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Translation and Processing of Mouse Hepatitis Virus Virion RNA in a Cell-Free System

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The first event after infection with mouse hepatitis virus strain A59 (MHV-A59) is presumed to be the synthesis of an RNA-dependent RNA polymerase from the input genomic RNA. The synthesis and processing of this putative polymerase protein was studied in a cell-free translation system utilizing 60S RNA from MHV-A59 virions. The polypeptide products of this reaction included two major species of 220 and 28 kilodaltons. Kinetics experiments indicated that both p220 and p28 appeared after 60 min of incubation and that protein p28 was synthesized initially as the N-terminal portion of a larger precursor protein. When the cell-free translation products were labeled with *N*-formyl[^{35}S]methionyl-tRNA$_i$, p28 was the predominant radioactive product, confirming its N-terminal location within a precursor protein. Translation in the presence of the protease inhibitors leupeptin and $ZnCl_2$ resulted in the disappearance of p28 and p220 and the appearance of a new protein, p250. This product, which approached the maximal size predicted for a protein synthesized from genomic RNA, was not routinely detected in the absence of inhibitors even under conditions which optimized the translation reaction for elongation of proteins. Subsequent chelation of $ZnCl_2$ resulted in the partial cleavage of the precursor protein and the reappearance of p28. One-dimensional peptide mapping with *Staphylococcus aureus* V-8 protease confirmed the precursor-product relationship of p250 and p28. The results show that MHV virion RNA, like many other viral RNAs, is translated into a large polyprotein, which is cleaved soon after synthesis into smaller, presumably functional proteins. This is in marked contrast to the synthesis of other MHV proteins, in which minimal proteolytic processing occurs.

The *Coronaviridae* are a group of RNA viruses with a unique replication strategy. The genome of mouse hepatitis virus (MHV), the most intensively studied coronavirus, is a single-stranded, infectious RNA molecule with a molecular size of approximately 6×10^6 daltons (14, 23, 26, 30). MHV-infected cells contain multiple polyadenylated subgenomic RNAs associated with polyribosomes (11, 19, 26). These mRNAs form a 3′ coterminal, nested set of sequences, and only the 5′ portion of each RNA, which is absent from the next smallest RNA, is translated into protein (3, 10, 13, 14, 20, 23, 27, 30).

Several of the subgenomic RNAs have been isolated and translated either in *Xenopus* oocytes or in an in vitro protein-synthesizing system from rabbit reticulocytes. RNA 2 codes for a 35,000-dalton nonstructural protein, RNA 3 codes for the surface glycoprotein E2, RNA 4 codes for a nonstructural protein of 14,000 molecular weight, RNA 5 codes for a nonstructural protein of 10,000 molecular weight, RNA 6 codes for the E1 transmembrane protein, and RNA 7 codes for the nucleocapsid protein. RNA 1, which is believed to code for the virus-induced RNA-dependent RNA polymerase, has been translated in vitro into a series of related polypeptides of approximately 200,000 molecular weight (13, 20, 22, 24, 25).

By analogy with other positive-stranded RNA viruses, such as Sindbis virus (5), these large proteins might be the precursors to the proteins actually present in the viral polymerase. However, it is difficult to study these proteins in infected cells because MHV does not inhibit host-cell protein synthesis efficiently, even at late times after infection. To circumvent this difficulty, we used a rabbit reticulocyte cell-free protein-synthesizing system to study the translation and processing of the protein products of the genomic RNA of MHV. Our results showed that virion RNA was translated in vitro into several large polypeptides, in agreement with a previous report (13), and that these large proteins were cleaved to a 28-kilodalton (kDa) protein.

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MATERIALS AND METHODS

Preparation of MHV-A59 virion RNA. MHV strain A59 (MHV-A59), used in all studies, was plaque-purified twice and propagated in either L-2 or BALB/c 3T3 clone 17 (17CL-1) cells in Dulbecco minimal essential medium supplemented with 2% bovine calf serum. The virus titer was 6×10^8 as determined by plaque assay on 17CL-1 cells. For preparation of virion RNA, 4×10^8 cells were infected at a multiplicity of infection of 0.04 and grown in Dulbecco minimal essential medium with 2% bovine calf serum and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES); pH 6.5) for 16 to 24 h, at which time approximately 50% of the cells were involved in syncytium formation.

