MURINE CORONAVIRUS GENE 1 POLYPROTEIN CONTAINS AN AUTOPROTEOLYTIC ACTIVITY

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SUMMARY

The 5' most gene of the murine coronavirus genome, gene 1, is presumed to encode the viral RNA-dependent RNA polymerase. cDNA clones representing this gene encompass more than 22 kilobases, suggesting that this region may encode multifunctional polyprotein(s). It has previously been shown that the N-terminal portion of this gene product is cleaved into a protein of 28 kilodaltons (p28). To identify possible functional domains of gene 1 and further understand the mechanism of synthesis of the p28 protein, cDNA clones representing the 5'-most 5.3 kilobases of the murine coronavirus mouse hepatitis virus strain JHM were subcloned into pT7 vectors from which RNAs were transcribed and translated in vitro. Although p28 is encoded from the first 1 kilobase at the 5'-end of the genome, translation of in vitro transcribed RNAs indicated that this protein was not detected unless the product of the entire 5.3 kilobase region was synthesized. This result suggests that the region close to 5.3 kilobases from the 5'-end of the genomic RNA is essential for the proteolytic cleavage and may contain an autoproteolytic activity. Addition of the protease inhibitor $ZnCl_2$ blocked cleavage of the p28 protein. Site-directed mutagenesis of Cys residue 1137 significantly reduced the cleavage of the p28 protein, indicating that this residue, probably in conjuction with a downstream domain, plays an essential role in the cleavage of p28. This Cys residue may be part of a papain-like autoprotease encoded by gene 1.

INTRODUCTION

Mouse hepatitis virus (MHV) contains an RNA genome of more than 6 x 106 molecular weight (15, 27). Upon infection of a susceptible cell, the viral genomic RNA is first translated, producing the viral RNA-dependent RNA polymerase. This polymerase transcribes the virion genomic RNA into a negative-stranded RNA, which is, in turn, transcribed into a positive-sensed genomic RNA and six subgenomic mRNAs (2, 3, 12, 14). These mRNAs form a nested-set structure and contain an identical 5'-end leader sequence of approximately 72 nucleotides, which is derived by a unique leader-primed transcription mechanism (13, 19, 21, 24).

In vitro translation studies of MHV mRNAs revealed three viral structural proteins which are the nucleocapsid protein, N, and two glycoproteins, M and S. Several small, nonstructural proteins are also encoded by subgenomic mRNAs (17, 22). The largest mRNA, which is of genomic length, is thought to encode the viral RNA-dependent RNA polymerase. Sequencing of the entire genome of another coronavirus, avian infectious bronchitis virus (IBV), suggests that this gene has a capacity to encode two very large proteins of larger than 300 kilodaltons (kDa) (1, 6). In vitro translation studies of murine coronavirus genomic RNA indicated that a large polyprotein of 250 kDa is synthesized and cleaved to 28 kDa and 220 kDa proteins (7, 23). The p28 protein has been detected in MHV-infected cells (8). Previous sequencing and translation studies had established that the p28 protein represents the N-terminal portion of the presumed RNA polymerase precursor (7, 23). To further elucidate the structure and mechanism of synthesis of the potential RNA-dependent RNA polymerase of MHV, cDNA clones representing the 5'-end 5.3 kilobase (kb) region of genomic RNA were translated in vitro. Translation studies of in vitro synthesized RNA derived from these cDNA clones showed that this protein may contain an autoproteolytic activity which is responsible for the cleavage of the p28 protein. The protein domain essential for this proteolytic activity is located in the region from 3.9 to 5.3

