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Cell-Free Translation of Murine Coronavirus RNA

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The coding assignments of the intracellular murine hepatitis virus-specific subgenomic RNA species and murine hepatitis virion RNA have been investigated by cell-free translation. The six murine hepatitis virus-specific subgenomic RNAs were partially purified by agarose gel electrophoresis and translated in an mRNA-dependent rabbit reticulocyte lysate, and the cell-free translation products were characterized by gel electrophoresis, immunoprecipitation, and tryptic peptide mapping. These studies have shown that RNA 7 codes for the nucleocapsid protein, RNA 6 codes for the E1 protein, RNA 3 codes for the E2 protein, and RNA 2 codes for a 35,000-dalton nonstructural protein. Genomic RNA directs the cell-free synthesis of three structurally related polypeptides of greater than 200,000 in molecular weight.

The coronaviridae are a group of RNA viruses which have been defined on morphological grounds. The virions are pleomorphic enveloped particles about 100 nm in diameter which have a "corona" of bulbous projections on their surfaces and mature by budding through the membranes of the endoplasmic reticulum rather than through the plasma membrane (35). Studies on the structure of the coronavirus genome have shown it to be a large, single-stranded RNA molecule which is polyadenylated (8, 11, 16, 17, 36) and infectious (15, 25) and therefore presumably is of positive polarity. Recent studies on the structure of the genome and on coronavirus-specific intracellular RNAs have confirmed this to be the case (10, 13, 14, 30, 31, 38).

We have been investigating the replication of one member of the coronaviridae, murine hepatitis virus (MHV). Cells infected with MHV contain at least seven virus-specific RNA species (14, 23, 29). These RNAs, having molecular weights ranging from 6.3×10^5 to 6.1×10^6, are polyadenylated and present on polyribosomes and, therefore, presumably function as mRNAs. The largest of the MHV-specific intracellular RNA species is of the same size and has the same oligonucleotide fingerprint as virion RNA (10, 13, 14). Recently, three of the six subgenomic RNAs have been translated in vitro (5, 13, 23, 26). In this report, we describe our in vitro translation studies on the subgenomic and genomic RNAs of MHV.

MATERIALS AND METHODS

Cells. The origin of the murine cell line 17 CL 1 has been described previously (34). Cells were grown in 32-ounce (960-ml) glass prescription bottles with Dulbecco modified Eagle medium containing 10% newborn calf serum as described previously (3).

Virus. The origin and growth of MHV-A59 and MHV-JHM have been described previously (21). For experiments, cells were removed from the substrate, infected in suspension at a multiplicity of 0.1 to 0.2 PFU per cell for 30 min, and plated into plastic culture dishes as described previously (3, 14). In experiments utilizing $^{32}P_i$ as label, the cells were resuspended and plated in phosphate-free medium.

Preparation of intracellular RNA. Cells were infected with MHV-A59 or mock infected as described above. At 4 h postinfection, actinomycin D (Calbiochem) was added to the cultures to a concentration of 5 μg/ml. After 15 min of incubation, cultures were labeled by the addition of $^{32}P_i$ (ICN) and incubated until 90% of the culture was involved in syncytia (7.5 to 8 h postinfection). The cultures were placed on ice, the medium was removed, and the cultures were rinsed once with phosphate-buffered saline (pH 7.4) and once with RSB (10 mM Tris–10 mM NaCl–1.5 mM $MgCl_2$ [pH 7.4]). A cytoplasmic extract was prepared by lysing the cells in RSB containing 0.5% Nonidet P-40 and 0.1% sodium dodecyl sulfate. This procedure leaves most of the nuclei in the culture dish. The cytoplasmic extract was then digested with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol as previously described (14).

Preparation of virion RNA. Cells were infected with MHV-A59 or MHV-JHM, incubated at 33°C, and labeled with [^{3}H]uridine (New England Nuclear Corp.) from 4 to 16 h postinfection. The cells were disrupted by three cycles of freeze-thawing, and virion RNA was purified as described previously (14). Briefly, the cell lysate was clarified at 10,000 × *g* for 30 min, and the virus was pelleted through a 0.5-ml, 15% (wt/wt) potassium tartrate pad and then partially purified by centrifugation at 35,000 rpm for 45 min in an SW41 rotor on a 5 to 25% (wt/wt) potassium tartrate gradient.