The cellular supernatants were harvested, combined, and centrifuged at $1,000 \times g$ for 5 min to remove cellular debris. The virus was concentrated by centrifugation for 90 min at $80,000 \times g$ in a Beckman SW27 rotor and suspended in 1 ml NTE buffer (0.1 M NaCl, 0.01 M Tris [pH 7.4], 0.001 M EDTA) by sonication. The virus was further purified by centrifugation through 5 to 40% sucrose in NTE buffer at $105,000 \times g$ for 45 min in a Beckman SW40 rotor. The visible virus band was harvested, diluted 1 : 1 with NTE buffer, made 1% in sodium dodecyl sulfate (SDS), and layered on a 15 to 30% sucrose gradient in NTE buffer containing 0.5% SDS. Virion RNA was separated by centrifugation at 45,000 $\times g$ for 16 h in a Beckman SW27 rotor, and the gradient was fractionated through a Gilford spectrophotometer. A single peak of RNA sedimenting at about 60S was collected, made 0.5 M in potassium acetate, and precipitated with 2 volumes

of 95% EtOH at −20°C. In some instances, the homogeneity of the preparation was confirmed by formaldehyde agarose gel electrophoresis.

In vitro translation system. Rabbit reticulocyte lysate (New England Nuclear Corp.) was used for all translation reactions. The translation reaction was usually 25 to 50 μl, and the concentrations of potassium acetate (180 mM) and magnesium acetate (1.5 mM) were optimized for the translation of MHV-A59 mRNA. Purified MHV-A59 virion RNA or polyadenylated intracellular RNA from MHV-A59-infected cells was added at a concentration of 8 μg/ml. [^{35}S]methionine (2,400 μCi/ml) or [^{3}H]leucine (1,000 μCi/ml) (New England Nuclear Corp.) was added as radiolabel. Incubations were for 90 min at 30°C unless otherwise indicated.

For experiments involving the inhibition of translation of capped RNA, the cap analog methyl-7 GTP (P-L Biochemicals, Inc.) was added before virion RNA at concentrations from 0 to 2 mM, keeping the total reaction volume constant. In pulse-chase experiments, unlabeled L-methionine was added to a final concentration of 10 mM.

Reactions were ended by the addition of a large excess of sample buffer (4% SDS, 40% glycerol, 0.125 M Tris [pH 7.4], 2% 2-mercaptoethanol, 0.01% bromphenol blue) (9) before boiling for 4 min at 100°C.

One-dimensional gel electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) of translation products was performed as described by Maizel (15) and modified by Hames (8), with 5 to 18% gradient polyacrylamide gels unless otherwise noted. The gels were run for 14 h at 50 V. The gels were fixed, stained, and processed for fluorography with either sodium salicylate (2) or 22% 2,5-diphenyloxazole in dimethyl sulfoxide (12). The treated gels were exposed to Kodak XAR X-ray film at −70°C.

For the determination of the molecular sizes of the cell-free products, samples were analyzed on 7 to 20% gradient polyacrylamide gels, on which log mobility is proportional to log molecular size in the range of 14 to 300 kDa (8).

Preparation and use of *N*-formyl[^{35}S]methionyl-tRNA. Preparation of *N*-formyl[^{35}S]methionyl-tRNA$_i$ was performed by the method of Stanley (28) by using an *Escherichia coli* S-100 fraction kindly provided by Thomas Conway of the Department of Biochemistry, University of Iowa. This fraction contains active tRNA synthetases and a tRNA transformylase, but under the conditions of the reaction, only methionyl-tRNA$_i$ is charged by the *E. coli* synthetase (28).

The final reaction mixture included the *E. coli* S-100 fraction, [^{35}S]methionine (2.0 mCi/ml), the formyl donor Ca leucovorin (0.02 mM; Lederle Laboratories), unfractionated calf liver tRNA (0.9 mg/ml; Boehringer Mannheim Biochemicals), 10 mM ATP, 15 mM $MgCl_2$, and 50 mM cacodylic acid (pH 7.4). After 30 min of incubation at 37°C, the tRNA was extracted with phenol and ether, precipitated with ethanol, and stored at −70°C. For use in the reticulocyte lysate translation system, *N*-formyl[^{35}S]methionyl-tRNA$_i$ was centrifuged, lyophilized, suspended in water, and added at a concentration of 80 μg/ml, along with a large excess of unlabeled L-methionine (10 mM) which eliminated the incorporation of any residual free [^{35}S]methionine (5). Incubations were for 90 min at 30°C, and the reaction products were analyzed as previously described.

