JOURNAL OF VIROLOGY, May 1991, p. 2595–2600
0022-538X/91/052595-06$02.00/0

Vol. 65, No. 5

Proteolytic Activity of Hepatitis A Virus 3C Protein

XI-YU JIA,[1] ELLIE EHRENFELD,[1,2]* AND DONALD F. SUMMERS[1]

Departments of Cellular, Viral, and Molecular Biology,[1] and Biochemistry,[2] University of Utah School of Medicine, Salt Lake City, Utah 84132

Received 10 December 1990/Accepted 14 February 1991

Although the genome organization and overall structure of hepatitis A virus are similar to those of other picornaviruses, nothing is known about the protein-processing pathways used by this virus to generate its capsid and nonstructural proteins from the polyprotein precursor. RNA transcripts of cloned hepatitis A virus cDNAs representing parts of the P2 and P3 regions of the genome were translated in rabbit reticulocyte lysates in vitro, and the translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before and after immunoprecipitation with specific antisera. Pulse-chase experiments demonstrated rapid cleavage at the P2-P3 junction, followed by further but incomplete processing at the 3C-3D junction. Mutation of the 3C coding sequence eliminated all cleavages. Efforts to demonstrate intermolecular cutting of the P2-P3 cleavage site by active 3C or 3CD sequences were unsuccessful; thus, it is likely that this cleavage occurs by intramolecular reaction, in *cis*.

Hepatitis A virus (HAV) is a member of the family *Picornaviridae* and appears to represent the only member of a new genus, distinct from entero-, rhino-, aphtho-, and cardioviruses. Little nucleotide sequence homology with these other virus groups has been observed, and numerous properties of HAV growth and replication, in cultured cells as well as in primate hosts, differ markedly from those of other members of the picornavirus family.

It is generally accepted that all picornaviruses express their genetic information by synthesis of a single polyprotein that is co- and posttranslationally cleaved to generate the functional viral gene products (for a review, see reference 11). In the cases studied thus far, the protease activities responsible for polyprotein processing are encoded by the viruses themselves. Although the proteases and their cleavage sites have been well characterized for some picornaviruses (14), no such data are available for HAV.

Several factors have made it difficult to study the proteolytic processing pathways for HAV. In infected cultured cells, the replication cycle is slow and asynchronous and often produces low virus yields (1, 6, 17). In addition, host cell protein synthesis is not inhibited by virus infection so that viral proteins are not detectable by radiolabeling during infection. In vitro translation of viral RNA or transcripts of infectious cDNA in rabbit reticulocyte lysates produces a complex pattern of proteins which results predominantly from aberrant internal initiation, and these interfere with analysis of protein processing (9). Thus, although HAV polyprotein cleavage sites have been predicted by computer-assisted amino acid alignments with other picornavirus sequences (2, 3, 14), no direct experimental support has been obtained for protease activity (or activities) within the polyprotein sequence or for the positions of most of the cleavage sites.

Members of the four classified genera of the *Picornaviridae* family differ in their protein-processing reactions. All members utilize 3C (or larger 3C-containing precursors) to catalyze both *cis* and *trans* cleavages, which account for most of the processing steps. The specific amino acid residues that constitute the cleavage sites, however, are variable (11, 14). In addition to 3C-mediated reactions, other proteolytic reactions are required for cleavage of the polyprotein. The entero- and rhinoviruses encode a second, related protease, 2A, which rapidly cleaves at its own N terminus prior to completion of translation. The aphthoviruses encode only 16 amino acids of the 2A sequence, but their genomes include a leader sequence upstream of the capsid-coding region, which encodes another protease. This foot-and-mouth disease virus leader protease cleaves at its own C terminus. The cardioviruses also have leader proteins, but no proteolytic functions have been detected for these. In both of the two latter groups of picornaviruses, the bond between P1 and 2A is cleaved by 3C rather than by 2A and cleavage between 2A and 2B occurs by an unidentified mechanism. Figure 1 shows a schematic summary of the proteolytic scissions in the four different groups of picornaviruses.

No corresponding information about protein processing in HAV is available. N-terminal sequencing data for two of the viral capsid proteins (13) suggest a 3C-like cleavage activity, but no protease activities have been identified directly and no nonstructural proteins have been observed in infected cells for analysis of their sizes or sequences. This report provides direct evidence that HAV 3C is a protease and suggests that *cis* cleavage at the P2-P3 junction is a preferred cleavage site. Although the precise junctions between most HAV proteins have not been experimentally confirmed, to facilitate data presentation in this report the junctions predicted by Cohen et al. (2) were assumed.