The virion RNA was extracted and further purified by sedimentation on a 10 to 30% (wt/wt) sucrose gradient in an SW50.1 rotor at 46,000 rpm for 107 min. RNA prepared in this manner is homogeneous by gel electrophoresis and is not contaminated with cellular RNAs detectable by hybridization (38).

Agarose gel electrophoresis. (i) Preparative electrophoresis. Intracellular RNA was extracted from infected cells, and polyadenylated RNA was selected by chromatography over polyuridylate-Sepharose (Sigma Chemical Co.) as described by Wilt (40). The polyadenylate-containing RNA was precipitated with ethanol, collected by centrifugation, denatured with 10 mM methylmercuric hydroxide, and electrophoresed in horizontal 0.8% agarose slab gels containing 5 mM methylmercuric hydroxide (2). Electrophoresis was carried out at 100 V for 6 h.

(ii) Analytical electrophoresis. RNA samples were precipitated with ethanol, collected by centrifugation, and denatured with glyoxal as previously described (14, 19). Electrophoresis (4 h, 100 V) was carried out in horizontal 0.8% agarose slab gels containing 10 mM phosphate buffer–2 mM EDTA (pH 7.0).

RNA elution from gels. After preparative electrophoresis, MHV-specific RNAs were located by autoradiography of wet gels wrapped in Saran and exposed to XR-5 or XAR-5 film (Eastman Kodak Co.) at 4°C in the presence of an intensifying screen (12). Agarose strips corresponding to the bands present in the autoradiograph were cut from the gel with a flamed scalpel and placed in sterile dialysis bags containing TA buffer (10 mM Tris–20 mM sodium acetate–5 mM EDTA [pH 7.3]), 1% 2-mercaptoethanol, and 10 μg of rabbit liver tRNA (Sigma). The MHV-specific RNAs were electroeluted from the gel at 280 mA for 2 h. The eluted RNA was extracted with phenol and precipitated with ethanol. The precipitated RNA was collected by centrifugation, taken up in water, and reprecipitated with ethanol a further three to five times. After the final ethanol precipitation, the RNA was dissolved in 2 to 10 μl of water and either used immediately for in vitro translation studies or stored at −70°C for later use.

In vitro translation. An mRNA-dependent rabbit reticulocyte lysate was prepared with micrococcal nuclease (P-L Biochemicals, Inc.) as described by Pelham and Jackson (20). A modification of the system described by Weiss et al. (37) was used for the in vitro translation of MHV virion and intracellular RNAs. Reaction mixtures were usually 25 μl. The final concentrations of reagents in the translation reactions were 10 mM HEPES (*N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.8), 2 mM GTP (Sigma), 1 mM ATP (Sigma), 10 mM creatinine phosphate (Calbiochem), 50 μg of creatinine phosphokinase (Calbiochem) per ml, 360 μM spermidine (Sigma), 78 mM KCl, 1.68 mM magnesium acetate, 80 μg of rabbit liver tRNA (Sigma) per ml, 50 μM all amino acids except methionine, and 700 to 900 μCi of [^{35}S]methionine (1,260 to 1,315 Ci/mmol; Amersham Corp.) per ml. Translation reactions were incubated at 29°C for 1 h.

Radioimmune precipitation. The preparation and properties of the mouse anti-MHV serum used in these studies have been described previously (3). Rabbit anti-E2 serum was kindly provided by K. Holmes, Uniformed Services University of the Health Sciences. The properties of this antiserum have been described previously (33). Portions of in vitro translation reactions were incubated on ice for 30 min with 20 μl of anti-MHV, anti-E2, or normal control serum. Antigen-antibody complexes were adsorbed to protein A-bearing *Staphylococcus aureus* (9) and washed five times in phosphate-buffered saline, and the immunoprecipitates were analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Maizel (18), and the gels were processed for fluorography using En^{3}Hance (New England Nuclear Corp.) or sodium salicylate (4).