One-dimensional peptide mapping. Partial digest mapping was performed by the method of Cleveland et al. (4). Individual [^{35}S]methionine-labeled protein bands of interest were excised from 5 to 18% gradient SDS-polyacrylamide gels which had been briefly stained and destained. They were equilibrated for 30 min in buffer containing 0.125 M Tris (pH 6.8), 0.1% SDS, and 1 mM EDTA and then placed on top of the stacking portion of a 15% polyacrylamide-SDS gel. The gel pieces were overlaid with more of the same solution containing 10% glycerol and 50 μg of *Staphylococcus aureus* V-8 protease per ml (Miles Laboratories, Inc.). Electrophoresis was performed as usual, except that the power was turned off for 30 min when the dye front neared the bottom of the stacking gel.

RESULTS

(i) Optimization of rabbit reticulocyte lysate for synthesis of high-molecular-weight proteins. Rabbit reticulocyte lysate was optimized for the translation of virion RNA into high-molecular-weight products as described in Materials and Methods. Incorporation of [^{35}S]methionine was directly proportional to the concentration of exogenous RNA added in the range from 2 to 16 μg/ml, and no difference in the size of the products was observed by SDS-PAGE analysis. A RNA concentration of 8 μg/ml was used in the experiments described below.

The efficiency of translation was calculated, assuming an average protein methionine content of 1.5% and assuming that the endogenous methionine concentration in the rabbit reticulocyte lysate was 10 μM (21). If all the radioactivity were incorporated into proteins of 220 kDa, three protein molecules would be synthesized from each RNA molecule per hour. Because many of the translation products were smaller than 220 kDa, the actual rate of initiation was probably in the range of five to seven times per hour. This rate of initiation is similar to that reported for other large viral proteins (5, 21).

(ii) Kinetics of appearance of >200- and 28-kDa proteins. The time course of appearance of radioactive protein products was determined by removing samples at various times for assay by SDS-PAGE (Fig. 1). Consistent with the theoretical rate of translation in the in vitro system (16), proteins with molecular sizes greater than 100 kDa appeared after approximately 30 min of synthesis, whereas larger polypeptides (>200 kDa) did not appear until 60 to 75 min. The most prominent large polypeptide had a molecular size of 220 kDa (p220), although several other bands were also observed. These discrete bands, with molecular sizes ranging from 69 to 200 kDa, were observed in all experiments and probably represented products of premature termination (see Fig. 8). In addition, a protein larger than 220 kDa was often detected, but because it was not present as reproducibly as was p220, it was not further analyzed.

Another major protein, with a molecular size of 28 kDa (p28), was first detected after 60 to 75 min of synthesis. The simultaneous late appearance of p220 and p28 suggested that p28 might result from the posttranslational processing of a larger protein, possibly while the nascent polypeptide chain was still attached to the polyribosome. The observations were not dependent on the radioactive amino acid, because identical results were seen when [^{35}S]methionine rather than [^{3}H]leucine was used as the source of radioactivity (Fig. 2).

(iii) Effect of cap analog on in vitro synthesis of viral proteins. MHV-A59 virion RNA requires a capped 5′ end for proper translation (11). To exclude the possibility that p28 was a late-appearing translation product of uncapped, degraded RNA, we translated virion RNA in the presence of increasing concentrations of methyl-7 GTP, a competitive inhibitor of cap-dependent initiation (1) (Fig. 2). Cap analog

added at a concentration of 1 mM before the addition of RNA inhibited the synthesis of p28 and larger proteins proportionally, with an overall 80% reduction of radiolabel incorporation, suggesting that all the proteins were products of translation of capped mRNA. The addition of 2 mM cap analog resulted in a 97% reduction in [^{35}S]methionine incorporation.

(iv) Processing of proteins after chase with unlabeled methionine. If larger proteins are precursors to p28, radioactivity should continue to accumulate in the smaller protein in the presence of a chase with an excess of unlabeled amino acid. We labeled proteins for 60 min with [^{35}S]methionine and then added a large excess of unlabeled methionine (final concentration, 10 mM) before an additional 90 min of incubation (Fig. 3). Both p220 and p28 were labeled by the end of the pulse period, and total acid-insoluble radioactivity did not increase over the subsequent 90 min of chase. In contrast, the radioactivity associated with p28 increased approximately 53% over this period, as determined by densitometry. This result suggested that p28 resulted from the cleavage of a larger precursor protein.

