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Polyprotein Processing in *cis* and in *trans* by Hepatitis A Virus 3C Protease Cloned and Expressed in *Escherichia coli*

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To determine the P3 region protein-processing sites cleaved by the hepatitis A virus 3C protease, a nested set of constructs containing a portion of 3A (3A* [the asterisk denotes an incomplete protein]), 3B, and 3C and various amounts of 3D, fused in frame to *Escherichia coli* TrpE-coding sequences under control of the tryptophan promoter, was made. Additional plasmids that encoded a portion of 2C (2C*) and the P3 proteins, including complete or incomplete 3D sequences, were constructed. After induction, *E. coli* containing these recombinant plasmids produced high levels of fusion proteins as insoluble aggregates. 3C-mediated cleavage products were identified by comparison of expression with a matching set of plasmids, containing an engineered mutation in 3C. Cleavage products were detected by immunoblot analyses by using antisera against the TrpE protein, against 3D*, and against 3CD*. Scissile bonds were determined by N-terminal amino acid sequencing of the proteins formed by cleavage. The results showed that when a portion of 2C was present, the primary cleavage by the 3C protease was between 2C and 3A, and the cleavage site was QG, as predicted by J. I. Cohen, J. R. Ticehurst, R. H. Purcell, A. Buckler-White, and B. M. Baroudy, J. Virol. 61:50–59, 1987. Very little further cleavage of the released P3 protein was detected. When the fusion protein contained no 2C and included only 3A*-to-3D sequences, efficient cleavage occurred between 3B and 3C, at the QS pair, also as predicted by Cohen et al. (J. Virol. 61:50–59, 1987). The latter proteins were also cleaved between 3C and 3D, but less efficiently than between 3B and 3C. Extracts of bacteria expressing proteins from 3A* to 3D also cleaved a radiolabelled hepatitis A virus substrate containing VP1*2ABC* sequences in *trans*.

Identification of hepatitis A virus (HAV) as a member of the family *Picornaviridae* (4, 17) and adaptation of virus isolated from human stool to growth in laboratory cell cultures (22) occurred more than a decade ago. Despite these accomplishments, the growth properties of this virus have made studies of the molecular biology of HAV replication difficult. Virus replication is slow and asynchronous, resulting in persistent infection rather than the rapid lytic cycles induced by most other picornaviruses (1, 5, 11, 26). Viral nonstructural proteins were not detected in cultured cells supporting virus replication, despite the application of sensitive immunologic reagents (25). Nevertheless, the determination of the genome sequences of several strains of HAV (3, 16, 18, 21) and the construction of an infectious cDNA clone derived from HAV HM175 (2) have opened new avenues of approach to the analysis of viral gene products and their role in HAV replication (24). Although the overall gene organization of HAV appears quite similar to those of the other picornaviruses, the sequences of individual proteins show only minimal similarities to those of all other members of the family.

All picornaviruses process their polyproteins via a series of *cis* and *trans* cleavages, catalyzed primarily by the 3C protein (14, 15, 19). Recently, the HAV 3C gene product has been shown to exhibit proteolytic activity, mediating cleavage at its own N and C termini (8) and at the proposed junction between 2C and 3A (12). These activities were observed for fusion proteins expressed from cloned cDNA in *Escherichia coli* (8) and in translation products directed by transcripts of HAV P3 region cDNA, produced in rabbit reticulocyte lysates (12). In both cases, 3C sequences containing mutations or deletions failed to catalyze the cleavage reactions. No intermolecular cleavages by 3C in *trans* were observed to occur in vitro.

In this study, the production of active 3C protease from cloned cDNAs in *E. coli* has been used to identify specific polyprotein cleavage sites and to study the processing reactions in the P3 region of the HAV polyprotein. N-terminal amino acid sequencing of some of the HAV proteins which resulted from cleavage by 3C confirmed the previously proposed (3) cleavage sites between 2C and 3A (QG) and between 3B and 3C (QS). We have also shown that 3C expressed in *E. coli* cleaves a radiolabelled HAV protein substrate containing VP1*2ABC* in *trans*. (The asterisk denotes an incomplete protein.)