Tryptic peptide analysis. Proteins were purified by polyacrylamide gel electrophoresis, and their positions were located by autoradiography of unfixed, dried gels. The bands of interest were excised, rehydrated, and electroeluted as described previously (39). Bovine gamma globulin (100 μg) was added as a carrier, and the proteins were precipitated with cold trichloroacetic acid (final concentration, 15%) for 1 h. The precipitate was collected by centrifugation, washed sequentially with a 1:1 mixture of ethanol and diethyl ether followed by ether, dried under vacuum, and then oxidized with performic acid (7). The sample was diluted with water, lyophilized, taken up in 0.5 ml of 0.05 M $(NH_4)_2CO_3$ buffer (pH 7.8), and digested with 30 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Diagnostics) for 4 h at 37°C. An additional 30 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin was then added, and the digestion was continued overnight at room temperature. The sample was lyophilized four times, dissolved in electrophoresis buffer (*n*-butanol–pyridine–acetic acid–water, 2:1:1:36, pH 4.7), relyophilized, and taken up in 10 μl of electrophoresis buffer. Samples were spotted onto cellulose thin-layer plates (20 by 20 cm by 0.1 mm; EM Laboratories) and electrophoresed at 1,000 V for 30 min. The plates were air dried and then chromatographed perpendicular to the direction of electrophoresis with butanol-pyridine-acetic acid-water (393:304:61:243). The thin-layer plates were dried, sprayed three times with En^{3}Hance spray (New England Nuclear Corp.), and exposed to preflashed film at −70°C.

For limited proteolysis mapping, bands containing the polypeptides of interest were excised from gels, treated with protease, and electrophoresed on a 15% polyacrylamide gel by the method of Cleveland et al. (6).

RESULTS

Cell-free translation of intracellular RNA. MHV-specific intracellular RNA was purified from infected cells by polyuridylate-Sepharose chromatography, followed by agarose gel electrophoresis. An autoradiograph of preparative and analytical gels is shown in Fig. 1. The regions of the preparative gel containing RNAs 2, 3, 4, 5, 6, and 7 were excised, and the RNA was eluted from the gel as described in Materials and Methods. Small portions of the RNA preparations were denatured with glyoxal and analyzed by gel electrophoresis. Preparations of RNAs 2, 3, and 7 were relatively free of other MHV-specific RNA species (Fig. 1). However,

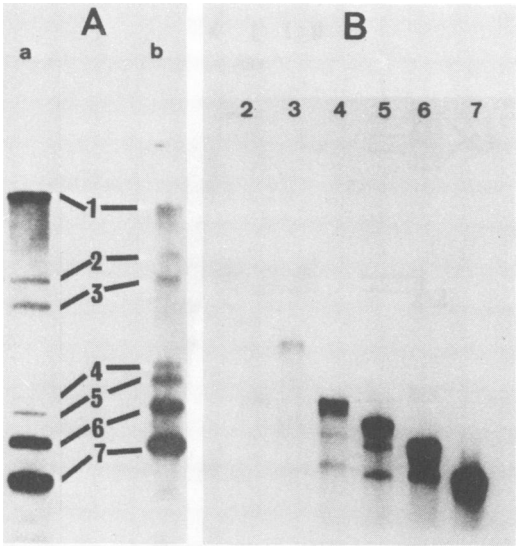

FIG. 1. Preparative and analytical agarose gel electrophoresis of MHV-specific RNA. (A) Infected cells (5×10^6) were labeled with $^{32}P_i$ in the presence of actinomycin D. The cytoplasmic RNA was extracted, precipitated with ethanol, denatured with glyoxal, and analyzed by electrophoresis in an 0.8% agarose gel. An autoradiograph of the dried gel is shown in lane a. ^{32}P-labeled MHV-specific RNA was extracted from 1.5×10^9 cells, selected by chromatography over polyuridylate-Sepharose, denatured with methylmercuric hydroxide, and electrophoresed in a preparative 0.8% agarose gel. An autoradiograph of one lane of the wet gel is shown in lane b. (B) Samples of partially purified MHV-specific RNAs were denatured with glyoxal and analyzed by electrophoresis in an 0.8% agarose gel. An autoradiograph of the dried gel is shown. The RNA species analyzed is indicated above the individual lanes.

small amounts of RNA 7 were sometimes detectable in preparations of RNAs 2 and 3. Preparations of RNA 4 contained a large amount of RNA 5 and smaller amounts of RNAs 6 and 7. Similarly, preparations of RNAs 5 and 6 contained varying amounts of smaller MHV-specific RNAs. The remainder of each RNA was translated in vitro in an mRNA-dependent reticulocyte lysate (20, 37).

Portions of translation reactions were analyzed by polyacrylamide gel electrophoresis (Fig. 2). RNA 7 directed the synthesis of a 60,000-molecular-weight (60K) polypeptide which comigrated with authentic MHV nucleocapsid (N) protein (Fig. 2, lane c). A more abundant 57K polypeptide was also synthesized in the cell-free reaction. This protein is related to MHV N protein (see below) and comigrated with a virus-specific intracellular protein that is also related to N protein (Bond et al., manuscript in preparation).