We then used a variable pulse-constant chase analysis to determine the relative position of p28 within the larger precursor proteins (Fig. 4). If p28 is located at the N terminus of the precursor protein, it should be synthesized within the first few minutes of incubation, even if it does not appear as a discrete protein until later. Proteins were labeled in the cell-free translation system for periods ranging from 5 to 30 min at 5-min intervals before chase with unlabeled methionine. Radioactivity associated with p28 was apparent after a 5-min label and 60-min chase, showing that the RNA encoding this protein was translated at very early times of incubation. Because p28 was not labeled in pulse experiments before 60 min of incubation, these results were consistent with an N-terminal location for p28 within a larger precursor protein.

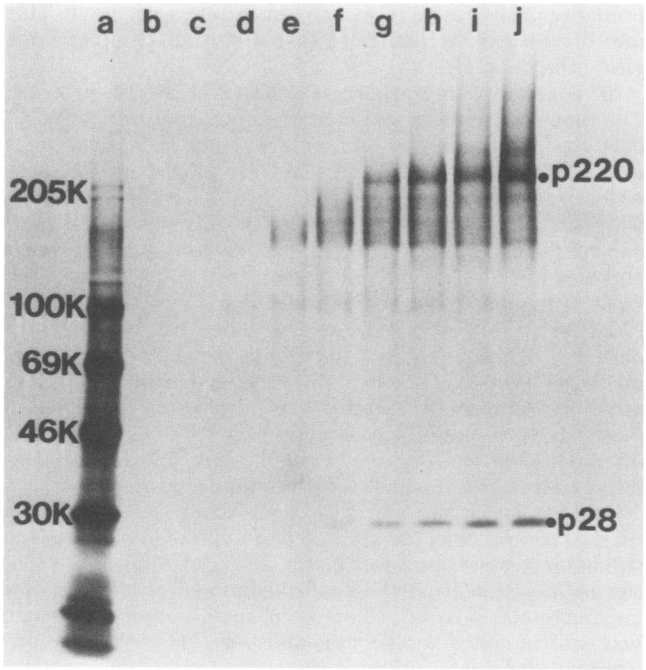

FIG. 1. Time course of appearance of [^{3}H]leucine-labeled polypeptides of MHV-A59 translation in vitro. MHV-A59 60S RNA (8 μg/ml) was added to the rabbit reticulocyte lysate cell-free translation system containing [^{3}H]leucine (1,000 μCi/ml). Aliquots were withdrawn at the times indicated and immediately processed for electrophoresis on a 5 to 18% gradient polyacrylamide gel. Lanes: c, 15 min; d, 30 min; e, 45 min; f, 60 min; g, 75 min; h, 90 min; i, 120 min; and j, 150 min. Lane a contains ^{14}C-labeled markers, and lane b shows the product of 150 min of incubation without added RNA.

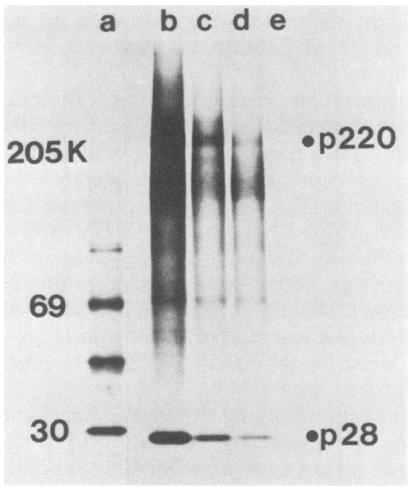

FIG. 2. Effect of cap analog on in vitro synthesis of [^{35}S]methionine-labeled polypeptides. A competitive inhibitor of capped initiation, methyl-7 GTP, was added to four parallel translation reactions at the concentrations indicated. Lanes: b, 0 mM; c, 0.4 mM; d, 1.0 mM; and e, 2.0 mM. [^{35}S]methionine was added as a radiolabel in these 90-min translations. Lane a contains ^{14}C-labeled markers. Equal amounts of each reaction were electrophoresed on a 5 to 18% gradient gel and analyzed by fluorography.

