresis was on 8–16% gradient gels.

with [^{35}S]methionine were analyzed by the same technique, a spot corresponding to the cell-free product was readily detectable (Fig. 6D). This protein comigrated with the cell-free product, as shown in Fig. 6E, in which the cell-free product and infected cell lysate were mixed prior to analysis. The ORF 2 protein was not present in uninfected cells, as shown in Figs. 6B and 6C.

The identity of the *in vivo* and cell-free products was confirmed using the antibody directed against the ORF 2 product. For these experiments, infected and uninfected cells were labeled with [^{35}S]cysteine and reacted with the anti-ORF 2 antibody. Precipitated labeled protein was isolated and analyzed by one-dimensional gel electrophoresis, as shown in Fig. 7. The anti-ORF 2 antibody reacted with a protein from infected cells (lane 4) which comigrated with the ORF 2 cell-free product (lane 5). This protein was not present in uninfected cells (lane 2), and was not detected when cells were labeled at early times (3–4 hr) after infection (lane 3). This protein could not be precipitated from infected cells with preimmune sera. A protein with the same approximate mobility could be detected in infected (lane 6) but not uninfected cell lysates without immune precipitation (data not shown).

DISCUSSION

In this paper we have constructed a plasmid encoding a fusion protein containing the second open reading frame of MHV-A59 gene 5. We have used the tribrid protein synthesized by *E. coli* carrying this plasmid to raise antisera to the protein encoded by this open reading frame. This antiserum was then used to dem-

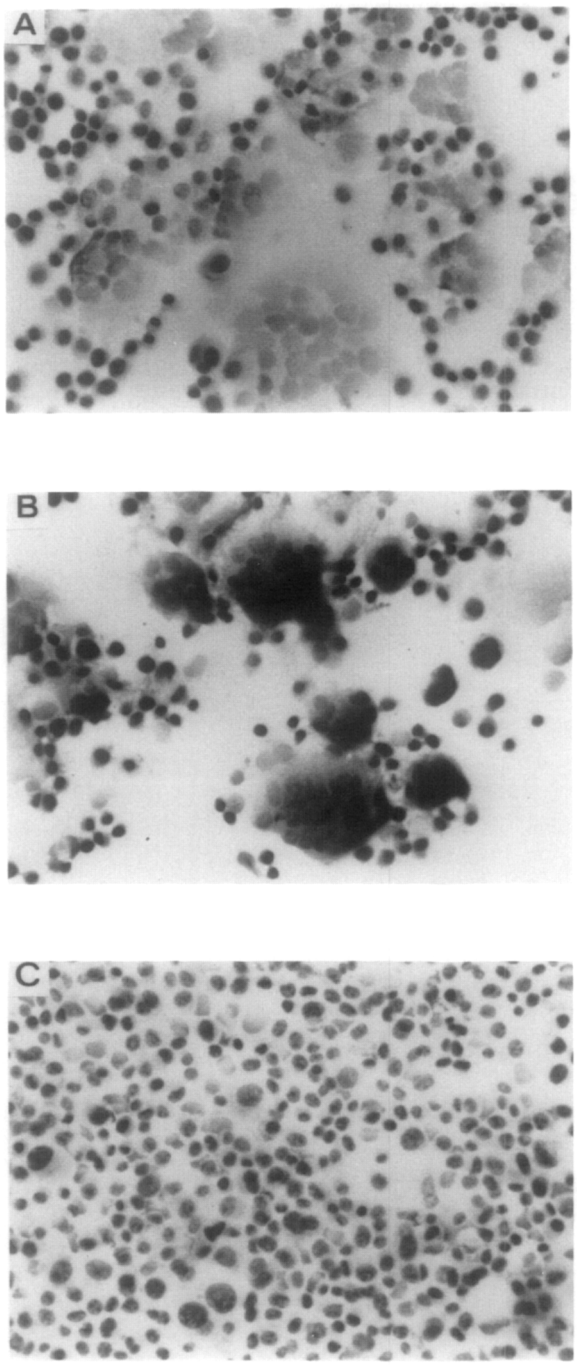

FIG. 5. Detection of ORF 2 protein product by immunoperoxidase staining. Infected and mock-infected cells were deposited on slides by cytocentrifugation and fixed by immersion in 5% paraformaldehyde for 5 min. Slides were stained by the ABC immunoperoxidase technique. (A) Infected cells stained with preimmune serum. (B) Infected cells stained with anti-ORF 2 serum. (C) Uninfected cells stained with anti-ORF 2 serum.

onstrate that the second open reading frame was translationally active during infection with MHV, resulting in the synthesis of a protein which could be demonstrated by immunocytochemistry. Furthermore, a protein which corresponds to the *in vitro* product of the second open reading frame, ORF 2, could be demonstrated by two-dimensional gel electrophoresis of infected cell extracts and by immunoprecipitation using anti-ORF 2 antibody. The use of antibodies to a protein derived in part from a MHV cDNA clone ensures that the 9.6-kDa protein identified by radioimmunoprecipitation is virus-encoded.

