

## Identification of the polymerase polyprotein products p72 and p65 of the murine coronavirus MHV-JHM

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### Abstract

The RNA polymerase gene of murine coronavirus MHV-JHM encodes a polyprotein of greater than 750 kDa. This polyprotein is proposed to be processed by two papain-like cysteine proteinases, PCP-1 and PCP-2, and a poliovirus 3C-like proteinase domain, 3C-pro, to generate protein products. The amino-terminal product of the MHV polymerase polyprotein, p28, is generated by cleavage of the polyprotein by PCP-1. To identify the viral products downstream of p28, we generated a fusion-protein specific antiserum directed against the region adjacent to p28 and used the antiserum to detect virus-specific proteins from MHV-JHM infected cells. When this antiserum was used to immunoprecipitate radiolabeled proteins from MHV-JHM infected cell lysates, virus-specific proteins of 72 and 65 kDa were detected. Furthermore, pulse and chase experiments demonstrated that p72 is likely a precursor to the mature protein product, p65. To investigate which viral proteinase may be responsible for generating p72 and p65, we expressed the 5'-region of the MHV-JHM RNA polymerase gene including the two papain-like cysteine proteinase domains in an in vitro transcription/translation system and analyzed the translation products for proteolytic processing. We also cloned and expressed the 72 kDa region immediately downstream from p28, and tested the ability of in vitro translated PCP-1 and PCP-2 to cleave p72 to p65 in trans. Our results indicate that neither viral proteinase domain PCP-1 nor PCP-2 is capable of cleavage of p72 to produce p65 in vitro. The role of MHV proteinases in the processing of p72 and p65 is discussed.

*Keywords:* Coronavirus; Polymerase polyprotein; Proteolytic processing

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Mouse hepatitis virus (MHV), a member of the family Coronaviridae, contains a single-stranded, positive sense RNA genome of approximately 32 kilobases (Pachuk et al., 1989; Lee et al., 1991).

The MHV genomic RNA is surrounded by a phosphoprotein to form a helical nucleocapsid structure within a lipid envelope. Upon infection, the MHV genomic RNA is translated to produce a viral RNA-dependent RNA polymerase which mediates the synthesis of negative strand RNA and subgenomic mRNAs. The subgenomic mRNAs form a 3'-coterminal nested set and are synthesized by a process that involves discontinuous transcription (Baric et al., 1983; Spaan et al., 1983). The discontinuous transcription of MHV RNA is thought to contribute to the high frequency of RNA recombination that is also observed in MHV-infected cells (Makino et al., 1986). The novel transcription mechanism proposed to generate MHV mRNAs and the high frequency of MHV RNA recombination implicate a polymerase enzyme with unusual discontinuous properties; however, that polymerase must also be capable of accurately replicating the 32 kb genomic RNA to produce infectious virus (reviewed by Lai et al., 1994; van der Most and Spaan, 1995). To understand the unique process of MHV RNA synthesis, we aim to identify the components of the RNA-dependent RNA polymerase, to determine how these proteins are generated and to elucidate their function in the complex MHV replication strategy.

The MHV RNA-dependent RNA polymerase is encoded by the 5'-most gene, gene 1. Gene 1 is 22 kb and contains two long open reading frames (ORFs) designated ORF 1a and ORF 1b (Pachuk et al., 1989; Lee et al., 1991; Bonilla et al., 1994). ORF 1a and ORF 1b overlap by 75 nucleotides and may be translated to produce a single polyprotein of approximately 750 kDa by a ribosomal frameshift mechanism (Brierly et al., 1987, 1989; Bredenbeek et al., 1990; Lee et al., 1991). This large polymerase polyprotein is proposed to be processed into mature protein products by viral proteinases encoded within the polyprotein, and perhaps by cellular proteinases as well. Three viral proteinase domains, including two papain-like cysteine proteinases (PCP-1 and PCP-2) and a poliovirus 3C-like proteinase domain (3C-pro), have been predicted by amino acid sequence comparison (Gorbalenya et al., 1989, 1991).