MATERIALS AND METHODS

Plasmid construction and mutations. The parental plasmid used to make all constructs was pHAV/7 (2), which was derived from HAV HM175 and which can be transcribed in vitro to generate infectious RNAs. A nested set of cDNA fragments was isolated, starting with the *Pst*I site in 3A (nucleotide [nt] 5129) resulting in constructs containing about 38% of the predicted carboxy (C) terminus of 3A (Fig. 1, plasmids 2 to 5). These constructs were designated 3A*, indicating the presence of a portion of 3A. All of these constructs contained all of the sequences of the predicted 3B and 3C sequences and various amounts of 3D; the percentages of 3D contained in four constructs were 8, 20, 82 and

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100 and are shown as subscripts in these designations. Constructs, including a portion of the 2C sequence, were also made by subcloning the *Bal*I fragment (nt 4578 to 6224); these included approximately 41% of the C-terminal sequences of 2C and 20% of the N-terminal sequences of 3D (Fig. 1, plasmid 1). In several of these plasmids, a mutated 3C (μ3C) region was inserted in place of the wild-type (wt) sequences; this mutation has been described previously (13). It contains two amino acid substitutions (His-151→Gly-151 and Gly-154→Arg-154) in the 3C-coding sequence.

The DNA fragments were fused in frame to *E. coli* TrpE-coding sequences under the control of the tryptophan promoter in pATH vectors (7) and transformed into *E. coli* DH5 cells. Some constructs were introduced into *E. coli* BL21 cells since this strain is reported to have a lower protease background (10). Figure 1 shows a summary of the clones used in this study.

Another construct (pE5HAVP2*) was made by inserting the *Nco*I-*Nhe*I fragment (nt 2814 to 4976) that contained HAV sequences VP1*2ABC* into the vector pE5LVPO (20), which was cut with the same two restriction enzymes. This placed the T7 promoter and the encephalomyocarditis virus 5′ untranslated region and translational start site directly upstream of the HAV cDNA. Translation of the T7 transcript from this construct yields a protein with a size of approximately 80 kDa. This protein was radiolabelled with [^{35}S]Met in a reticulocyte lysate and was used as substrate for 3C protease assays as described below.

Expression and analyses of HAV proteins. Bacterial cultures were grown in M9 medium supplemented with 0.5% Casamino Acids and ampicillin and were induced with 15 μg of indoleacrylic acid per ml. After 4 h of induction, cells were collected by centrifugation, washed two times in 10 mM sodium phosphate buffer (pH 7), and disrupted by sonication. After centrifugation at 10,000 × *g* for 10 min, the supernatant and pellet were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. HAV proteins were detected with rabbit antisera against the TrpE protein, against 3D* (25), and against 3CD*. The latter antiserum was produced by using the 3CD* product of clone 2 as an immunogen. 3CD* was separated by SDS-PAGE, electrotransferred to Westran membrane (Schleicher & Schuell), and eluted from the membrane (23) prior to injection of rabbits as described previously (13). 3C-mediated cleavage products were identified by comparison with proteins from a matching set of plasmids containing mutated 3C.

To characterize cleavage sites, N-terminal amino acid sequence analyses of some polypeptides were performed. Proteins in bacterial extracts were separated by SDS-PAGE, transferred to Westran membrane, and stained with amido black. The appropriate membrane segments were cut out and subjected to N-terminal sequence analysis (12) in an ABI 477A pulsed liquid sequencer.

***trans* cleavage assays.** Extracts of *E. coli* containing HAV proteins were treated with 100 μg of RNase A per ml for 15 min at 37 or 30°C. The substrate was [^{35}S]Met-labelled VP1*2ABC*, produced by in vitro translation of transcripts of pE5HAVP2* in rabbit reticulocyte lysates (Promega). Samples were analyzed by SDS-PAGE and autoradiography.