RNA 6 directed the cell-free synthesis of a 20K polypeptide that comigrated with an intracellular precursor to the MHV E1 virion glycoprotein (Fig. 2, lane b) (27, 32; Bond et al., manuscript in preparation). Some N-related 57K polypeptide was also synthesized in this reaction, probably owing to the preparation of RNA 6 containing small amounts of RNA 7 (see Fig. 1 and Discussion).

RNA 5 directed the synthesis of a 14K polypeptide in addition to the 20K E1-related polypeptide and 57K N polypeptide (Fig. 2, lane d). An MHV-specific 14K polypeptide has been observed in infected cells (22, 27). RNA 4 directed the synthesis of the same polypeptides, only in lesser amounts (data not shown). RNAs 4 and 5 were difficult to purify, and thus it was difficult to assign their protein products (see Discussion).

RNA 3 directed the synthesis of a 120K polypeptide that is related to MHV E2 protein (see below). This polypeptide is not observed in infected cells but is approximately of the same molecular weight as the unglycosylated precursor of the E2 polypeptide synthesized in the presence of tunicamycin (22).

RNA 2 directed the cell-free translation of a 35K polypeptide that comigrates with a 35K MHV-specific intracellular protein previously described (3, 27).

Radioimmune precipitation. Samples of the translation products of RNAs 2, 5, 6, and 7 were immunoprecipitated with mouse antiserum to MHV (3) or with a serum from a preimmune mouse, and the immunoprecipitates were analyzed by polyacrylamide gel electrophoresis. This mouse antiserum reacts strongly with the N and N-related proteins, somewhat less with the E1 and E2 proteins, weakly with the 35K nonvirion protein, and very weakly with the 14K nonvirion protein (3; unpublished data). The 20K putative El polypeptide, the 60K and 57K putative N polypeptides, and the 35K polypeptides from the cell-free reactions were all specifically immunoprecipitated by antiserum against MHV (Fig. 3). The large amount of N-related polypeptide relative to 35K polypeptide found in the immunoprecipitates of RNA 2 (Fig. 3, lane a) is probably due to the higher titer of the sera against N (compare total products of RNA 2 in Fig. 2 with immunoprecipitates in Fig. 3). The 14K cell-free product of RNA 5 was not immunoprecipitated by this antiserum, probably owing to the low reactivity of the serum with intracellular 14K polypeptide. The 120K polypeptide was specifically immunoprecipitated by antisera directed against viral E2 polypeptide (lanes g and h), further suggesting that the polypeptide is related to MHV E2 glycoprotein.

Tryptic map studies. The 60K and 57K cell-free translation products of RNA 7, the 57K

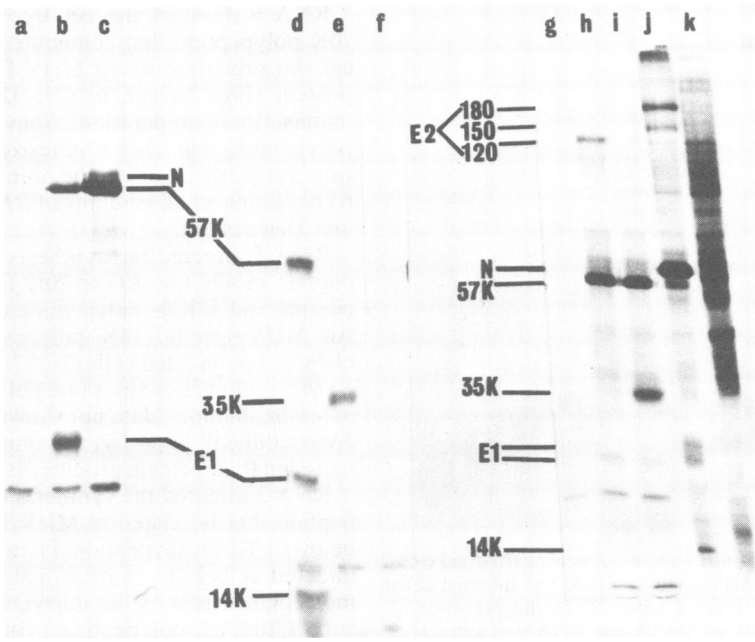

FIG. 2. In vitro translation of MHV-specific RNA. MHV-specific RNA was purified and translated in a rabbit reticulocyte lysate as described in the text. Five microliters of translation reactions were analyzed by polyacrylamide gel electrophoresis. The figure is a composite of three experiments. Samples a through c were analyzed on a 10% polyacrylamide gel, and samples d through f and g through k were analyzed on 7.5 to 15% gradient gels. Translation reactions containing no added RNA are shown in lanes a, f, and g. The translation products of RNA 6 are shown in lane b; those of RNA 7, in lane c; those of RNA 5, in lane d; those of RNA 2, in lanes e and i; and those of RNA 3, in lane h. Infected and mock-infected cell lysates were electrophoresed (lanes j and k, respectively). The positions of the MHV-specific intracellular proteins are indicated to the left of lane g.