The first direct evidence for proteolytic processing of the polymerase polyprotein came from *in vitro* translation studies of MHV genomic RNA. Denison and Perlman showed that *in vitro* translation of viral genomic RNA produced p28 and p220, and that production of p28 was inhibited by the addition of a proteinase inhibitor, zinc chloride (Denison and Perlman, 1986). Using an antiserum generated against a synthetic peptide representing a portion of p28, the p28 protein was detected in MHV-infected cells and shown to be the amino-terminal protein of the polymerase polyprotein (Denison and Perlman, 1987; Soe et al., 1987). Translation of RNAs representing the 5' end of the viral genome revealed that the PCP-1 domain was required for the autoproteolytic cleavage of p28 (Baker et al., 1989). The cleavage of p28 by PCP-1 was inhibited by leupeptin in both *in vivo* and *in vitro* reactions (Denison and Perlman, 1986; Baker et al., 1989). Furthermore, the catalytic cysteine and histidine residues of the PCP-1 domain have been identified (Baker et al., 1993). The cleavage site recognized by PCP-1 for the cleavage of p28 has been determined to be the Gly-247/Val-248 dipeptide bond (Dong and Baker, 1994; Hughes et al., 1995). A number of potential PCP-1 cleavage sites are predicted, but only the p28 cleavage site has been experimentally confirmed.

A second, independent cleavage event has recently been implicated in the release of a 65 kDa product in MHV-A59 infected cells (Denison et al., 1995). Using an antiserum which was directed against a fusion protein encoding the entire p28 domain and the region adjacent to the p28 cleavage site, Denison and co-workers immunoprecipitated a 65 kDa protein from MHV-A59 infected cells. Additional *in vitro* experiments using cloned cDNAs of MHV-A59 suggest that p65 may be cleaved by the 5'-most proteinase PCP-1 (Bonilla et al., 1995). However, Kim et al. (1995) have shown that E64d, an irreversible inhibitor of cysteine proteinases, specifically blocks the *in vivo* cleavage of p65 but surprisingly has very little effect on the cleavage of p28. This suggests that a proteinase other than PCP-1, which cleaves p28, is responsible for the liberation of p65. In this study, we aimed to identify the proteolytic product ad-

acent to p28 in the MHV-JHM polymerase polyprotein and to determine how that protein is processed.

To examine the synthesis and processing of the MHV-JHM polymerase polyprotein, we used antisera directed against the amino-terminal domains of the polymerase, anti-p28 and anti-647, to immunoprecipitate [<sup>35</sup>S]methionine-labeled proteins from MHV-JHM infected cells. The anti-p28 serum was generated using a synthetic peptide representing amino acids 78–93 of MHV-JHM and has been previously described (Baker et al., 1989). For this study, the anti-647 serum was generated against a bacterial GST-MHV fusion protein which encodes a 647 basepair domain that is adjacent to p28 (see Fig. 1). The bacterial expression vector, pGEX-KG, which contains the glutathione S-transferase (GST) gene under the control of a tac promoter, was kindly provided by Dr. Steven Broyles (Purdue University, West Lafayette, IN). To generate a GST-MHV fusion protein, a 647 basepair *Xho*I fragment (nt 1128–1775 of MHV JHM-X), was isolated from pT7-NBgl plasmid DNA (Baker et al., 1989) and ligated into the *Xho*I site of pGEX-KG. The resulting expression plasmid which encodes a GST-MHV fusion protein with a predicted molec-