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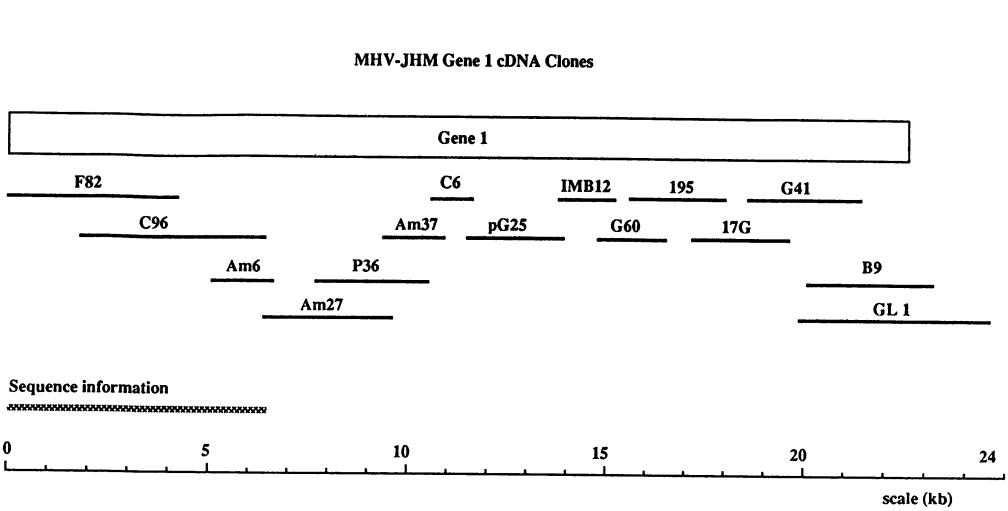


Fig. 1. Schematic diagram of cDNA clones representing MHV-JHM gene 1. Lengths are expressed in kilobase pairs (kb).

kb from the 5'-end of the genomic RNA. Site-directed mutagenesis of this cDNA suggests that Cys 1137 interacts with this domain and is essential for the cleavage of p28.

MATERIALS AND METHODS

cDNA Clones. cDNA clones representing the entire genome of MHV-JHM were generated by priming for first-strand cDNA synthesis with specific oligodeoxyribonucleotides as previously described (21, 23). Fig. 1 shows the positions of the cDNA clones relative to MHV-JHM genomic RNA.

In vitro transcription and translation. Plasmids pT7-NH, pT7-N2H, and pT7-NBgl consist of MHV-JHM gene 1 sequence from the Nar I site (nucleotide 187) to the first Hind III site (nucleotide 1989), the second Hind III site (nucleotide 3880), and the Bgl II site (nucleotide 5273), respectively, inserted downstream from the T7 polymerase promoter (26). DNA was linearized in the polylinker region following the MHV sequence by digestion with restriction enzyme Eco RI and transcribed in vitro with T7 RNA polymerase as previously described (23). The resulting RNA was translated in an mRNA-dependent rabbit reticulocyte lysate (Promega Biotech) under conditions optimized for MHV RNA translation (7).

Immunoprecipitation of in vitro translated proteins. Anti-p28 serum was generated in rabbits directed against a synthetic peptide representing amino acids 78-93 (NH_3-R-D-I-F-V-D-E-D-P-Q-K-V-E-A-S-T-COOH) of the p28 protein (23). Immunoprecipitation was performed by the method of Kessler (11) and proteins were analyzed by electrophoresis on 5 to 12.5% SDS-polyacrylamide gels (18).

Mutagenesis of plasmid pT7-NBgl. Site-directed mutagenesis was accomplished following the procedure of Higuchi et al using oligonucleotides containing specific mismatches and the polymerase chain reaction to amplify mutated DNA (10). Briefly, oligo #98 (5'-GTGAGGCATGCGACAGGGAA-3'), encompassing the unique Sph I site at nucleotide 3339, and oligo #99 (5'-CGAAGCCAAGAATTAGTACG-3') containing a mismatch at nucleotide 3624, were used to prime DNA synthesis from plasmid pT7-NBgl in the polymerase chain reaction (PCR). Likewise, oligo #101 (5'-CGCTCTTAACTAGTTTGTCC-3'), encompassing a unique Spe I site at nucleotide 3743 and oligo #100 (5'-CGTACTAATTCTTGGCTGCG-3') also containing a mismatch at nucleotide 3624 were used to prime DNA synthesis from pT7-NBgl. The product DNAs from these two reactions were then mixed, denatured and reassembled, and oligos #98 and #101 added to again prime DNA synthesis by PCR. The resulting PCR product DNA consisted of MHV genomic cDNA sequence from nucleotide 3334 to 3756 with a specific mutation (G to C) at nucleotide 3624 which would result in an amino acid change from cysteine to serine at residue 1137. The DNA was digested with Sph I and Spe I and inserted into the Sph I to Spe I site of pT7-NBgl and the resultant plasmid designated pT7-NB.1. The specific mutation was confirmed by sequencing the double stranded plasmid DNA using the Sequenase system (US Biochemicals).