(v) N-terminal labeling of in vitro proteins. *N*-Formyl[^{35}S]methionyl-tRNA$_i$ is incorporated only into the N terminus of primary translation products (28). Thus, its presence in a protein indicates that the protein is either a primary translation product or its N-terminal fragment. To confirm the N-terminal location of p28 within the precursor protein, the cell-free translation products of MHV-A59 virion RNA were labeled with *N*-formyl[^{35}S]methionyl-tRNA$_i$ (Fig. 5). A large proportion of the radioactivity was present in p28 (lanes d and e), in marked contrast to the results obtained when [^{35}S]methionine was the radioactive precursor (lane b). The observation that little radioactivity was associated with proteins of >200 kDa indicated that p28 was cleaved from most of the precursor proteins of this size.

(vi) Effect of proteolysis inhibition. Because p28 appeared to result from the proteolytic cleavage of larger precursors, we examined the products synthesized in the presence of known protease inhibitors (Fig. 6). We determined that processing was most effectively inhibited by using either $ZnCl_2$ (1.0 mM) or leupeptin (1.0 mM), both of which are general inhibitors of thiol and serine proteases (32). Both inhibitors had two effects on the protein patterns seen by SDS-PAGE analysis. First, the accumulation of p28 was selectively inhibited without a major effect on the accumulation of the higher-molecular-weight proteins. Second, the major band, p220, was reduced in amount, and a new band of 250 kDa (p250) appeared. Many of the other proteins also appeared to increase in molecular size by 20 to 30 kDa.

The inhibition of proteolysis by $ZnCl_2$ should be reversible by a chelating agent such as EDTA. In the next experiment, proteins were labeled for 60 min in the presence of $ZnCl_2$ so

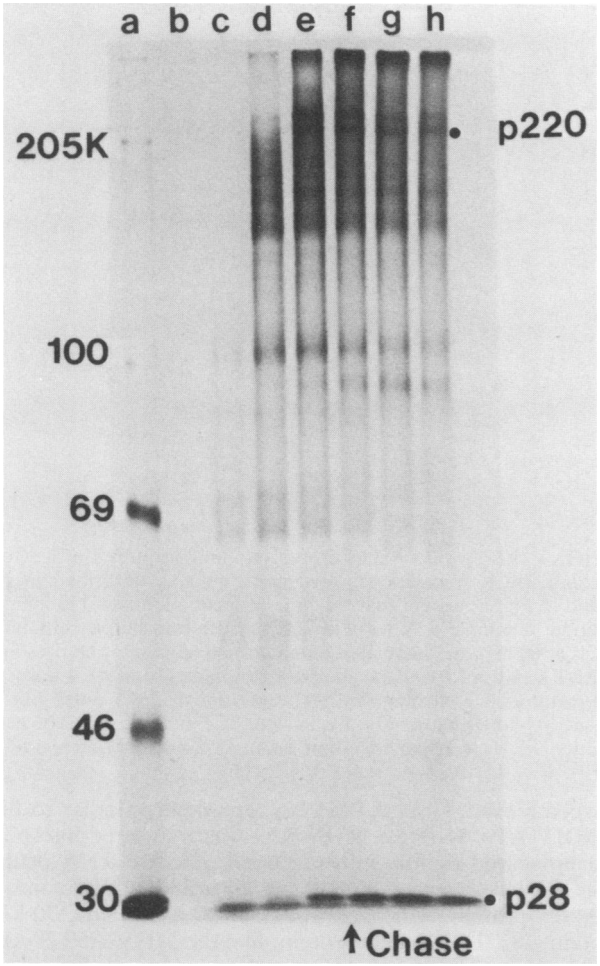

FIG. 3. SDS-PAGE analysis of polypeptides synthesized in constant pulse-variable chase experiment. [^{35}S]methionine and MHV-A59 virion RNA were added to the cell-free system and incubated for 60 min with samples withdrawn at the times indicated. Lanes: b, 0 min; c, 15 min; d, 30 min; and e, 60 min. Excess unlabeled methionine (10 mM) was then added for an additional 90 min, with samples withdrawn at 90 min (lane f) 120 min (lane g), and 150 min (lane h). Lane a contains ^{14}C-labeled marker proteins. All samples were electrophoresed on a 7.5% polyacrylamide gel.

that no p28 was detectable (Fig. 7). A portion of the incubation mixture was then incubated with 1 mM EDTA and unlabeled methionine for an additional 60 min. The addition of EDTA to the $ZnCl_2$-treated sample resulted in the appearance of p28 (Fig. 7), suggesting that reactivation of the protease caused the resumption of processing. Only a small fraction of the usual amount of p28 was observed after the EDTA treatment, suggesting either that the reversal of inhibition was incomplete or that the precursor proteins were less efficiently processed after release from polyribosomes.