ular mass of 50 kDa, was designated pGEX-647. The fusion protein was induced and isolated following the method of Guan (Guan and Dixon, 1991), and injected into rabbits to generate polyclonal antiserum. To identify MHV gene products, a mouse fibroblast cell line, 17Cl-1, was infected with MHV-JHM at a multiplicity of infection (m.o.i.) of 10 and labeled at 6.5 h post-infection (p.i.) with [<sup>35</sup>S]methionine for 120 min. Whole cell lysates were prepared and immunoprecipitated as previously described (Baker et al., 1989) using protein A-sepharose beads and anti-p28, anti-647 or pre-immune serum, and analyzed by electrophoresis in a 10% SDS-polyacrylamide gel (Fig. 2). The amino-terminal product of the polymerase polyprotein, p28, was detected in MHV-JHM infected cells using anti-p28 (Lane 6), demonstrating that translation and processing of the polymerase polyprotein was occurring during the labeling period. The anti-647 serum specifically precipitated a protein with an apparent molecular mass of 65 kDa (Lane 4), which was not detected in mock infected cells (Lane 3) or from mock or MHV-JHM infected cell lysates immunoprecipitated with pre-immune serum (Lanes 1 and 2). Two additional MHV-JHM specific proteins of 72 kDa and greater than 300 kDa were also detected using anti-647 serum.

To determine if the p72 and/or 300 kDa proteins were precursors to p65, we performed pulse and chase experiments. MHV-JHM infected cells were pulse-labeled with [<sup>35</sup>S]methionine from 7.0 to 7.5 h p.i. Following the pulse, the radiolabeled proteins were chased by replacing the labeling media with media containing excess unlabeled methionine. Whole cell lysates were prepared at various times during the chase and immunoprecipitated with anti-p28 and anti-647, and products were analyzed by electrophoresis in 10% SDS-polyacrylamide gels (Fig. 3(A,B)). The p28 protein is detected immediately after the 30 min pulse and no precursor protein is observed (Fig. 3(A)). The p28 protein also appears to be stable throughout the 120 min chase. This result is consistent with previous data demonstrating that p28 is cleaved in *cis* and is stable in MHV-infected cells (Denison and Perlman, 1987; Baker et al., 1989). Immunoprecipitation of the MHV-JHM

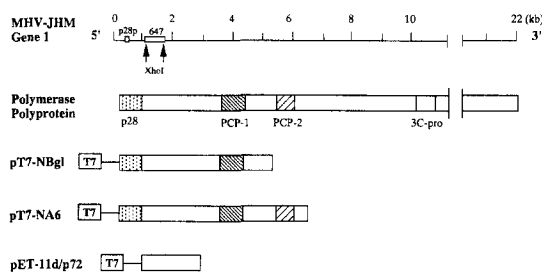


Fig. 1. Schematic representation of the polymerase gene and open reading frame (ORF) of mouse hepatitis virus strain JHM (MHV-JHM). The positions of p28, the p28 peptide (p28p) used to generate the anti-p28 antiserum, the 647 nucleotide *Xho*I fragment used to generate the GST-fusion antiserum, the papain like cysteine proteinase domain 1 (PCP-1), the predicted papain like cysteine proteinase domain 2 (PCP-2) and the putative polio virus 3C-like proteinase domain (3C-pro) are shown. Plasmids pT7-NBgl and pT7-NA6 contain 5.3 kb and 6.8 kb of sequences respectively, from the 5'-end of MHV-JHM gene 1. Plasmid pET-11d/p72 contains nt 956–2896 of MHV-JHM gene 1.