RESULTS

Cleavage between 2C and 3A. Bacteria harboring the plasmid which encoded the HAV protein sequence 2C*3ABCD* (Fig. 1, plasmid 1) expressed several polypeptides not detected in bacteria containing the pATH vector alone (Fig. 2A, lanes V and 1). The largest of these was approximately 97 kDa, which was the size predicted for the complete fusion protein TrpE 2C*3ABCD*. Plasmids engineered to contain a mutated 3C sequence, previously shown to inactivate 3C-mediated proteolysis (13), accumulated large amounts of the 97-kDa fusion protein (Fig. 2A, lane 1-μ3C), suggesting that the fusion product containing wt 3C sequences underwent cleavage. The 97-kDa intact fusion protein was readily visualized by immunoblot with anti-TrpE serum (Fig. 2B) and anti-3D* serum (Fig. 2C). Another HAV-specific polypeptide with a size of approximately 53 kDa was present in extracts containing wt but not mutant 3C sequences (Fig. 2A). This band also reacted with anti-TrpE serum (Fig. 2B, lane 1) but not with anti-3D serum (Fig. 2C, lane 1). Its mobility is consistent with that expected for Trp2C*, suggesting that cleavage occurred between 2C and 3A. The remaining 3ABCD* portion of the fusion protein that would result from this cleavage was identified in lane 1 but not in lane 1-μ3C by immunoblotting with anti-3D* serum (Fig. 2C) and by determinating the N-terminal amino acid sequence of the isolated polypeptide. The sequence was GISDD, which is the predicted N terminus of protein 3A (3). These results demonstrate fairly efficient cleavage (approximately 50%) at a Q-G bond in *E. coli*. Since the cleavage products were not detected from constructs containing mutant 3C, we conclude that cleavage between 2C and 3A was catalyzed by HAV 3C sequences.

Additional differences between the proteins derived from wt and mutant 3C constructs were observed for gels stained with Coomassie blue (Fig. 2A) or immunoblotted (Fig. 2B and C). Proteins with sizes of approximately 20 kDa from plasmid 1 and approximately 25 kDa from plasmid 1-μ3C were isolated and subjected to N-terminal sequence analysis. Both contained the N terminus, MEEKAT. This sequence is located at position 137 to 142 of 3C, and the proteins are apparently the products of internal initiation of translation directed by a fortuitous Shine-Dalgarno sequence in the preceding HAV RNA. Gauss-Müller et al. (8) have previously identified a protein produced in *E. coli* from the same internal initiation site. It is not known why the product from the 1-μ3C plasmid migrates as a larger protein. Plasmid 1 also produced a 29-kDa protein, with an N-terminal sequence of MMEFY. This sequence is located at amino acid residues 54 to 58 of 3C-3D and thus is also likely to represent an internal initiation product. A band of approximately 34 kDa is apparent in the 1-μ3C lane.

Other than the 3C-mediated cleavage between 2C and 3A, no other cleavages were observed in the protein produced by plasmid 1. Attempts to detect scission between 3B and 3C sequences or between 3C and 3D sequences were negative. To ensure that no nucleotide changes or rearrangements which might have altered or eliminated these putative cleavage sites had occurred, the nucleotide sequence surrounding the 3B-3C-coding junction was determined and was found to be identical to that of the parental pHAV/7 (data not shown).

Cleavage between 3B and 3C. A series of constructs in a pATH vector which contained HAV P3 region-coding sequences was prepared, starting downstream of the 2C-3A junction. Each plasmid encoded the C-terminal 30% of 3A, all of 3B and 3C, and various amounts of 3D sequences (Fig. 1, plasmids 2 to 5). Induction of these clones yielded TrpE fusion proteins of the predicted sizes, visible after the gel was stained with Coomassie blue (Fig. 3A): each clone expressed a major band with an approximate size of 70 kDa