Our results are consistent with those of Skinner *et al.* (1985) who showed that a 9- to 10-kDa polypeptide was present in MHV-JHM-infected cells but not in uninfected controls. Cell-free translation of size fractionated RNA isolated from infected cells was consistent with this protein being encoded in gene 5, although it could not be definitively determined that this protein was virus-encoded, in part due to the unavailability of specific antisera.

Eukaryotic mRNAs, in general, are monocistronic and initiate protein synthesis at the most 5′ AUG. Although the utilization of a downstream open reading frame, as we have documented for MHV gene 5, is unusual, it is not unique. Other examples of RNA viruses which utilize a downstream reading frame are reovirus (Ernst and Shatkin, 1985), vesicular stomatitis virus (Herman, 1986), and Sendai virus (Curran *et al.*, 1986). In these cases the upstream reading frame is also utilized. At present there are no data indicating that the gene 5 upstream reading frame is expressed in MHV-infected cells.

The ORF 2 polypeptide appears to be expressed in relatively low amounts during the MHV replication cycle (Fig. 7, lanes 6 and 7). This could be due to regulation at the transcriptional and/or translational level. MHV RNA 5 is one of the less abundant viral

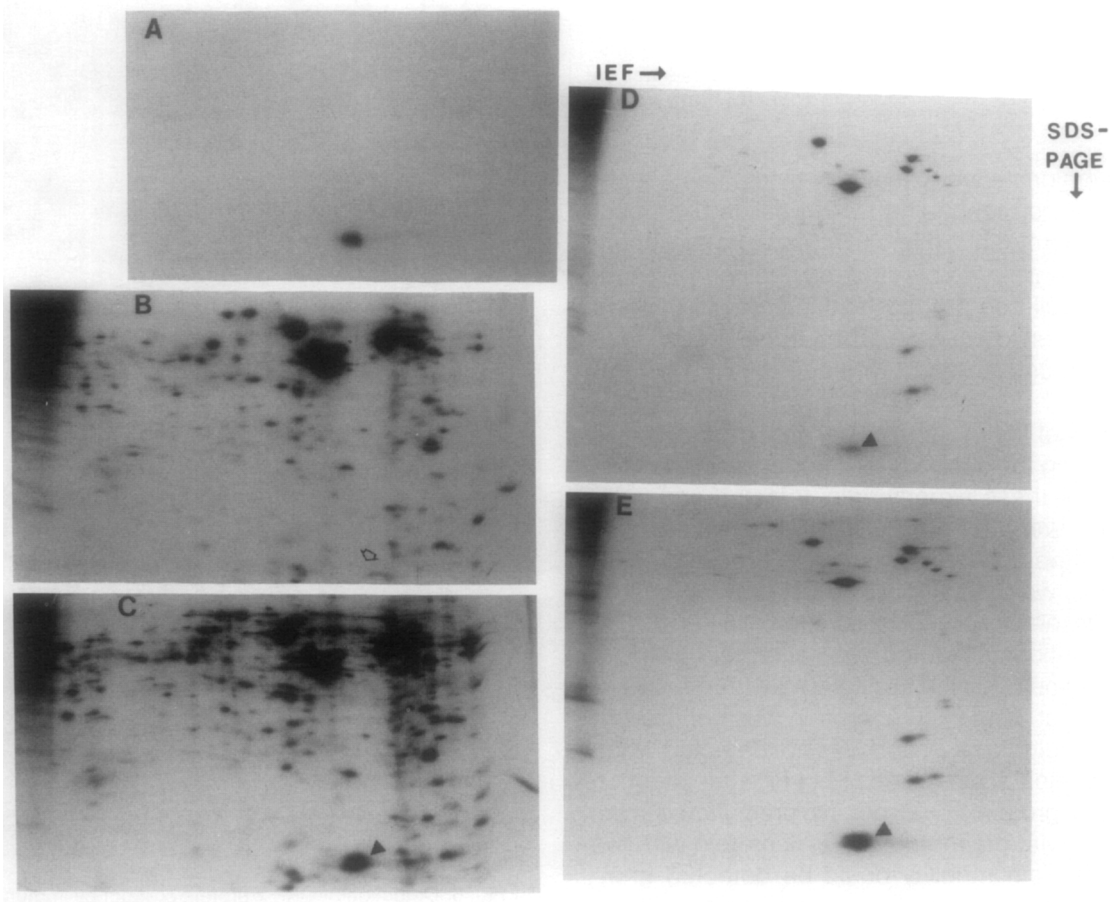

FIG. 6. Two-dimensional gel electrophoresis of intracellular proteins. MHV-infected and uninfected L-2 cells were resuspended in DMEM lacking methionine supplemented with 2% fetal bovine serum at 5 hr postinfection. Ten minutes later, cells were labeled with [^{35}S]methionine (100 μCi/ml) for 1 hr. Cells were processed as described under Materials and Methods. (A) [^{35}S]methionine-labeled cell-free translation product of gene 5 ORF 2. (B) Uninfected cell lysate. (C) Mixture of (A) and (B). (D) Infected cell lysate. (E) Mixture of (A) and (D). Solid arrows in (C), (D), and (E) indicate the position of the gene 5 ORF 2 polypeptide. The open arrow in (B) indicates where this protein would be located if it were present in this sample.

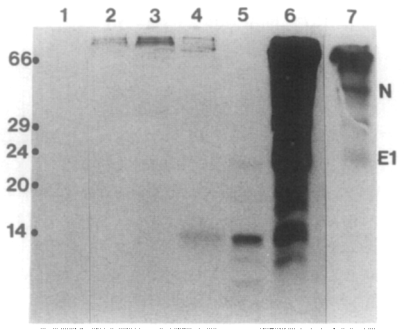

FIG. 7. Immunoprecipitation of gene 5 ORF 2 product from infected cells. MHV-infected cells and uninfected L-2 cells were resuspended in DMEM lacking cysteine at the times indicated below and labeled for 1 hr with [^{35}S]cysteine. Cytoplasm was then prepared and reacted with antibody as described under Materials and Methods prior to analysis by electrophoresis on 15% SDS–polyacrylamide gels. Lane 1, infected cell extract (labeled 6.5–7.5 hr p.i.) precipitated with preimmune serum. Lane 2, uninfected cell extract precipitated with anti-ORF 2 serum. Lanes 3 and 4, infected cell extracts labeled at 3.5–4.5 hr p.i. and 6.5–7.5 hr p.i., respectively, and precipitated with anti-ORF 2 serum. Lane 5, cell-free translation product of gene 5 ORF 2 gene. Lanes 6 and 7, total cytoplasmic extract of cells labeled at 6.5–7.5 hr p.i. The positions of molecular weight markers are indicated on the left and the E1 and N viral structural proteins are indicated on the right. Lane 7 was exposed for 1/10 as long as the rest of the gel.