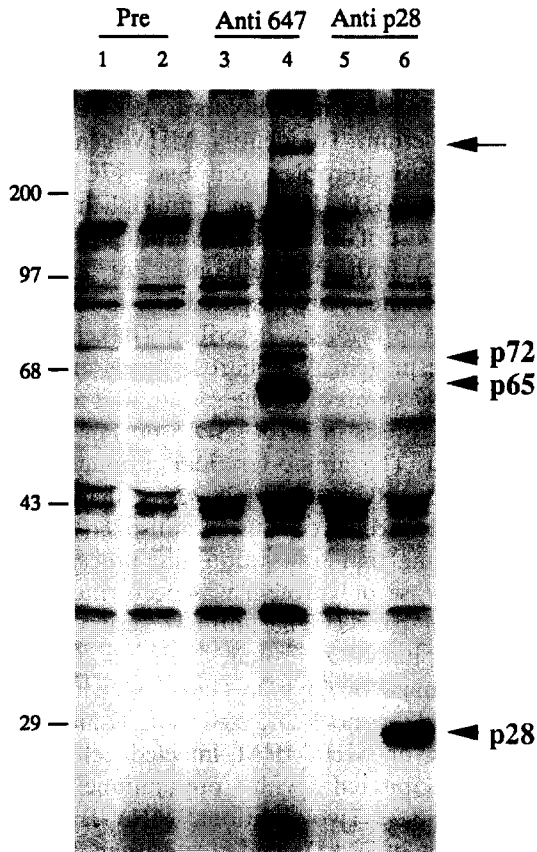


Fig. 2. Detection of viral proteins from MHV-JHM infected 17Cl-1 cells by immunoprecipitation using anti-647 antiserum. Immunoprecipitates of [ $^{35}$ S]methionine-labeled, MHV-JHM infected 17Cl-1 cells or mock-infected cells were analyzed by 10% SDS-PAGE. Lanes: products immunoprecipitated from mock-infected cells (Lanes 1, 3 and 5) or from MHV-JHM infected cells (Lanes 2, 4 and 6) with preimmune serum, anti-647 or anti-p28 as indicated. Arrowheads indicate viral proteins p72, p65 and p28. The arrow indicates a viral protein of greater than 300 kDa. Molecular weight markers are labeled in kilodaltons on the left of the gel.

infected lysates with anti-647 reveals a precursor-product relationship of p72 and p65 (Fig. 3(B)). The p72 protein is the predominant viral protein precipitated from the pulse-labeled cells. The p65 protein is detected by 15 min of chase and gradually accumulates with a concomitant decrease in p72. These results indicate that the p72 is likely the precursor to the p65 protein. The p72 and p65 proteins may share either amino- or carboxy-terminal regions and be recognized by the anti-647

serum. The high molecular weight protein (greater than 300 kDa) was also detected at very low levels in the pulse-chase experiment. This large protein likely represents a primary translation product of ORF 1a. This precursor polyprotein may be cleaved to produce p72/p65 and the p290 which was detected by Denison and co-workers (Denison et al., 1992).

Analysis of the MHV strain A59 polymerase gene products has also identified a 65 kDa protein as the mature proteolytic product adjacent to p28 (Denison et al., 1995). The MHV-A59 p65 protein was identified using a fusion protein antiserum (UP102) that was generated against the entire p28 coding region and approximately 1 kb of adjacent sequence. Consistent with our results, Denison and co-workers demonstrated that p28 is detected immediately after a 30 min pulse, and that p65 is detected after approximately 20 min of chase. However, no precursor protein of 72 kDa was detected from MHV-A59 infected cells using antiserum UP102 (Denison et al., 1995). There are several possible explanations for this difference. First, because the UP102 and 647 antisera were generated against different virus strains and in different fusion protein constructs, the antisera may recognize different epitopes. The p72 precursor protein may be folded in a conformation that is not efficiently recognized by the UP102 antiserum. A second possible explanation is that the proteolytic processing of the MHV-A59 strain and the MHV-JHM strain may differ in this region.

To determine how p72 and p65 are generated from the polymerase polyprotein, we expressed cDNA clones encoding the 5'-end of ORF 1a, including the 2 papain-like cysteine proteinase domains, and analyzed the translation reactions for the presence of proteolytic products. The cDNA clones pT7-NA6 and pT7-NBgl, representing the 5'-end 6.8 and 5.3 kb, respectively (see Fig. 1; Baker et al., 1989; Lee et al., 1991), were expressed in vitro in the presence of [ $^{35}$ S]methionine in a coupled transcription/translation system according to the manufacturer's instructions (TNT system, Promega, Madison, WI). The translation products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