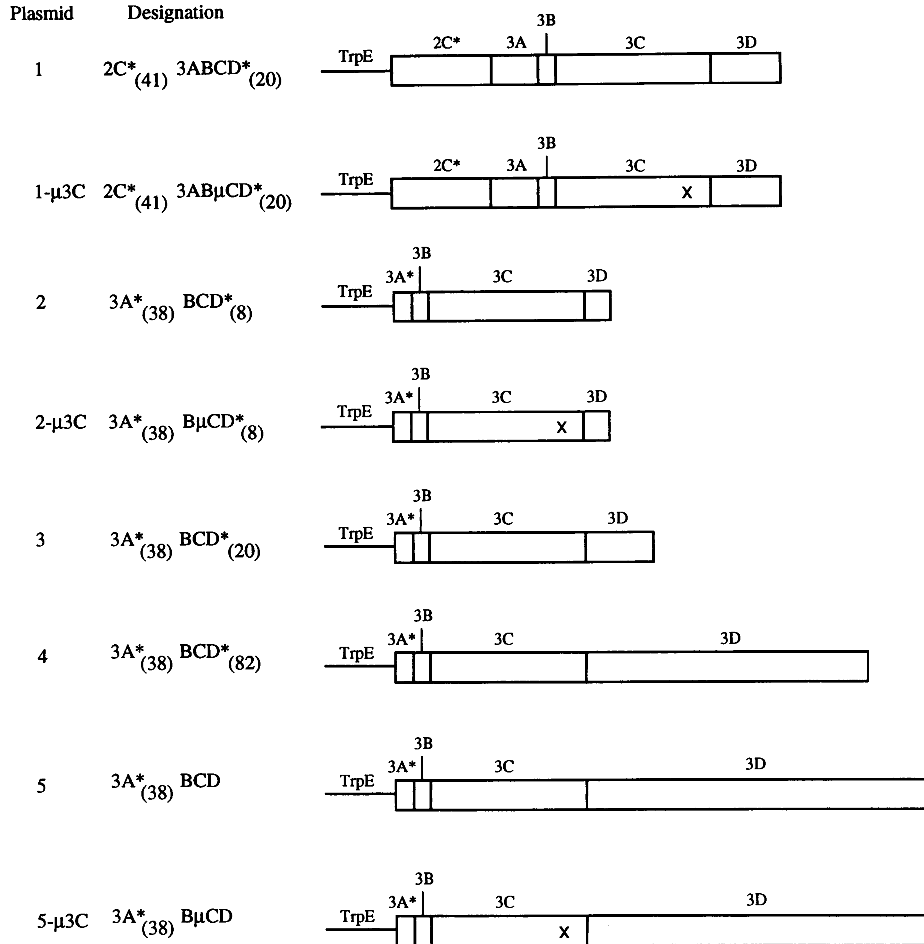


FIG. 1. Plasmids containing HAV cDNA. Each plasmid contains the indicated HAV-coding sequences fused in frame to *E. coli* TrpE-coding sequences under control of the tryptophan promoter. Plasmid 1 was constructed by using the *Bal*I fragment (nt 4578 to 6224) from pHAV/7; in plasmid 1-μ3C, a mutated 3C was inserted in place of wt sequences. The set of constructs 2 to 5 was made by using the *Pst*I site (nt 5129), and for construction of the following plasmids, the indicated sites were used: for plasmid 2, another *Pst*I site (nt 6053); for plasmid 3, an *Apa*I site (nt 6236); for plasmid 4, a *Bgl*II site (nt 7138); and for plasmid 5, an *Eco*RI site (nt 7528). Mutated 3C sequences were inserted in place of wt sequences in plasmids 2 and 5. An asterisk denotes an incomplete protein, and the numbers in parentheses indicate the approximate percentage of the coding sequence present. μ3C designates a mutated 3C, and x indicates the approximate location of the mutation.

(clone 2), 77 kDa (clone 3), 110 kDa (clone 4), or 120 kDa (clone 5). Comparisons of the proteins produced by clones 2 and 5 with those of their corresponding clones containing mutant 3C sequences showed a major protein with a size of approximately 42 kDa present in all extracts with a wt 3C sequence. This band was immunoreactive with anti-TrpE serum and had a molecular weight corresponding to that of Trp3A*B (Fig. 3B).