In other experiments, *N*-formyl[^{35}S]methionyl-tRNA$_i$ was used to label selectively the N-terminal methionine of the proteins synthesized in the presence of leupeptin. In contrast to the results shown in Fig. 5, most of the radioactivity was incorporated into p250 and other high-molecular-weight proteins, with little incorporated into p28 (data not shown).

(vii) *S. aureus* V-8 protease mapping of in vitro products. To establish more clearly the relationship between the multiple large protein products and p28, we performed one-dimensional peptide mapping with *S. aureus* V-8 protease (Fig. 8). The products of the in vitro translation were fractionated by SDS-PAGE, and the individual proteins of interest were excised before treatment with V-8 protease in situ (4). We anticipated that the peptides present in p28 would not be found in the p220 protein but would be present in p250 synthesized in the presence of leupeptin or $ZnCl_2$. However, most of the peptides present in p28 were also found in both p220 and p250 (Fig. 8), although at least one of the peptides appeared unique to p28 (indicated by arrow). In addition, p220 and p250 were very similar by partial peptide mapping, with no unique peptides observed in p250. The experiment also showed that the multiple proteins observed between p28 and p220 were related, suggesting that they were premature termination products.

These results demonstrated that all of the high-molecular-weight proteins are related and confirmed the precursor-product relationship of these proteins and p28. The same results were obtained when the cell-free translation products were analyzed by tryptic peptide mapping (data not shown).

DISCUSSION

Using cell-free translation of MHV-A59 virion RNA, we identified two previously undescribed products, p28 and p250. p250 appeared to be the primary translation product of virion RNA and was reproducibly detectable only when proteolysis was inhibited. The data indicated that p28 is cleaved from the N-terminal portions of p250, as well as from premature termination products. Cleavage occurred either from nascent polypeptides during translation or from

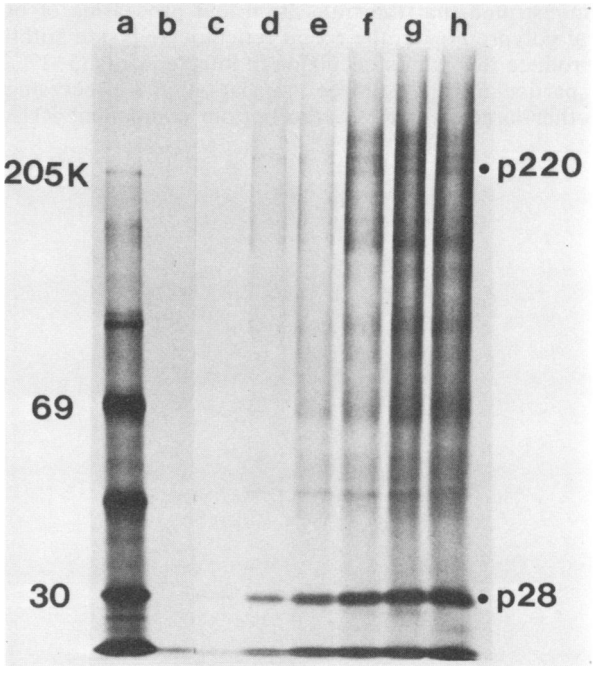

FIG. 4. SDS-PAGE analysis of proteins synthesized in variable pulse-constant chase experiment. In this pulse-chase experiment, samples were withdrawn from the translation reaction at the indicated times and incubated an additional 60 min at 30°C in the presence of excess unlabeled methionine (10 mM). Lanes: c, 5 min; d, 10 min; e, 15 min; f, 20 min; g, 25 min; and h, 30 min. Lane a contains ^{14}C-labeled markers, and lane b contains the products of incubation without virion RNA. All products were analyzed on a 5 to 12.5% gradient gel.