RESULTS

cDNA cloning of MHV-JHM. To understand the structure and biochemical properties of the probable RNA polymerase of MHV, cDNA clones representing the entire gene were prepared (Fig. 1). From preliminary sequence information, we estimate the size of gene 1 to be greater than 22 kilobases, making the genomic RNA

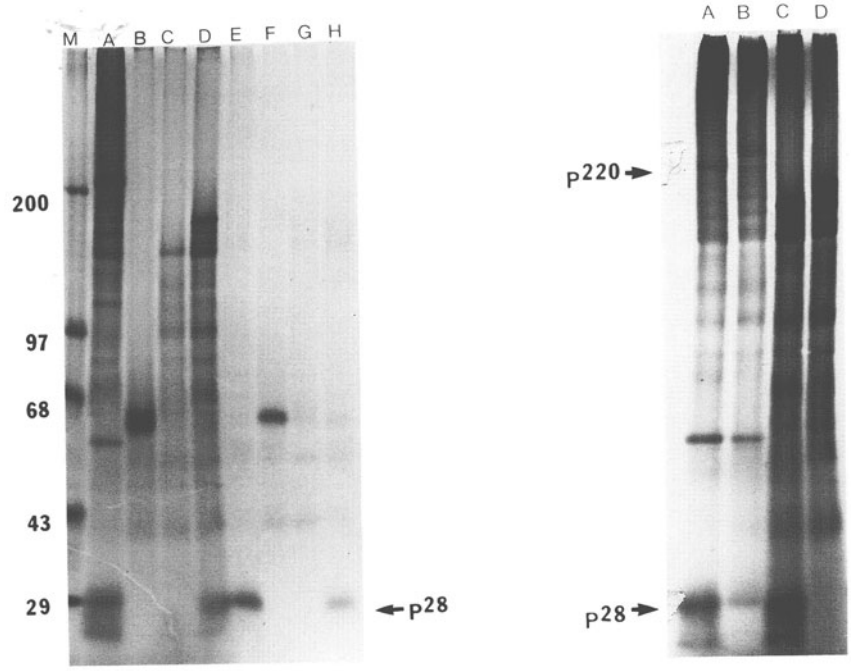


Fig. 2. Translation and immunoprecipitation of MHV-JHM gene 1 polypeptides (left). Capped RNA was synthesized from linearized plasmids pT7-NH, pT7-N2H, and pT7-NBgl with T7 RNA polymerase. The RNA was translated in vitro in rabbit reticulocyte lysates in the presence of (^{35}S)-methionine. The protein products were analyzed on a 3 to 12.5% gradient SDS-polyacrylamide gel. Lanes A, B, C, and D, respectively, show translation products of MHV-JHM genomic RNA, pT7-NH generated RNA, pT7-N2H generated RNA, and pT7-NBgl generated RNA. Lanes E, F, G and H show the products of immunoprecipitation with antiserum to p28. Lane M contained (^{14}C)-labeled marker polypeptides; molecular masses are given in kilodaltons on the left.

Fig. 3. Effects of $ZnCl_2$ on cell-free translation of gene 1 RNA (right). Zinc Chloride (1 mM) was added to the reticulocyte lysate before the addition of RNA, and translation reactions were incubated for 90 min in the presence of (^{35}S)-methionine. Lanes: A, control translation of MHV-JHM genomic RNA; B, with $ZnCl_2$; C, translation of pT7-NBgl-generated RNA; D, with $ZnCl_2$.

of murine coronavirus approximately 32 kb. The sequence of the 5'-most 2.0 kb region encoding the p28 protein has been reported previously (23). We have further extended the sequence for an additional 4.5 kb downstream (data not shown).