The extracts from each clone were examined for the presence of the remaining portions of the fusion proteins, assuming cleavage had occurred between 3B and 3C. In clone 2, which contained only 8% of the 3D sequence, there was a 28-kDa protein of the expected size of 3CD*, which was absent in clone 2-μ3C (Fig. 3A). This band did not react with anti-TrpE serum or with anti-3D* serum, which was raised against a 3D immunogen lacking the N-terminal 8% of the 3D sequence (25) (Fig. 3B and C). It was visualized, however, with anti-3CD* serum (data not shown). When the protein was isolated and subjected to N-terminal amino acid sequencing, it proved to be STLEIG, confirming the identification of 3CD* and demonstrating the cleavage between 3B and 3C at the Q-S bond predicted by Cohen et al. (3). Clone 3, containing 20% of 3D sequences, produced a corresponding 3CD* with a size of approximately 35 kDa, which appeared as the bottom of a doublet, just underlying a bacterial protein (Fig. 3A, lane 3, marked by an x). This protein also failed to react with our anti-3D* serum (Fig. 3C) but did react with anti-3CD* serum (data not shown). We were unable to isolate this polypeptide for N-terminal amino acid sequence analysis. Identification of possible 3CD* proteins in clones 4 and 5 was complicated by large amounts of fusion protein degradation, apparently induced by the increasing amounts of the 3D sequence present in these proteins. The degradation products are visualized with anti-TrpE serum (Fig. 3B) and with anti-3D* serum (Fig. 3C). Attempts to reduce the degradation of the chimeric protein by introducing the plasmids into *E. coli* BL21, which is deficient in an outer membrane protease (10), failed to alter the degradation pattern.