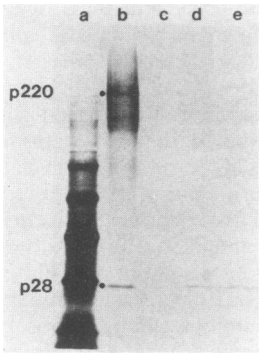

FIG. 5. Analysis of N-terminal labeling of cell-free translation products. Either N-formyl[^{35}S]methionyl-tRNA$_i$ or [^{35}S]methionine was added to translation reactions and incubated for 90 min. Samples were then run on a single 5 to 18% gradient polyacrylamide gel. Lanes: b, [^{35}S]methionine-labeled proteins; c, *N*-formyl [^{35}S]methionyl-tRNA$_i$ without added RNA; d and e, incorporation from charged or charged and formylated [^{35}S]methionyl-tRNA$_i$, respectively. Lane a contains ^{14}C-labeled markers. Products in lane b were diluted 1:10 in SDS sample buffer, and 4% of the translation mixture was applied. Products in lanes c, d, and e were diluted 1:1, and the entire translation reaction was applied to the gel. The two left lanes were exposed to Kodak XAR film at −70°C for 6 days, and the three right lanes were exposed for 17 days.

newly completed proteins soon after release from polyribosomes, because p250 was not detected in the absence of protease inhibitors and p220 and p28 appeared nearly simultaneously in the cell-free lysate.

Experiments with alphaviruses and picornaviruses have demonstrated that the translation and processing of other viral polyproteins in the rabbit reticulocyte lysate faithfully reproduce the events occurring in infected cells (5, 17, 21). In particular, the cell-free translation and processing of another large viral RNA, the bottom component RNA of

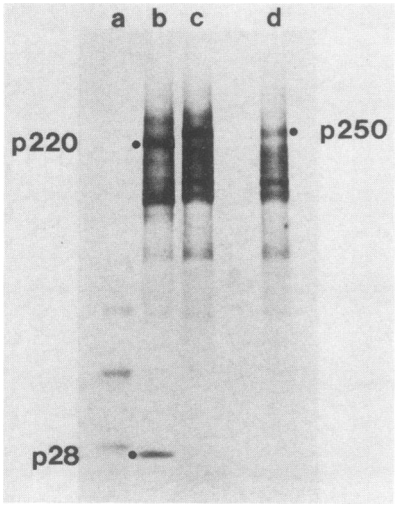

FIG. 6. Effect of protease inhibitors on cell-free translation of MHV-A59 virion RNA. Either leupeptin or zinc chloride was added to the lysate before the addition of MHV-A59 mRNA, and translation reactions were incubated for 90 min in the presence of [^{35}S]methionine: Lanes: b, control translation without protease inhibitors; c, addition of leupeptin (1.0 mM); and d, addition of $ZnCl_2$ (1.0 mM). ^{14}C-labeled markers are in lane a. Samples were analyzed on a 5 to 18% gradient polyacrylamide gel.

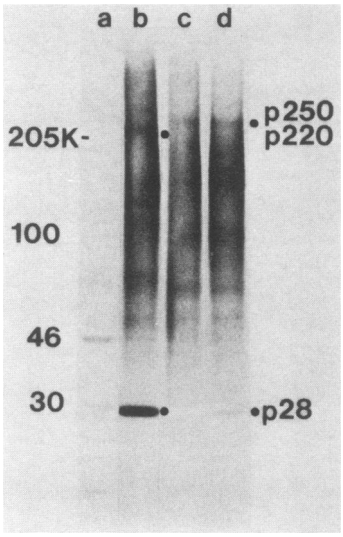

FIG. 7. EDTA reversal of proteolysis inhibition by $ZnCl_2$. Control and $ZnCl_2$ translations were performed as described in the legend to Fig. 6, except that the time of incubation was 120 min. In addition, a sample was withdrawn from the translation containing $ZnCl_2$ at 60 min and incubated an additional 60 min in the presence of EDTA (1.0 mM) and unlabeled methionine (10 mM). Lanes: b, [^{35}S]methionine control; c, $ZnCl_2$ (1 mM); and d, $ZnCl_2$ pulse-EDTA chase. ^{14}C-labeled markers are in lane a. Equal amounts of each reaction were electrophoresed on a 5 to 18% polyacrylamide gel.

cowpea mosaic virus (CPMV) is remarkably similar to that of MHV-A59. Whereas MHV-A59 was translated into p250 and processed rapidly into p220 and p28, the RNA of this plant virus is translated both in vivo and in vitro into a 200-kDa protein, which is processed to a 32- and 170-kDa protein (18). The 32-kDa protein, like the MHV-A59 28-kDa protein, is released from the N terminus of nascent polypeptides chains (6), because it appears in the lysate at approximately the same time as does the 200-kDa protein.