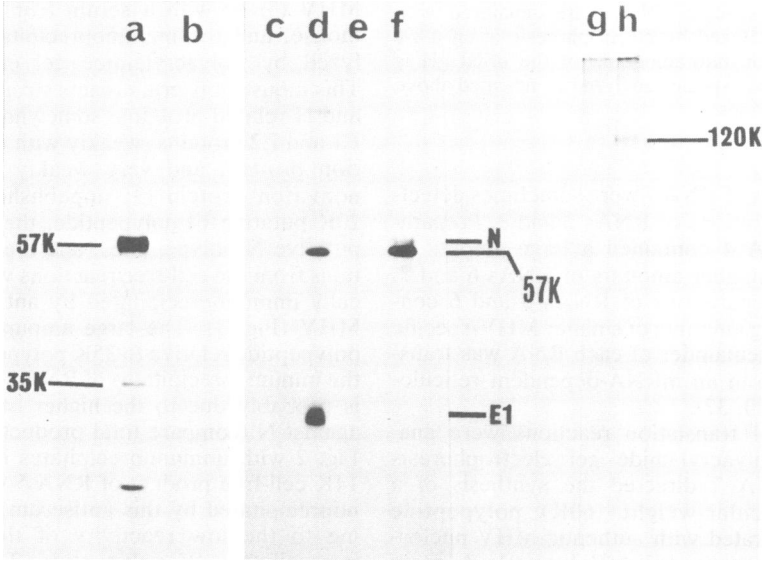

FIG. 3. Radioimmune precipitation of in vitro translation products. Samples of in vitro translation products were immunoprecipitated and analyzed by electrophoresis on 7.5 to 15% polyacrylamide gels. Lanes a and b, immunoprecipitates of the translation products of RNA 2 with immune and nonimmune mouse sera, respectively; lanes c and d, immunoprecipitates of the products of RNA 6 with nonimmune or immune mouse sera, respectively; lanes e and f, immunoprecipitates of the translation products of RNA 7 with nonimmune or immune mouse sera, respectively; lanes g and h, immunoprecipitates of the translation products of RNA 3 with normal rabbit serum or rabbit anti-E2 serum, respectively.

translation product of RNA 6, and in vivo labeled N protein were purified by polyacrylamide gel electrophoresis and digested with trypsin, and the tryptic peptides were separated by two-dimensional thin-layer electrophoresis and chromatography. These four proteins have identical [^{35}S]methionine tryptic peptide maps (Fig. 4).

A similar analysis was performed on the 20K protein synthesized in the cell-free system from RNA 6 and on the E1 protein isolated from purified virions. These two preparations have identical [^{35}S]methionine tryptic maps (Fig. 5).

Translation of virion RNA. Intact, full-length virion RNA was purified as a 57S RNA on sucrose gradients as described in Materials and Methods. Polyacrylamide gel analyses of the products of cell-free translation directed by four different preparations of virion RNA demonstrated three major translation products with molecular weights greater than 200K (Fig. 6). These polypeptides do not coelectrophorese with any known MHV-specific protein. Limited proteolysis peptide mapping (6) showed these three proteins to be structurally related to each other (Fig. 7).

DISCUSSION

Cell infected with MHV contain at least seven virus-specific RNA species (14, 29). The largest of these RNAs has been shown to be identical to virion RNA as judged by electrophoretic mobility and RNAse T1 fingerprint studies (14). The six subgenomic RNAs make up a nested set with common 3′ ends in which every RNA contains the sequence present in any smaller RNA and additional sequences consistent with a larger size (5, 10, 14, 38) in an arrangement similar to that described for another coronavirus, avian infectious bronchitis virus (30, 31).