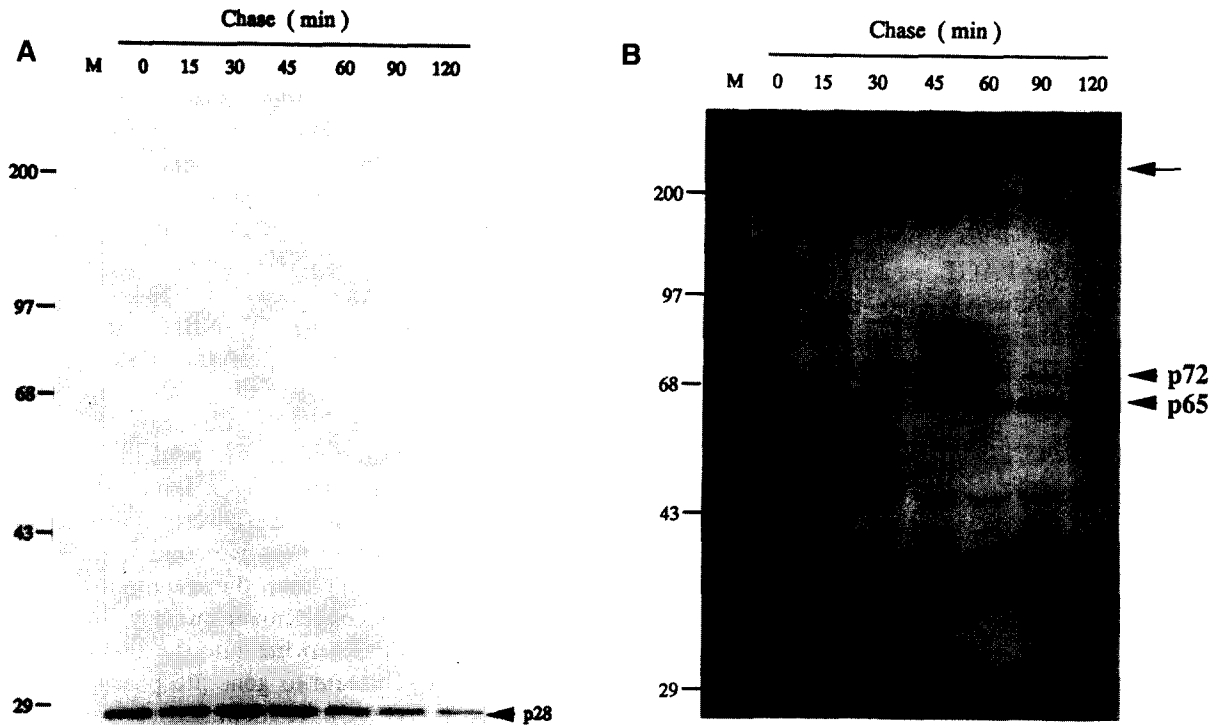


Fig. 3. Synthesis and processing of p72 in MHV-JHM infected 17Cl-1 cells. MHV-JHM infected cells were pulse labeled for 30 min with [ $^{35}$ S]methionine at 7.0 h post infection; the labeling media was then replaced with media containing excess methionine and cell extracts were prepared at the times indicated. The cell extracts were immunoprecipitated with anti-p28 (A) or anti-647 (B). The immunoprecipitation products were analyzed by 10% SDS-PAGE. 'M' denotes immunoprecipitation products from pulse-labeled mock infected cell extracts. Arrowheads indicate the specific immunoprecipitation products, p72 and p65. The arrow indicates the additional protein band immunoprecipitated by anti-647. Molecular weight markers are labeled in kilodaltons on the left of the gel.

directly (Fig. 4, Lanes 1 and 2) or after immunoprecipitation with anti-647 (Lanes 4 and 5) or anti-p28 (Lanes 6 and 7). As expected, p28 is detected from the primary translation products of both pT7-NA6 and pT7-NBgl, since both encode PCP-1, and it has been shown that PCP-1 is responsible for the cleavage of p28 (Baker et al., 1989). Clone pT7-NA6 contains an extension of the 5'-end of the polymerase gene which includes the putative PCP-2 domain. Analysis of pT7-NA6 in vitro translation products reveals protein products of approximately 195 and 28 kDa. No additional proteolytic products were specifically detected either in the primary translation products (Fig. 4, Lane 1) or after immunoprecipitation with anti-647 (Lane 4). These results indicate that although PCP-1 can cleave p28 from the

polyprotein representing the 5' 6.8 kb of ORF-1a, neither PCP-1 nor PCP-2 can cleave p72 or p65 from the precursor polyprotein in vitro.