No significant cleavage between 3C and 3D was observed

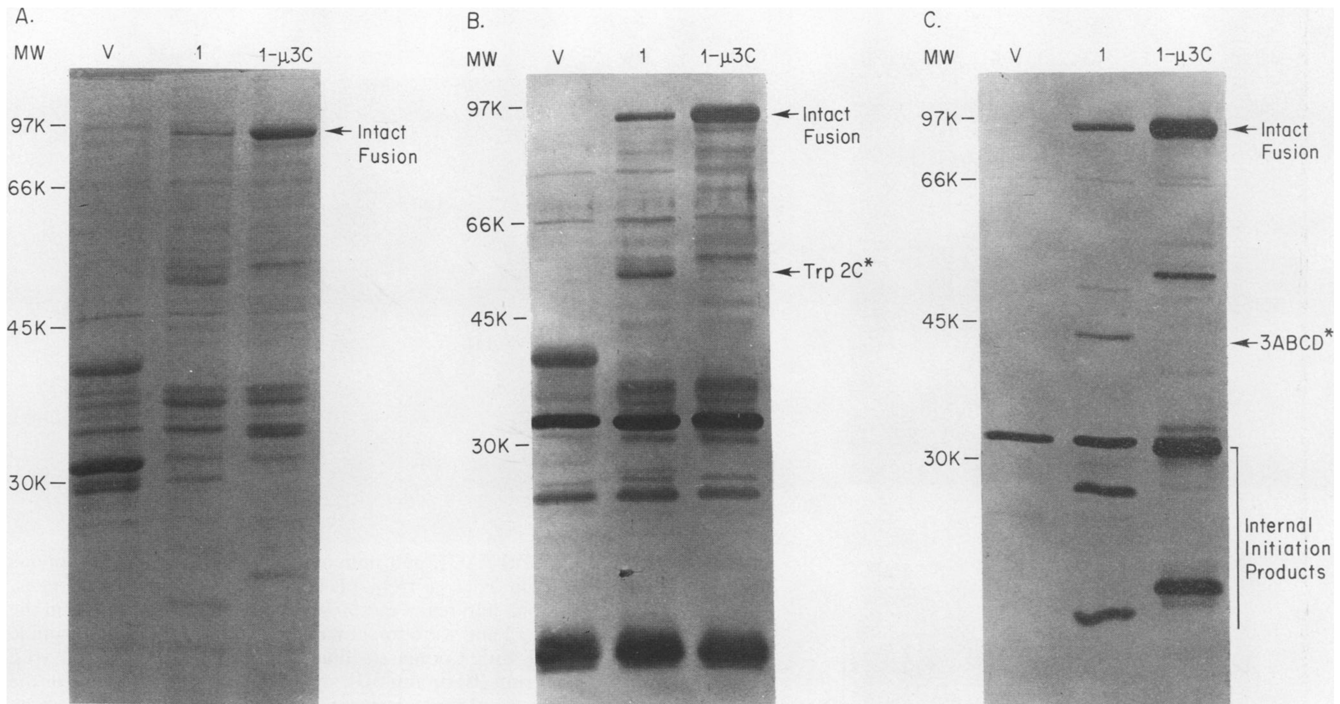


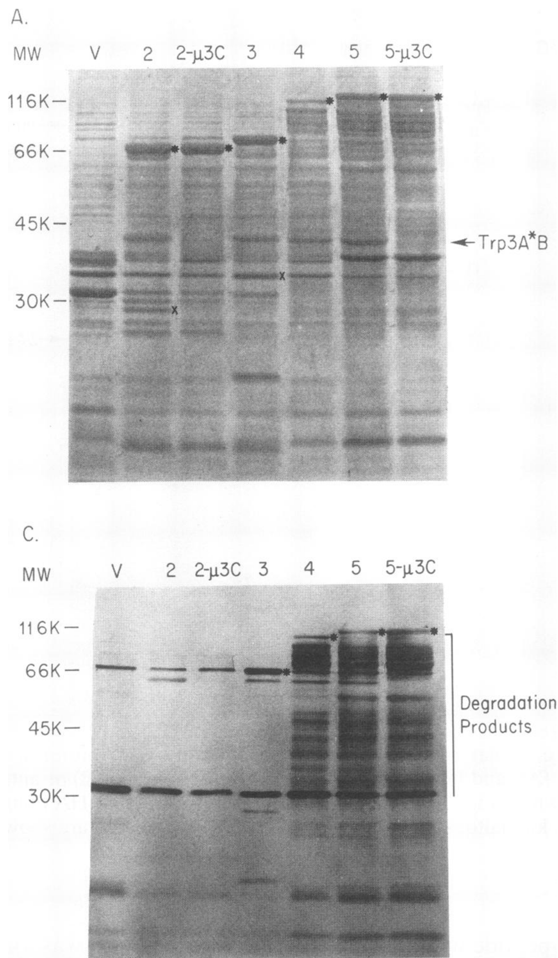
FIG. 2. SDS-PAGE and immunoblot analysis of HAV proteins synthesized in *E. coli* from plasmids containing P2 and P3 sequences. Induced cultures of *E. coli* were sonicated and centrifuged at 10,000 × *g*. Pellet fractions, enriched for recombinant proteins, were fractionated on SDS–10% polyacrylamide gels, stained with Coomassie blue (A), and immunoblotted with anti-TrpE serum (B) or anti-3D* serum (C). The designations of the lanes refer to plasmid designations shown in Fig. 1. V represents vector only, with no HAV inserts. Putative identifications of HAV proteins are indicated on the right. Sizes (in kilodaltons) of protein molecular size standards are shown on the left.

for any of these clones; thus, the scissile band generating the putative 3D protein could not be determined. An additional protein with a size of approximately 60 kDa was produced by bacteria containing wt 3C sequences (Fig. 3A and C) but was not characterized. Clone 3 produced proteins with sizes of approximately 27 and 20 kDa which reacted with anti-3D* serum (Fig. 3C, lane 3), but these proteins were not identified.

***trans* cleavage by 3C.** Complete processing of the HAV polyprotein likely requires cleavages in *trans* as well as in *cis*. For example, other picornaviruses release their capsid protein precursors from the polyprotein during translation and then generate the capsid proteins in an intermolecular cleavage reaction catalyzed by 3C. To determine whether HAV 3C sequences produced in *E. coli* by these recombinant plasmids were able to cleave HAV proteins in *trans*, a radiolabelled potential substrate containing HAV sequences VP1*2ABC* was synthesized in a rabbit reticulocyte lysate programmed with transcripts of pE5HAVP2*. This plasmid contains the encephalomyocarditis virus 5′ noncoding region and translational start site fused in frame to HAV sequences coding for the C-terminal 96 amino acids of VP1 and 2AB and all but the C-terminal 6 amino acids of 2C. An upstream T7 promoter allows synthesis of the RNA transcript in vitro. Translation of these transcripts yields a protein with a size of approximately 80 kDa. After incubation of the translation mixture with buffer or with an extract of *E. coli* transformed with a control pATH vector, no change in the mobility of the 80-kDa protein was seen (Fig. 4, lanes B and V). When the translation mixture was incubated with extracts of *E. coli* containing plasmid 2 or 5, with wt 3C sequences, a new polypeptide with a size of approximately 67 kDa was formed (Fig. 4, lanes 2 and 5). This product likely represents 2ABC* or 2BC*, depending on where the processing event occurs. The residual portion of the substrate was also seen as a rapidly migrating band of 10 to 15 kDa but had migrated off the gel shown in Fig. 4. Mutation of the 3C sequences, as in plasmid 2-μ3C or 5-μ3C, eliminated the cleavage activity in the *E. coli* extract (Fig. 4).