In this paper, we have shown that at least five

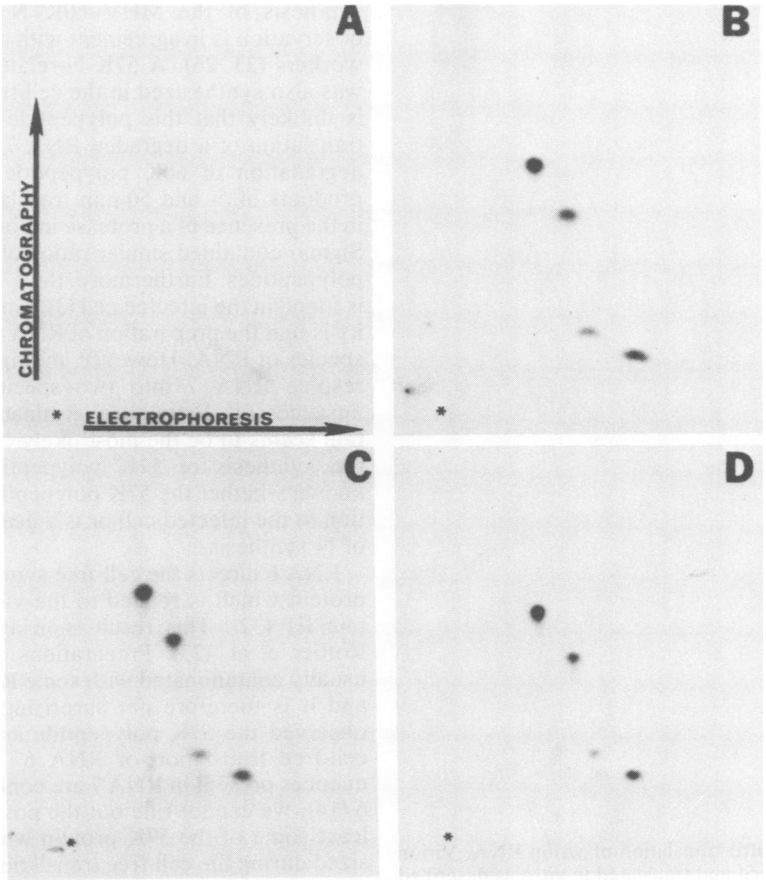

FIG. 4. Tryptic maps of in vitro synthesized N protein. The [^{35}S]methionine-labeled in vitro translation products of RNA 7 were purified by polyacrylamide gel electrophoresis, digested with trypsin, and analyzed as described in the text. (A) Tryptic map of the N protein; (B) map of the 57K protein; (C) tryptic map of the 57K translation product of RNA 6; (D) tryptic map of in vivo [^{35}S]methionine-labeled N protein purified from infected cells by gel electrophoresis.

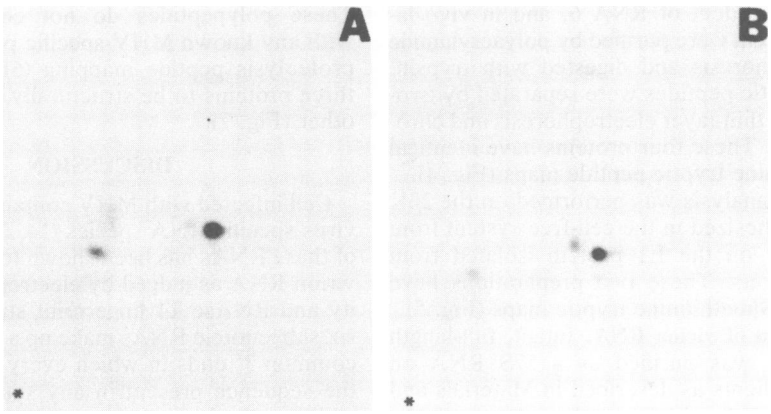

FIG. 5. Tryptic map of E1 protein synthesized in vitro. The translation product of RNA 6 which coelectrophoresed with E1 protein was eluted from a polyacrylamide gel and digested with trypsin, and a tryptic map was prepared (A). E1 protein from purified virus was similarly analyzed (B).