Translation and processing of the MHV Gene 1 protein products. In vitro translation studies of MHV genomic RNA have revealed two primary protein products, p28 and p220 (7). It has previously been shown that p28 is an amino-terminal cleavage product of the MHV gene 1 protein (23). To obtain more information on the translation and processing of gene 1 protein products, we subcloned into pT7 vectors various fragments of cDNA clones containing gene 1 sequences. These plasmids represent gene 1 sequences starting from nucleotide 187, which is just upstream of the initiator AUG (nucleotide 215), and extend to 2.0, 3.9, and 5.3 kb from the 5'-end of the genome. RNA was transcribed from linearized plasmids and translated in the presence of (^{35}S)-methionine in rabbit reticulocyte lysates. The protein products were analyzed by polyacrylamide gel electrophoresis as shown in Fig. 2. In vitro translation of MHV-JHM genomic RNA resulted in the synthesis of the p28 and p220 proteins (Fig. 2, lane A), in agreement with the previously published results (7, 23). The identity of p28 was confirmed by its precipitation by an antiserum specific to a peptide of p28 (Fig. 2, lane E). In contrast, translation of an RNA representing the first 2.0 kb of gene 1 yielded only a primary translation product of approximately 65 kDa (Fig. 2, lane B). Translation of an RNA extending 3.7 kb into the gene 1-coding region resulted in the translation of a primary protein product of approximately 135 kDa (Fig. 2, lane C). No p28 was detected in either of these two translation reactions even though both primary translation products are predicted to contain p28 sequence. However, translation of an RNA extending to 5.3 kb of the gene 1 sequence yielded a translation product of approximately 160 kDa and the p28 protein (Fig. 2, lane D). The identity of p28 was confirmed by its precipitation by the specific antiserum against the peptide of p28

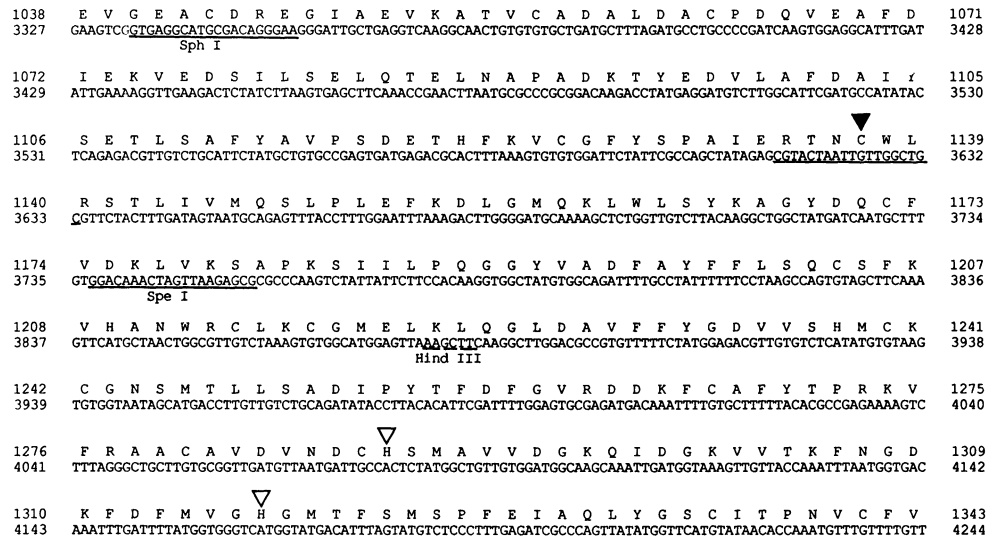


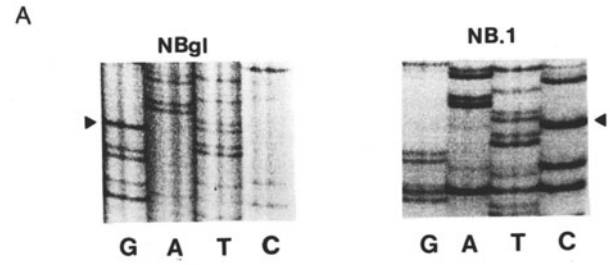
Fig 4. Sequence of the putative protease domain of MHV-JHM gene 1. A translation of the long open reading frame is shown in single-letter amino acid code. The cysteine residue proposed to be essential for protease activity is indicated by the filled triangle. Histidine residues which may be important for protease activity are indicated by open triangles. Sequences used to generate oligonucleotides used for site-directed mutagenesis are underlined.