DISCUSSION

Although the polyprotein processing patterns and cleavage sites have been characterized to various extents for members of each of the four genera of picornaviruses (14, 15, 19), the pattern of generation of HAV-specific proteins has not been determined. A comparison of nucleotide sequences among HAV strains shows a high degree of similarity to each other but only limited homology to other picornaviruses. Nevertheless, several investigators have predicted the cleavage sites and the resulting HAV protein products, on the basis of computer alignments of the deduced amino acid sequences (3, 6, 19). Direct amino acid analyses have been performed to identify the N-terminal amino acids of two capsid proteins, VP1 (9, 16) and VP2 (9). More recently, Gauss-Müller et al. (8) analyzed the protein products generated from a recombinant β-galactosidase fusion protein containing HAV 3A*BCD* sequences in *E. coli* and identified cleavages at the same Q-S bond observed in the present study. A second smaller protein, which was suggested to result from cleavage between 3C and 3D* sequences, was observed, but the scissile bond was not determined.



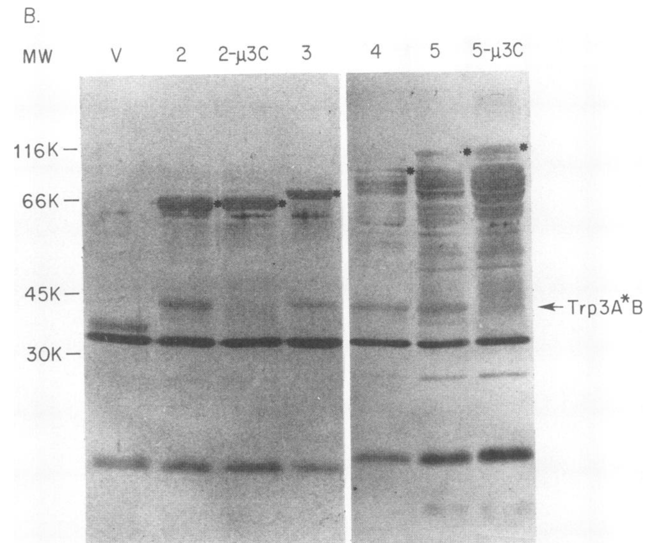


FIG. 3. SDS-PAGE and immunoblot analysis of HAV proteins synthesized in *E. coli* from plasmids containing P3 sequences. Fractions from cultures of *E. coli* were prepared as described in the legend to Fig. 2 and were fractionated on SDS–10% polyacrylamide gels, stained with Coomassie blue (A), and immunoblotted with anti-TrpE serum (B) or anti-3D* serum (C). The designations of the lanes refer to the plasmid designations shown in Fig. 1. V represents vector only, with no HAV inserts. Putative identifications of HAV proteins are indicated on the right side. Sizes (in kilodaltons) of protein molecular size standards are shown on the left. *, intact fusion protein; x, 3CD*.