Although the viral proteinase domains PCP-1 and PCP-2 do not cleave p72 or p65 from the precursor polyproteins described above, they may still be responsible for the cleavage of p65 if the appropriate precursor, p72, is presented. Alternatively, p72 may be processed by MHV proteinase 3C-pro or some other unidentified viral proteinase, to generate p65. To address this possibility we asked the following question: If p72 is presented independently, will a viral proteinase domain recognize and cleave it to produce p65? For this purpose, we reverse-transcribed and polymerase chain reaction (PCR) amplified the 72 kDa coding region adjacent to p28 starting at

nucleotide 956, encoding the valine in the P1' position of the p28 cleavage site, and ending precisely at a potential 3C-pro E/S cleavage site, at nucleotide 2896. This region was cloned into the expression vector pET-11d (Novagen, Madison, WI), generating plasmid pET-11d/p72 (Fig. 1). In vitro transcription/translation of this plasmid DNA yields a protein which, when analyzed by SDS-PAGE, is approximately 72 kDa (Fig. 5(A), Lane 1). To determine if MHV proteinase domains PCP-1 or PCP-2 act in trans to cleave p72, we generated radiolabeled p72 protein and unlabeled pT7-NBgl and pT7-NA6

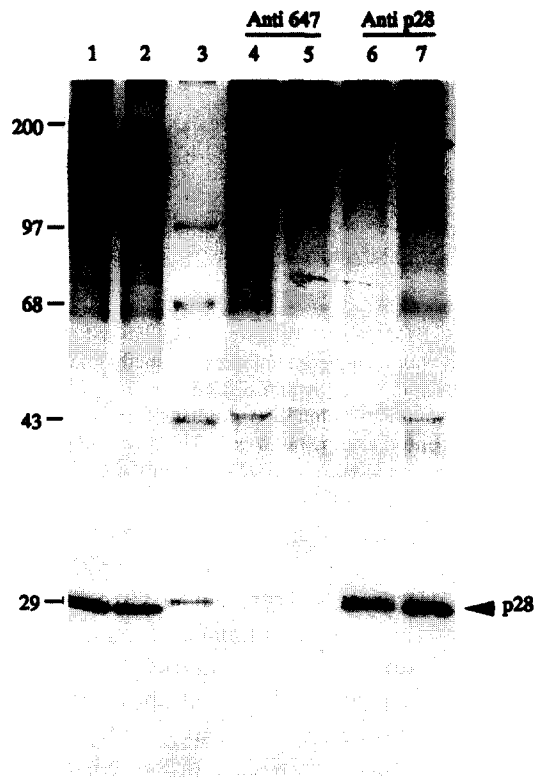


Fig. 4. In vitro translation and immunoprecipitation of MHV-JHM polymerase gene products. Linearized plasmids pT7-NA6 and pT7-NBgl were in vitro transcribed and translated in the presence of [ $^{35}$ S]methionine. The translation products were analyzed by 10% SDS-PAGE directly (Lanes 1 and 2) or following immunoprecipitation with anti-647 (Lanes 4 and 5) or anti-p28 (Lanes 6 and 7). Dots highlight higher molecular weight immunoprecipitated products. Lane 3 contains  $^{14}$ C-labeled molecular marker polypeptides. Molecular mass standards are given in kilodaltons.