The 200-kDa CPMV protein is cleaved to the 170- and 32-kDa proteins in vitro by an endogenous protease of the rabbit reticulocyte lysate; a cellular enzyme is presumed responsible for the cleavage in infected cells (31). The protease responsible for the cleavage of the MHV-A59 precursor proteins was sensitive to leupeptin and to $ZnCl_2$, but whether it is viral or host in origin is not known.

The 170-kDa CPMV protein is further cleaved in vivo at one of two locations to produce either a 110- to 60-kDa pair or a 87- to 84-kDa pair of proteins. This secondary processing occurs in vitro, but only after prolonged incubation (18 h) of the rabbit reticulocyte lysate (6). In our system, no further processing of the cell-free products of MHV-A59 virion RNA was observed after 150 min of incubation.

The 32-kDa CPMV protein has protease activity and cleaves at a glutamine-methionine (7, 33). This protein probably does not catalyze its own cleavage from a precursor protein but is important in the processing of the viral capsid proteins. Each of the MHV-A59 proteins is translated from a separate mRNA (13, 20, 22), and further processing, except for the envelope glycoprotein E2 (23, 30) and perhaps the viral polymerase, does not require a virus-induced protease. Thus, although the processing of the MHV-A59 translation product is similar to that of the CPMV p200, there is less need for a protease in the replication strategy as thus far elucidated.

We were unable to detect p28 in MHV-A59-infected cell

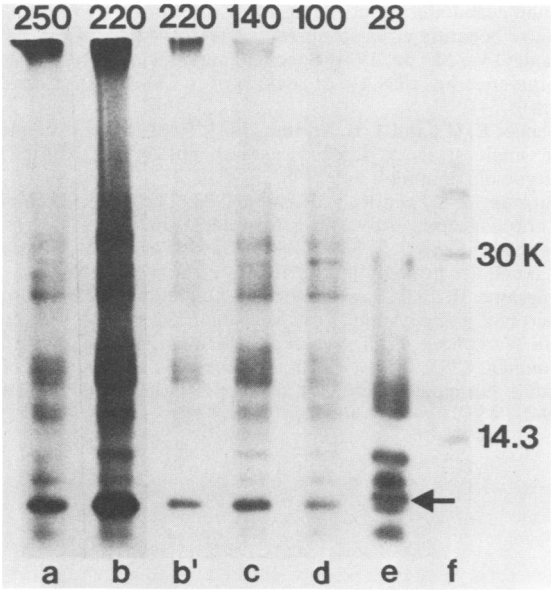

FIG. 8. One-dimensional peptide mapping of in vitro products. Virion RNA was translated as described in the legend to Fig. 6 for 90 min in the presence and absence of leupeptin. Samples were electrophoresed on a 5 to 18% polyacrylamide gel, and the bands of interest were excised and treated with *S. aureus* V-8 protease as described in Materials and Methods. Lanes: a, leupeptin p250 band; b, control p220 band; b', control p220 band; c, control p140 band; d, control p100 band; e, control p28 band; and f, ^{14}C-labeled markers. Lanes a and b and b' through f were exposed for 11 and 5 days, respectively. A peptide unique to p28 is indicated by the arrow.

lysates at early or late times after infection by either one- or two-dimensional gel electrophoresis, most likely because it is produced in quantities below the limits of detection of the assay procedure. This is not surprising, because in cells infected with other viruses, viral polymerase is usually present as a minor protein (29), thereby making identification of the specific protein difficult. MHV-A59 does not shut off host cell protein synthesis efficiently, even at late times after infection, making detection of p28 more difficult.

The peptide mapping experiments confirmed the precursor-product relationship between p250 and p28. However, they also indicated that p220 contained all the peptides present in p28. This was unanticipated, because the kinetics analyses suggested that p220 and p28 were both cleavage products of p250. The most likely explanation for this result is incomplete cleavage of p28 from the larger precursor proteins. The experiments in which the cell-free products were labeled with *N*-formyl[^{35}S]methionyl-tRNA$_i$ were consistent with this explanation, because small amounts of radioactivity were associated with the larger proteins, suggesting incomplete processing.

A putative viral polymerase must be able to synthesize complementary-strand RNA, leader RNA, and genomic and subgenomic positive-strand RNA. Although the enzymatic activities of p28 and p220 have not been determined, these proteins or other cleavage products are candidates for such functions.

ACKNOWLEDGMENTS

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