To further characterize HAV polyprotein processing by the HAV 3C protease, we constructed a series of plasmids coding for TrpE fusion proteins with a portion of either 2C or 3A at the amino terminus of the HAV sequence and various amounts of 3D (8 to 100%) at the carboxy terminus. All constructs contained a complete 3C sequence; two constructs (2-μ3C and 5-μ3C) contained a mutated 3C sequence for comparative analyses. When these proteins were expressed in *E. coli*, autocatalytic cleavage of the fusion proteins occurred between 2C and 3A when this junction was present and between 3B and 3C when 2C* sequences were not present. The cleavage products were identified by their mobilities in SDS-PAGE and by immunoblotting with rabbit anti-TrpE, anti-3D*, and anti-3CD* sera. In addition, the precise cleavage sites were determined by direct N-terminal amino acid sequencing of the isolated protein products. Both cleavage sites were as predicted by Cohen et al. (3), and the 3B-3C junction was as shown previously by Gauss-Müller et al. (8). No cleavages occurred when the 3C sequence was altered at amino acid residues 151 and 154 (13). It is puzzling that bacteria expressing 2C*3ABCD* protein failed to cleave at the 3B-3C junction following cleavage at the 2C-3A junction, since cleavage at the former site occurred readily in 3A*BCD* proteins. The nucleotide sequence coding for the 3B-3C junction was shown to be unaltered in these clones. We have previously demonstrated rapid and efficient cleavage at the 2C-3A junction in proteins synthesized in rabbit reticulocyte lysates programmed with RNAs coding for 2C*3ABCD* (12). These proteins underwent further cleavage to 3ABC and D*, but this step was slow and incomplete and the protein products were unstable. Expression of these proteins in *E. coli* yielded sufficient

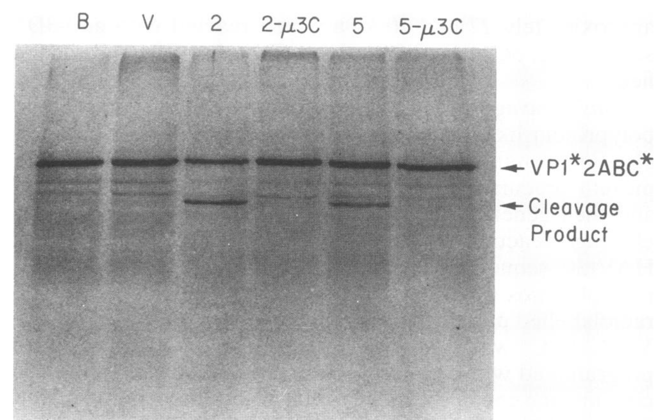


FIG. 4. *trans* cleavage of VP1*2ABC* by 3C protease. Fractions of cultures of *E. coli* were prepared as described in the legend to Fig. 2 and incubated with RNase and then with radiolabelled VP1*2ABC* in vitro translation product for 1 h at 37°C. Another aliquot of translation product was incubated with phosphate-buffered saline (B lane). The products were analyzed directly by SDS–10% PAGE. HAV proteins are designated on the right; the cleavage product probably represents 2ABC* or 2BC*, depending on where the processing event occurs. The designations of the lanes refer to the plasmid designations shown in Fig. 1. V represents vector only, with no HAV inserts.

quantities for N-terminal sequence analysis, whereas in vitro translation generally did not.

Previous attempts to demonstrate intermolecular cleavage, in *trans*, by the HAV 3C protease expressed in *E. coli* (8) or translated in rabbit reticulocyte lysates (12) failed. These studies utilized partial HAV polyprotein substrates from either the P1 or the P3 region. In the present study, we utilized a substrate from the P1-P2 region of the HAV polyprotein and were able to demonstrate relatively efficient cleavage in *trans* by wt 3C sequences in vitro. We have also observed cleavage in *trans* between VP3 and VP2 in a partial P1 substrate (data not shown). The precise amino acid pair cleaved by 3C in *trans* was not determined. The cleavage was catalyzed with similar efficiencies regardless of whether the complete 3D sequence was present in the plasmid (plasmid 5) or whether only a small portion of 3D was present (plasmid 2); thus, only 3C, and not 3CD, is required for the observed activity. The availability of clones of *E. coli* whose extracts can cleave HAV polyproteins in *trans* may permit further definitions of the HAV protein processing pattern.

To date, 3C is the only HAV protease identified. Whereas other picornaviruses manifest proteolytic scission of the polyprotein either at the N or C terminus of 2A by several different mechanisms (19), no evidence for a 2A protease cleavage or for non-3C-mediated cleavages exist for HAV. The complete pattern of HAV protein processing and the identification of the HAV nonstructural proteins present in infected cells still await further investigation.

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