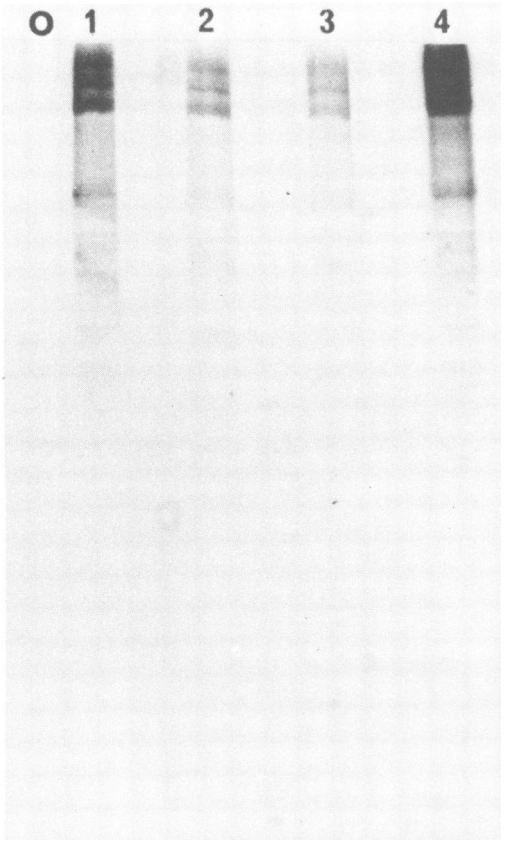

FIG. 6. In vitro translation of virion RNA. Virion RNA was purified and translated in vitro as described in the text. The translation products were analyzed by polyacrylamide gel electrophoresis. Analyses of a translation with no added RNA (lane O), with three different preparations of MHV-A59 RNA (lanes 1, 3, and 4), and with MHV-JHM RNA (lane 2) are shown.

MHV-specific subgenomic RNAs are mRNAs. RNA 7, the smallest RNA, directed the cell-free synthesis of the MHV 60K N protein. This observation is in agreement with results of other workers (23, 26). A 57K N-related polypeptide was also synthesized in the cell-free reaction. It is unlikely that this polypeptide is a result of translation of a degraded RNA 7 or an in vitro degradation of 60K polypeptide, because the products of 5- and 50-min translation reactions in the presence of a protease inhibitor (aprotinin; Sigma) contained similar ratios of 60K and 57K polypeptides. Furthermore, the 57K polypeptide is found in the infected cell (3). Another possibility is that the preparation of RNA 7 contains two species of RNA. However, multiple attempts to resolve RNA 7 into two species have been unsuccessful. Premature termination of translation seems to be the most likely explanation for the synthesis of 57K polypeptide. It is not known whether the 57K polypeptide has a function in the infected cell or is a dead end product of N synthesis.

RNA 6 directs the cell-free synthesis of a 20K protein which is related to the virion glycoprotein E1 (32). This result is in agreement with Rottier et al. (23). Preparations of RNA 6 are usually contaminated with some RNA 7 (Fig. 1), and it is therefore not surprising that we also observed the 57K polypeptide as a product of cell-free translation of RNA 6. Since the sequences present in RNA 7 are contained in RNA 6 (14), we cannot rule out the possibility that at least some of the 57K protein which is synthesized during the cell-free translation of RNA 6 is due to internal initiation of protein synthesis. This is unlikely, as internal initiation has not been observed in the translation of eucaryotic mRNAs.

RNA 3 directs the synthesis of a 120K poly-

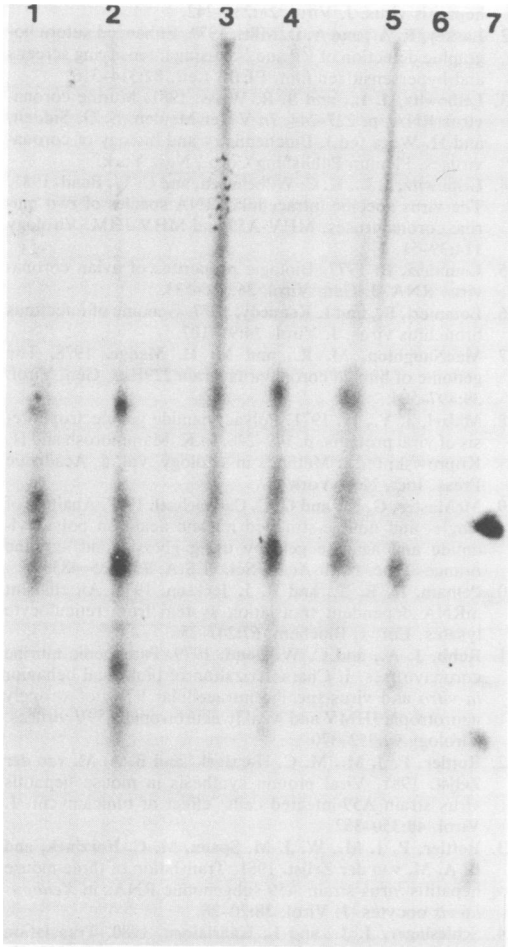

FIG. 7. Partial proteolysis maps of virion RNA in vitro translation products. The three high-molecular-weight bands shown in Fig. 6, lane 1, were cut out of a dried gel and electrophoresed into a 15% polyacrylamide gel with either 10 μg of V8 protease (lanes 1, 3, and 5) or 100 μg of V8 protease (lanes 2, 4, and 6) by the procedure of Cleveland et al. (6). A partial proteolysis map of virion N protein is shown for comparison (lane 7; digested with 100 μg of V8 protease).