translation products, mixed the proteins and incubated the mixtures for 60 or 120 min. The proteins were then analyzed by electrophoresis on an SDS-polyacrylamide gel and visualized by autoradiography (Fig. 5). In samples where fully-translated proteins are mixed and incubated together for an additional 60 min, p72 is not cleaved into p65 by PCP-1 and PCP-2-containing proteins (translated from pT7-NBgl and pT7-NA6, respectively) (Fig. 5(A), Lanes 3–6). Similarly, when p72 is included in the pT7-NBgl and pT7-NA6 translation mixtures to test the possibility of a co-translational activity of PCP-1 and PCP-2 (Fig. 5(A), Lanes 7–12), p72 also remains un-cleaved. Incubation of the p72 protein with rabbit reticulocyte lysates (Fig. 5(A), Lanes 1 and 2), a major component in the transcription/translation reactions, serves as a negative control and reflects p72 stability throughout the incubation period. Results from this experiment indicate that neither PCP-1 nor PCP-2 can mediate the trans-cleavage of p72 as a substrate to generate p65 in vitro.

In summary, we have identified two proteolytic products, p65 and p72 of the MHV-JHM polymerase polyprotein, from MHV-JHM infected cells. A 65 kDa protein has also been identified for MHV-A59 by Denison et al. (1995), but the p72 protein has not previously been reported for any coronavirus. Our pulse-chase experiments indicate that the 72 kDa protein is likely a precursor to the p65 protein (Fig. 3). Using both *cis* and *trans*-cleavage assays, we showed that neither the PCP-1 nor PCP-2 domain mediates the cleavage of p72 or p65 in vitro (Figs. 4 and 5). These results suggest that a proteolytic pathway distinct from that used to generate p28 is used to generate p72 and p65 in MHV-JHM infected cells.

It is likely that a number of distinct proteolytic events are required to generate the functional MHV RNA polymerase. To date, a number of proteins have been identified from MHV infected cells, including p28, p72, p65, p50, p240 and p290 (Denison and Perlman, 1987; Denison et al., 1992, 1995; this report), but only the proteolytic pathway for generating p28 has been elucidated (Baker et al., 1993; Dong and Baker, 1994; Hughes et al., 1995). There has been no report of the cleavage activity of the PCP-2 domain. Re-