MATERIALS AND METHODS

Plasmid constructions and mutations. The parental plasmid used to make all constructs was pT7-HAV1. Its construction has been described previously (5). It contains the complete HAV cDNA sequence juxtaposed to a T7 promoter in pGEM-2 (Promega). Transcription by T7 RNA polymerase generates plus-strand HAV RNA with five extra nucleotides at the 5′ terminus.

pTHAV/P3 and its mutated derivative PTHAV/P3-μ3C have already been described (9). Both of these plasmids contain the T7 promoter adjacent to the HAV 5′ noncoding region through the initiating ATG, fused to the HAV P3

* Corresponding author.

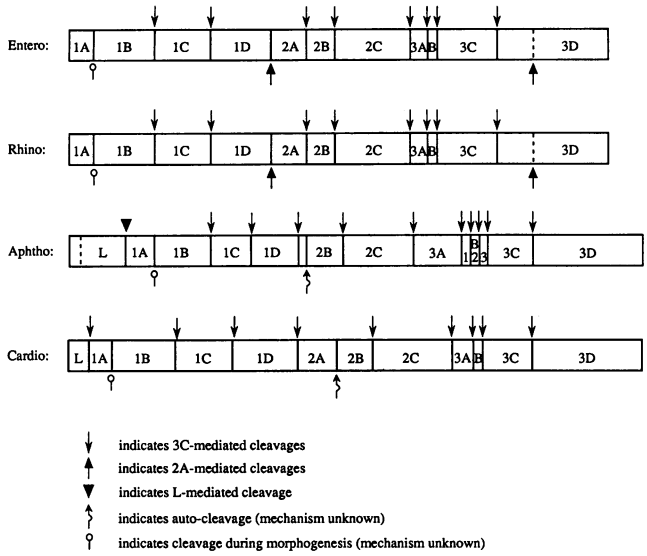


FIG. 1. Polyprotein cleavage maps of four genera of picornaviruses. References and discussions are in references 11 and 14.

region. Translation of T7 transcripts of these plasmids generates proteins containing 11 amino acids of vector sequences and 8 amino acids from the predicted C terminus of HAV protein 2C fused to the P3 protein (see Fig. 2A). The mutated plasmid, pTHAV/P3-μ3C, contains a six-nucleotide linker insertion at the *Bst*XI site (nucleotide 5862), which results in insertion of two amino acids after amino acid residue 191 of protein 3C (see Fig. 2B).

pTHAV/2C*P3 was constructed by cutting pT7-HAV1 with *Xba*I just downstream of the initiating ATG (nucleotide 735) and with *Bal*I, which cuts in the middle of the 2C gene, at nucleotide 4583. The 3′ overhangs from the *Xba*I cut were filled in with the Klenow fragment of DNA polymerase, and the two blunt ends were ligated. This generated a plasmid containing the HAV 5′ noncoding region and translational start fused to the coding sequence for the C-terminal 138 amino acids of 2C and the entire P3 protein. The mutated version of this plasmid, pTHAV/2C*P3-μ3C, was made as described above for pTHAV/P3-μ3C and generates a protein with a two-amino-acid insertion near the C terminus of 3C.

Plasmids pTHAV/P3* and pTHAV/2C*P3* are the same as their parents, pTHAV/P3 and pTHAV/2C*P3, except that sequences coding for the C-terminal three-fourths of the 3D portion of P3 are deleted. This was accomplished by cleavage of the parental plasmids with *Bgl*II, which cuts at nucleotides 6218 and 7143, followed by religation of the large fragment. This deletion caused a frameshift which generated five codons before a stop codon, thereby truncating the 3D-coding sequence.

In vitro transcription and translation. HAV RNAs were transcribed with T7 RNA polymerase (Promega Biotec) from 1 μg of linearized plasmid DNA in a 30-μl reaction, in accordance with manufacturer instructions. Nucleic acids were extracted with phenol and precipitated with ethanol, and a portion was analyzed by agarose gel electrophoresis, followed by staining with ethidium bromide. Translation of 40 to 100 ng of RNA in 10-μl reactions was performed in a rabbit reticulocyte lysate (Promega) at 30°C for 1 h. Samples (0.5 μl) were diluted with 30 μl of gel sample buffer, boiled for 3 min, and analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels.

Immunoprecipitation of translation products. Three-microliter portions of translation reactions were diluted to 40 μl with SDS-polyacrylamide gel sample buffer (10), boiled for 2 min, and then diluted 10-fold with cold IP buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40). Appropriate antisera were added (1 μl), and the samples were rotated overnight at 4°C. After addition of 25 μl of a 50% suspension of protein A-Sepharose (Pharmacia), samples were rotated for 40 min at 4°C. Protein A-antibody-antigen complexes were collected by centrifugation and washed three times with 0.5 ml of wash buffer (50 mM Tris-HCl [pH 7.4], 0.5 M NaCl, 5 mM EDTA, 5% sucrose, 1% Nonidet P-40) and once with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The complexes were finally suspended in 50 μl of gel sample buffer, boiled for 3 min, and clarified by centrifugation, and 40 μl of the supernatant was analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE).