peptide. This polypeptide is similar in molecular weight to the unglycosylated precursor to the E2 protein, which is synthesized in infected cells in the presence of tunicamycin (22). Furthermore, this 120K cell-free product is specifically immunoprecipitated with an antiserum specific for the E2 protein. We are therefore confident that RNA 3 encodes the E2 protein. A similar conclusion was reached by Rottier et al. (23), who demonstrated that RNA 3 directed translation of a glycosylated, 150K form of the E2 protein in *Xenopus laevis* oocytes.

In addition, we have demonstrated that the 35K nonstructural polypeptide observed in MHV-infected cells (3, 27) is encoded in RNA 2. RNAs 4 and 5 are present in low concentrations and are difficult to separate from each other and from larger amounts of RNAs 6 and 7. Thus, it is difficult to obtain translation products free of E1 and N, and to determine whether it is RNA 4 or 5 that encodes the 14K polypeptide. RNA 5 and, to a lesser extent, RNA 4 direct the synthesis of a 14K polypeptide which coelectrophoreses with an MHV-specific intracellular polypeptide previously reported (22, 27). The 14K translation product was not immunoprecipitable with anti-MHV serum and therefore can only tentatively be identified as a virus-specific product. Failure to react with antiserum is probably based on the low titer of this serum against the 14K protein. Recently, Siddell et al. (28) used gradient centrifugation to partially purify the intracellular RNA species synthesized by MHV-JHM-infected cells. Cell-free translation of these partially purified RNAs led these workers to propose similar coding assignments for MHV-JHM as those which we determined for MHV-A59-specific intracellular RNA.

The in vitro translation of virion RNA results in the synthesis of three structurally related polypeptides of greater than 200K. It is highly likely that these are virus-specific polypeptides, since cDNA probe made from such purified virion RNA does not hybridize with very large quantities of RNA from uninfected cells (38). This cell-free synthesis of large polypeptides directed by genuine RNA is similar to results obtained with alphaviruses (24). We have not, as yet, been successful in demonstrating these very large polypeptides in infected cells, owing possibly to rapid processing of this protein into smaller polypeptides, as has been described for the alphaviruses (24), or to their presence in very small amounts.

The replication strategy of MHV and other coronaviruses appears to be similar in many respects to that of other positive strand viruses, such as alphaviruses. Cell-free translation suggests that, upon infecting a susceptible cell, the virion RNA is uncoated, and large (greater than 200K) polypeptides are translated from the 5′ portion of the genome. These polypeptides, or proteolytic cleavage products of these polypeptides, might function as a virus-specific, RNA-dependent RNA polymerase, as has been shown for alphaviruses. Subsequently, the intracellular virus-specific RNAs are synthesized. The genomic size RNA is presumably incorporated into progeny virions. The subgenomic RNA species serve as mRNAs for both virion and nonstructural proteins.

The nested set structure of the MHV-specific subgenomic RNAs (10, 14) suggests that the RNAs are translated in the following manner.

RNA 7 is translated nearly in its entirety to give rise to the 60K N protein and the 57K N-related protein. The additional 5′ sequences present in RNA 6 and not in RNA 7 encode the E1 protein. Similarly, the additional 5′ sequences present in RNA 5, or possibly RNA 4, encode the 14K polypeptide; the additional 5′ sequences in RNA 3 are translated to the E2 protein, and the 35K polypeptide is translated from the additional 5′ sequences present in RNA 2. The remaining 5′ portion of the virion RNA approximates half of the genome and is translated into putative polymerase polypeptides. The sizes of these RNAs are fully compatible with this hypothesis. Thus, translation of MHV-specific RNAs would occur from the 5′ end, as is the case for most, if not all, eucaryotic mRNAs. Although the outlines of the replication strategy of coronaviruses are beginning to be defined, it is clear that a great deal more work remains to be done.

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