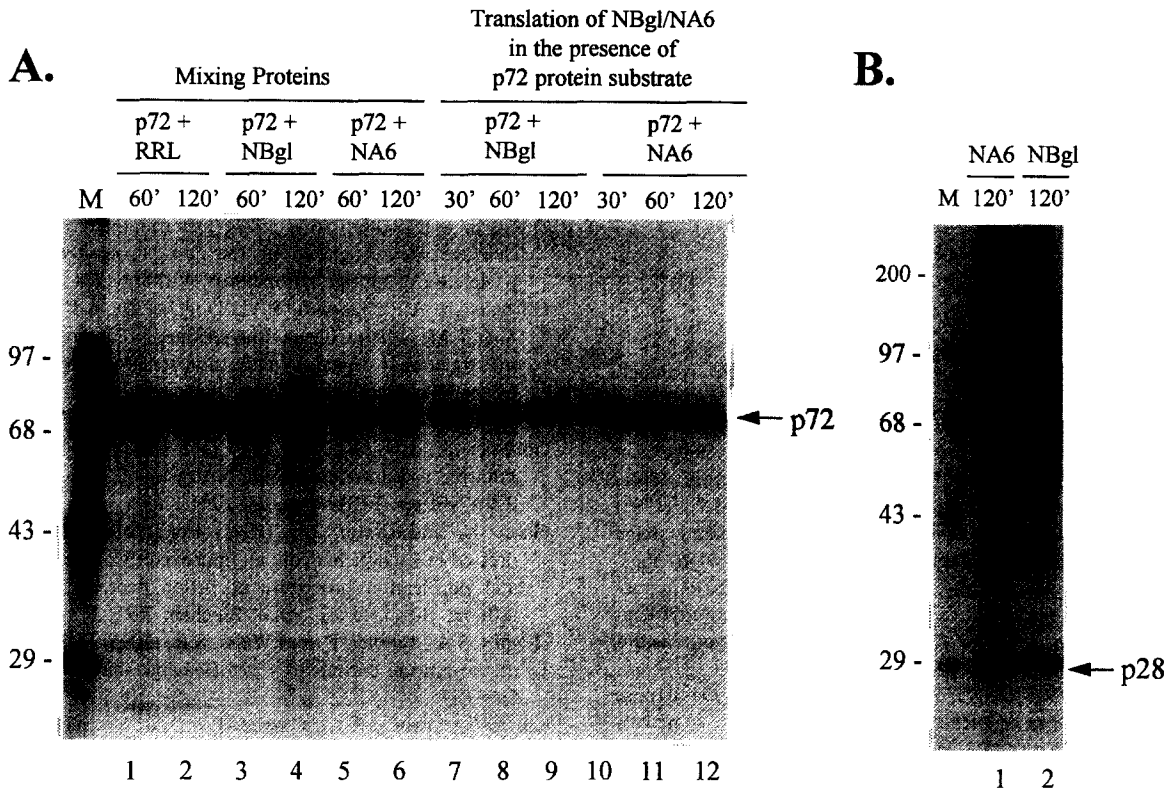


Fig. 5. *Trans*-cleavage analysis of p72 by pT7-NBgl and pT7-NA6 translation products. (A) For all samples, pET-11d/p72 plasmid DNA was transcribed and translated in the presence of [<sup>35</sup>S]methionine for 120 min. The p72 translation product was then incubated with unlabeled transcription/translation products generated from: rabbit reticulocyte lysates alone (Lanes 1 and 2), pT7-NBgl plasmid DNA (Lanes 3 and 4) or pT7-NA6 plasmid DNA (Lanes 5 and 6). To test the potential co-translational activity of pT7-NBgl and pT7-NA6 translation products to cleave p72 in *trans*, plasmid DNAs were transcribed and translated in the absence of [<sup>35</sup>S]methionine, but in the presence of [<sup>35</sup>S]methionine radiolabeled p72 protein (Lanes 7–12). The *trans*-cleavage assay products were analyzed by electrophoresis on a 10% SDS–polyacrylamide gel and visualized by autoradiography. Molecular weight markers are indicated in kilodaltons on the left of the gel. (B) *In vitro* transcription/translation products generated from plasmid DNA pT7-NA6 (Lane 1) and pT7-NBgl (Lane 2) in the presence of [<sup>35</sup>S]methionine as a control for parallel unlabeled translation reactions. Products were analyzed on a 10% SDS–polyacrylamide gel and visualized by autoradiography. The position of the p28 *cis*-cleavage product is indicated by the arrow on the right.

cently, the activity of the coronavirus 3C-pro domain has been identified for MHV-A59 (Lu et al., 1995), human coronavirus 229-E (Ziebuhr et al., 1995) and the avian coronavirus infectious bronchitis virus (Liu and Brown, 1995; Liu et al., 1995; Tibbles et al., 1996). Using bacterial expression vectors, it has been shown that the coronavirus 3C-like proteinases recognize predicted cleavage sites, Q/S for MHV-A59 and IBV, and Q/A for 229E, to release the 3C domain from the polyprotein precursor. These cleavage sites are consistent with the consensus recognition sequence

of Q, E/G, S, A for 3C proteinases (Dougherty and Semler, 1993; Krausslich and Wimmer, 1988; Palmenberg, 1990). The picornavirus 3C proteinases recognize multiple sites in their polyprotein and cleave both in *cis* and in *trans*. The MHV-JHM 3C-pro may also have multiple cleavage sites, including a putative E/S cleavage site in the region predicted for cleavage of p72. We are currently investigating whether the 3C-pro domain is in fact responsible for cleavage of p72 or p65, or if an as yet unidentified proteinase domain is involved in these processing events.

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