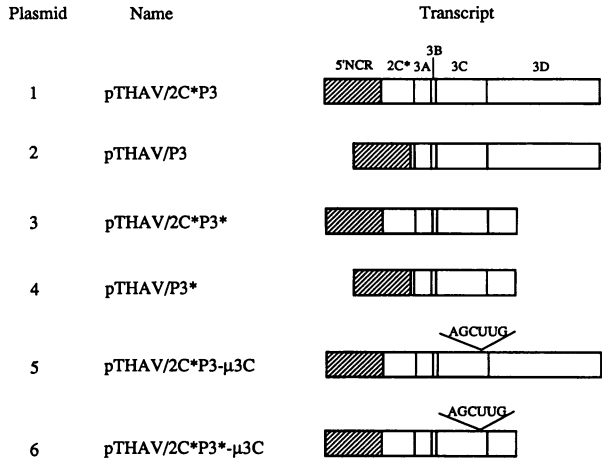
The antisera used were (i) anti-2C, collected from a rabbit immunized by M. Tesar in this laboratory, with a fusion protein containing the immunoglobulin G-binding site of Staph A protein encoded in the vector pRIT2T (Pharmacia) fused to the predicted sequences of HAV 2C; (ii) anti-2C3A, a generous gift from V. Gauss-Müller, University of Lübeck, Lübeck, Germany, which was raised in rabbits against a β-galactosidase fusion protein that contained the C-terminal 30 amino acids of HAV 2B, all of 2C, and approximately half (29 amino acids) of 3A, expressed in *Escherichia coli*; (iii) anti-3C, also received from V. Gauss-Müller, collected from a rabbit immunized with a β-galactosidase fusion protein containing the C-terminal 30 amino acids of 3A, all of 3B, 3C, and the N-terminal 26 amino acids of 3D; and (iv) anti-3D, prepared in this laboratory by W. Updike, from a rabbit immunized with the C-terminal four-fifths of HAV protein 3D expressed in *E. coli*.

RESULTS

In vitro translation of HAV RNAs. Previous work in this laboratory on the translation of full-length HAV RNA in rabbit reticulocyte lysates revealed frequent utilization of numerous internal AUG codons for initiation of protein synthesis in vitro (9). The P3 region contained most of these preferred aberrant initiation sites, and the resulting polypeptides were not substrates for 3C-mediated protein processing. To enable us to detect any putative processing by 3C-containing proteins, several plasmids whose transcripts would direct the synthesis of different 3C-containing proteins were constructed (Fig. 2A). All plasmids contain a T7 promoter juxtaposed to the complete HAV 5′ noncoding region, up to and including the AUG codon thought to represent the start of the coding sequence. This segment is indicated by the designation THAV in the plasmid name. Following this designation, the approximate predicted protein sequences encoded in the plasmid are indicated; an asterisk indicates an incomplete protein-coding sequence. For example, plasmids 1 and 3 contain cDNA coding for 138 C-terminal amino acids of 2C preceding P3 region sequences, whereas plasmids 2 and 4 contain coding sequences for only 8 amino acids of 2C. Plasmids 1 and 2 contain the entire P3 region, including all of the 3D sequences, whereas plasmids 3 and 4 encode only 13 kDa of the 3D N-terminal sequence. Plasmids 5 and 6 are identical to 1 and 3, except that they contain a 6-bp insertion near the 3′ end of the 3C coding sequence.

Each of these plasmids was transcribed in vitro, and the

A.



B.

		188									195
wt	3C	G	I	H	V			A	G	G	N
μ	3C	G	I	H	P	S	L	A	G	G	N

FIG. 2. Plasmids and transcripts of the HAV P3 region. (A) Plasmids 1 to 6 are described in Materials and Methods. Their transcripts were synthesized by T7 RNA polymerase. (B) The 6-bp insertion indicated in plasmids 5 and 6 alters the wild-type amino acid sequence (wt 3C) between predicted residues 188 and 195, as indicated by the insertion of a serine-lysine pair between predicted residues 191 and 192 (μ 3C).