mRNAs (Leibowitz *et al.*, 1981). Further evidence for transcriptional regulation is the greatly decreased amount of RNA 5 in MHV-JHM-infected cells when compared to cells infected with MHV-A59 (Leibowitz *et al.*, 1981). In regard to translational regulation, the sequence found near the initiation site of protein synthesis in gene 5 ORF 2, GAAAUGU, is not a good match for the Kozak eukaryotic consensus initiation sequence A(G)CCAUGG (Kozak, 1984). The most important divergence is at the strongly conserved −3 position. A is present at this position with a frequency of 79%, and G with a frequency of 18% in eukaryotic mRNAs. However, other MHV genes are better matches. Gene 6 (E1) and gene 7 (N) have initiation codons embedded in the sequences AUUAUGA and AGGAUGA (Spaan *et al.*, 1983), respectively. These are sequences which are frequently used by eukaryotic mRNAs.

The role of the MHV gene 5 ORF 2 protein in the infected cell is unknown. The coronavirus IBV is predicted to direct the synthesis of two small proteins encoded by overlapping reading frames in a fashion similar to that of MHV gene 5 (Boursnell and Brown, 1984). One of these is quite hydrophobic and is a likely counterpart to the protein encoded in MHV gene 5 ORF 2. Other families of RNA viruses have recently been demonstrated to encode small hydrophobic proteins as well. Influenza virus induces the synthesis of the nonstructural M_2 protein, an integral membrane protein (Zebedee *et al.*, 1985). The paramyxovirus SV5 also encodes a small extremely hydrophobic protein (Hiebert *et al.*, 1985). The functions of all of these proteins in viral replication are unknown at present.

The approach that we used in this paper to construct a plasmid which directed the synthesis of a tribrid protein suitable for raising antibodies to ORF 2 required knowledge of the sequence of gene 5. We have recently modified our approach to make it more generally useful, even when the sequence of the gene in question is not known. Randomizing the ends of the insert and vector by homopolymer tailing or by nuclease digestion prior to ligation will produce the appropriate gene fusion in about 6% of the transformants obtained. Furthermore, the ability to probe for the presence of the correct N-terminus of the tribrid protein by immunoblot analysis with anti-RecA antibody, the correct C-terminus by the presence of β-galactosidase activity, and the insert by Southern hybridization provides assurance that the correct construction has been achieved.

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