(Fig. 2, lane H). These data indicated that the translation of a region between 3.9 kb and 5.3 kb from the 5'-end of the genomic RNA is required for the cleavage of the N-terminal portion, p28, from the primary gene 1 protein. The estimated molecular mass of the primary translation products of the three in vitro transcribed RNAs is 66 kDa, 136 kDa, and 186 kDa, respectively. This is in close agreement with the estimated molecular mass of the translation products detected on the gels: 65 kDa for pT7-NH, 135 kDa for pT7-N2H, and 160 kDa plus 28 kDa for pT7-NBgl.

Interestingly, the 65 kDa protein from the pT7-NH-generated translation reaction was precipitated by p28-specific serum (Fig. 2, lane F), while the 135 kDa protein from the pT7-N2H translation reaction was not (Fig. 2, lane G), indicating that the p28 epitope is either unavailable for binding or absent from that protein. As predicted, p160 was not precipitated since it does not contain the p28 sequence. The above data indicated that the cleavage of the p28 protein occurred only after the translation of a region more than 3.9 kb from the 5'-end of the viral RNA. This suggests that the region between 3.9 and 5.3 kb may contain an autoproteolytic activity or that it induces a conformational change in the protein which allows cleavage of p28.

To identify the primary translation product of pT7-NBgl RNA, we performed in vitro translation in the presence of protease inhibitors. As has been shown previously (7), the cleavage of p28 from the primary translation product (250 kDa) of MHV genomic RNA could be inhibited by addition of 1 mM $ZnCl_2$ to the translation reaction (Fig. 3, lane B). When the pT7-NBgl RNA was translated in the presence of 1 mM $ZnCl_2$, a protein of 185 kDa, but no p28, was obtained (Fig. 3, lane D). This result is in agreement with the predicted molecular weight of the primary translation product of this open reading frame.

Identification of a putative protease domain. Analysis of the amino acid sequence of the gene 1 polyprotein revealed a homology to the thiol family of proteases (Gorbalenya, A. E. and E. V. Koonin, personal communication). Thiol proteases such as papain, act by the juxtaposition of a cysteine (Cys) and a histidine (His) residue brought together by secondary structure to form a catalytic site. For MHV gene 1 polyprotein, Cys 1137 and either His 1288 or His 1317 were identified as the probable amino acids that may be involved in the protease activity (Gorbalenya, A.T. and Koonin, E.V. personal communication). The positions of these amino acids are shown in Fig. 4. The Hind III site at the end of plasmid pT7-N2H falls between the Cys and His residues. The failure of pT7-N2H polyprotein to cleave p28 suggests that both Cys and His residues are required for the catalytic site. Site-directed mutagenesis was used to directly test the role of Cys 1137 in the protease activity of the gene 1 polyprotein. Mutagenesis of this residue in pT7.NBgl was accomplished by the method of Higuchi (10), using synthetic oligonucleotides and polymerase chain reaction as described in the Materials and Methods section. Nucleotide 3625, G, was converted to C, resulting in the amino acid change at position 1137 from Cys to serine (Ser). The mutant plasmid was designated pT7-NB.1. Mutagenesis was confirmed by sequencing the region of the mutation in both the parent and mutant plasmids (Fig. 5).



B

T N C W L

NBgl: ACTAATTGTTGGCTG

T N S W L

NB.1: ACTAATTCTTGGCTG

Fig. 5. Sequence of region encompassing the Cys to Ser mutation in plasmid pT7-NB.1. (A) DNA sequence of plasmids pT7-NBgl and pT7-NB.1 from nucleotide 3619 to 3632. (B) Translation of the open reading frame is shown in single-letter amino acid code. The G to C change is underlined.

In vitro translation of RNA synthesized from pT7-NB.1 resulted in the synthesis of a large primary product of approximately 185 kDa (Fig. 6, lane B) in contrast to the pT7-NBgl-generated RNA which yield a smaller protein, p160, likely representing the processed protein (Fig. 6, lane A). As expected, when these translation reactions were precipitated with p28-specific antiserum, p28 was detected in the translation products of pT7 NBgl but not pT7-NB.1. Interestingly, the large primary protein product of 185 kDa was also precipitated from the pT7-NB.1 translation product (Fig. 6, lane D). This indicated that the conformation of this protein was such that the p28 epitope was available to bind to the antibody. Cys residue 1137 may be essential for both a conformational and catalytic role in the protease activity of the gene 1 polyprotein.

DISCUSSION

Theoretical considerations suggest that the 5'-most gene of the RNA genome of MHV, gene 1, codes for MHV-specific RNA-dependent RNA polymerases (23). We estimate the size of this genetic region to be greater than 22 kb; so far, only the amino-terminal protein, p28, of the potential gene product has been detected in virus-infected cells (8). This large genetic region is probably expressed as a single protein since there is only one mRNA species corresponding to gene 1. According to the nested-set structure of coronavirus mRNAs (12), only one translation initiation site at the 5'-end of each mRNA is utilized and, thus, only one functional protein is probably expressed from this entire gene. Significantly, the sequence of the avian coronavirus IBV genome reveals the presence of two overlapping ORFs in this region, which are likely translated into a polyprotein by a ribosomal frameshifting mechanism (4, 5). The exceptionally large size of this gene product suggests that the protein may have many different functional domains. Alternatively, the primary product of this large gene may undergo post-translational processing into smaller proteins. This possibility is comparable to other positive-stranded RNA viruses in which several nonstructural proteins are generated from post-translational cleavage of a single polyprotein (20, 25). Indeed, it has been shown that the 28 kDa protein is the N-terminal cleavage product of the gene 1 polyprotein (7, 23). The data presented in this report further show that this polyprotein may contain an autoproteolytic activity which is responsible for the cleavage of p28 from the primary translation product. A potential papain-like protease domain was identified, which corresponds to approximately 3.6 to 4.2 kb from the 5'-end of the genomic RNA. Interestingly, IBV also contains a papain-like protease region at 4.3 to 4.9 kb from the 5'-end of the genomic RNA (1). The presence of an autoprotease activity may be a common feature for all coronaviruses.

Mutagenesis of Cys residue 1137 indicated that it was essential for the autoprotease activity. This Cys residue probably interacts with a downstream region, since the 135 kDa protein containing amino acids 1-1222 is unable to cleave p28 (Fig. 2). Cys 1137 may also be important for maintaining the conformation of

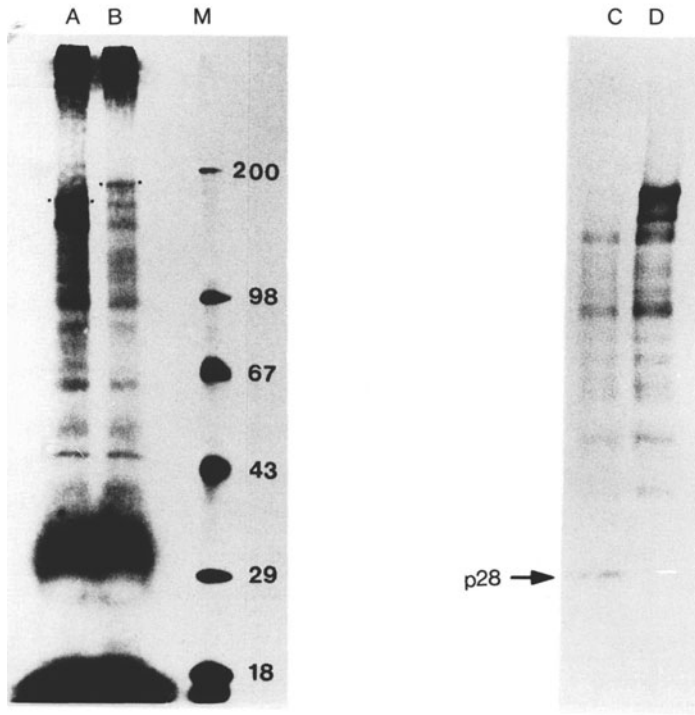


Fig. 6. Translation and immunoprecipitation of pT7-NBgl and pT7-NB.1 polypeptides. Capped RNA was synthesized from linearized plasmids pT7-NBgl and pT7-NB.1 with T7 RNA polymerase. The RNA was translated in vitro in rabbit reticulocyte lysates in the presence of (^{35}S)-methionine. The protein products were analyzed on a 3 to 12.5% gradient SDS-polyacrylamide gel. Lanes A and B, respectively , show translation products of pT7-NBgl and pT7-NB.1 generated RNA. Lanes C and D show the products of immunoprecipitation with antiserum to p28. Lane M contained (^{14}C)-labeled marker polypeptides; molecular masses are given in kilodaltons.

the protein. The primary translation product p135 was not precipitated by anti-p28 serum, indicating that the epitope was unavailable. In contrast, the pT7-NB.1 translation product, p185, which had a Ser at 1137, was precipitated with anti-p28 serum.

The gene 1 product of MHV may be a polyprotein analogous to that of picornaviruses, alphaviruses or flaviviruses (20, 25). In these viruses, the proteases are an integral part of the polyprotein and cleave the polyprotein at several sites. It is likely that the proteolytic activity of the MHV gene 1 product may also cleave itself at several additional sites. These cleavage products may perform various functions directly or indirectly involved in RNA synthesis. Each cleavage product of the gene 1 polyprotein may belong to a separate complementation group (16). By computer analysis of the IBV gene 1 sequence, it has been suggested that this protein may contain different domains for RNA polymerase, helicase, nucleotide binding activities and several proteases (9). Indeed, the complex mechanism of MHV RNA synthesis may require all of these enzymatic activities. The approach demonstrated in this paper may help us identify the enzymatic activities associated with these proteins. We are currently working to determine the role of His residues in the proteolytic activity and to identify the minimal region of the protease domain.

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LITERATURE CITED

1. Boursnell, M. E. G., T. D. K. Brown, I. J. Foulds, P. F. Green, F. M. Tomley, and M. M. Binns. 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J. Gen.Virol. 68:57-77.

2. Brayton, P. R., M. M. C. Lai, C. D. Patton, and S. A. Stohlman. 1982. Characterization of two RNA polymerase activities induced by mouse hepatitis virus. J. Virol. 42:847-853.
3. Brayton, P. R., S. A. Stohlman, and M. M. C. Lai. 1984. Further characterization of mouse hepatitis virus RNA dependent RNA polymerases. Virology 133:197-201.
4. Brierly, I., M.E.G. Boursnell, M.M. Binns, B. Bilimoria, V.C. Blok, T.D.K. Brown and S.C. Inglis. 1987. An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. EMBO 6:3779-3785.
5. Brierley, I. P. Digard and S. C. Inglis. Characterization of an efficient coronavirus ribosomal frameshift signal: requirement for an RNA pseudoknot. Cell 57: 537-547.
6. Brown, T. D. K., M. E. G. Boursnell, M. M. Binns, and F. M. Tomley. 1986. Cloning and sequencing of 5' terminal sequences from avian infectious bronchitis virus genomic RNA. J. Gen. Virol. 67:221-228.
7. Denison, M. R., and S. Perlman. 1986. Translation and processing of mouse hepatitis virus virion RNA in a cell-free system. J. Virol. 60:12-18.
8. Denison, M. R., and S. Perlman. 1987. Identification of a putative polymerase gene product in cells infected with murine coronavirus A59. Virology 157:565-568.
9. Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov. 1989. Coronavirus genome: prediction of putative functional domains in the non-structural polyprotein by comparative amino acid sequence analysis. Nuc. Acids Res. 17:4847-4861.
10. Higuchi, R., B. Krummel, and R. K. Saiki. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nuc. Acids Res. 16: 7351-7367.
11. Kessler, S. W. (1981). Use of protein A bearing staphylococci for the immunoprecipitation and isolation of antigens from cells. In "Methods in Enzymology" (J.J. Longone and H. Van Vunakis, Eds.), Vol. 73, pp. 442-459. Academic Press, New York.
12. Lai, M. M. C. 1988. Replication of Coronavirus RNA. In "RNA Genetics." (E. Domingo, J.J. Holland, and P. Ahlquist, Eds.), Vol. I, pp. 115-136. CRC Press, Inc., Boca Raton, Florida.
13. Lai, M. M. C., R. S. Baric, P. R. Brayton, and S. A. Stohlman. 1984. Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus-a cytoplasmic RNA virus. Proc. Natl. Acad. Sci. USA 81:3626-3630.
14. Lai, M. M. C., P. R. Brayton, R. C. Armen, C. D. Patton, C. Pugh, and S. A. Stohlman. 1981. Mouse hepatitis virus A59: mRNA structure and genetic localization of the sequence divergence from hepatotropic strain MHV-3. J. Virol. 39:823-834.
15. Lai, M. M. C., and S. A. Stohlman. 1978. RNA of mouse hepatitis virus. J. Virol. 26:236-242.
16. Leibowitz, J. L., J. R. DeVries, and M.V. Haspel. 1982. Genetic analysis of murine hepatitis virus strain JHM. J. Virol. 42:1080-1088.
17. Leibowitz, J. L., S. R. Weiss, E. Paavola, and C. W. Bond. 1982. Cell-free translation of murine coronavirus RNA. J. Virol. 43:905-913.
18. Maizel, J. 1971. Polyacrylamide gel electrophoresis of viral proteins. Methods Virol. 5:176-246.
19. Makino, S., S. A. Stohlman, and M. M. C. Lai. 1986. Leader sequences of murine coronavirus mRNAs can be freely reassorted: evidence for the role of free leader RNA in transcription. Proc. Natl. Acad. Sci. USA 83:4204-4208.
20. Semler, B. L., R. J. Kuhn, and E. Wimmer. 1988. Replication of the poliovirus genome. In "RNA Genetics." (E. Domingo, J.J. Holland, and P. Ahlquist, Eds.), Vol. I, pp. 23-48. CRC Press, Inc., Boca Raton, Florida.
21. Shieh, C.-K., L.H. Soe, S. Makino, M.-F. Chang, S. A. Stohlman, and M. M. C. Lai. 1987. The 5'-end sequence of the murine coronavirus genome: implications for multiple fusion sites in leader-primed transcription. Virology 156:321-330.
22. Siddell, S. G. 1983. Coronavirus JHM: coding assignments of subgenomic mRNAs. J. Gen. Virol. 64:113-125.
23. Soe, L. H., C.-K. Shieh, S. C. Baker, M.-F. Chang, and M. M. C. Lai. 1987. Sequence and translation of the murine coronavirus 5'-end genomic RNA reveals the N-terminal structure of the putative RNA polymerase. J. Virol. 61:3968-3976.
24. Spaan, W. J. M., H. Delius, M. Skinner, J. Armstrong, P. Rottier, S. Smeekens, B. A. M. van der Zeijst, and S. G. Siddell. 1983. Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. EMBO J. 2:1839-1844.
25. Strauss, J. H. and E. G. Strauss. 1988. Replication of the RNAs of alphaviruses and flaviviruses. In "RNA Genetics." (E. Domingo, J.J. Holland, and P. Ahlquist, Eds.), Vol. I, pp. 71-90. CRC Press, Inc., Boca Raton, Florida.
26. Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074-1078.
27. Wege, H., A. Muller, and V. ter Meulen. 1978. Genomic RNA of the murine coronavirus JHM. J. Gen. Virol. 41:217-227.