resulting transcripts were used to program cell-free translation reactions in rabbit reticulocyte lysates. The translation products are shown in Fig. 3. Each lane is numbered to correspond to the plasmid number defined in Fig. 2. The profile of polypeptides translated from 2C and P3 sequences is relatively complex, as expected from our previous work that showed high levels of protein synthesis initiation from multiple internal sites in the P3 region (9). The largest proteins observed in all cases, however, correspond to the molecular weights expected from translation of the entire open reading frame in each construct without protein processing. Comparison of lanes 1 and 2 (Fig. 3) showed that the patterns were identical, except for the largest band. The slower mobility of the largest protein translated from transcripts of plasmid 1, pTHAV/2C*P3, reflects the additional 2C-coding sequences in this plasmid, compared with pTHAV/P3, in lane 2. The same difference was observed upon comparison of lanes 3 and 4, also reflecting the larger portion of 2C encoded in pTHAV/2C*P3* than in pTHAV/P3*. The largest primary translation product in lane 1 did not accumulate significantly; rather, a product of 89 kDa, which is the predicted size of P3, accumulated. This suggested that cleavage occurred at the 2C-P3 junction. Consistent with this hypothesis was the appearance in lane 1 of an ~16-kDa polypeptide, which is the expected size of the 2C* fragment that would be released from 2C*P3 following the postulated cleavage. An 89-kDa protein was also found after translation of pTHAV/P3 transcripts (lane 2), moving just slightly faster than the primary translation product from this transcript. The largest product from this translation would contain ~2 kDa of sequences from 2C and the vector at the N terminus of the P3 sequences. If these were cleaved from the adjacent P3 sequences, the ~2-kDa peptide (8 amino acids from 2C plus 11 amino acids from vector sequences) predicted to be released in lane 2 would not be detectable on the 10% polyacrylamide gel. As expected, no 16-kDa 2C* fragment appeared in lane 2. The same results were seen upon comparison of lanes 3 and 4. Transcripts from both of these truncated 3D plasmids produced proteins of 48 kDa, the predicted size of P3*, whereas only the transcript encoding 138 amino acids of 2C* generated the 16-kDa band (lane 3).

The above analyses of the translation products of transcripts from plasmids containing HAV cDNA coding for P3 sequences with variable lengths of 3D or flanking 2C protein (Fig. 2, plasmids 1 to 4) were most readily interpreted as indicating cleavage between 2C and 3A. If this were the case, and if the cleavage were mediated by 3C sequences, as occurs with other picornaviruses, then inactivation of 3C by mutagenesis should increase accumulation of the largest, primary translation products and simultaneously prevent the appearance of 2C* and P3 or P3* among the translation products of transcripts of the mutated plasmids. 3C was inactivated by an in-frame linker insertion which encoded two amino acids adjacent to a histidine residue near the C terminus of HAV 3C (Fig. 2B). This His residue corresponds, by amino acid sequence alignment (14), to a His residue in poliovirus 3C which has been shown previously to be essential for protease activity (7). Figure 3, lanes 5 and 6, shows the results of translations of transcripts from plasmids pTHAV/2C*P3-μ3C and pTHAV/2C*P3*-μ3C (Fig. 2, plasmids 5 and 6), carrying the linker insertions. Comparison of lanes 5 and 1 shows, as predicted, increased accumulation of the largest, intact translation product and the absence of bands corresponding to cleaved 2C* and P3. Similarly, comparison of lane 6 with lane 3 shows increased accumulation of the largest product and no production of cleaved P3*. Another mutant 3C gene constructed in this laboratory, which resulted in His-to-Gly and Gly-to-Arg substitutions at predicted 3C amino acid residues 151 and 155, also elimi-

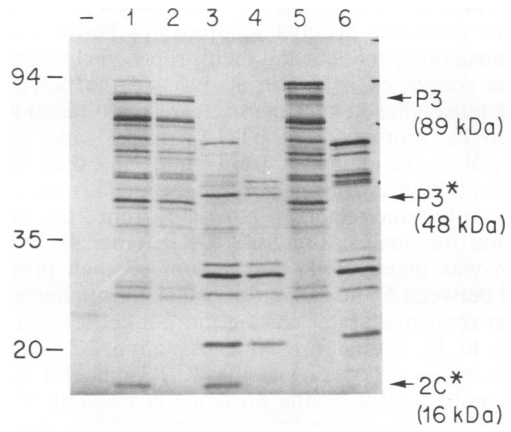


FIG. 3. Translation products of HAV P3 RNAs. Transcripts shown in Fig. 2 were translated in rabbit reticulocyte lysate, and the products were analyzed by SDS–10% PAGE. Each lane is numbered to correspond to the plasmid number defined in Fig. 2. The minus-labeled lane shows lysate with no added RNA. The molecular masses (kilodaltons) of brome mosaic virus proteins translated from brome mosaic virus RNA (Promega) in the same lysate are indicated on the left. Putative HAV proteins and their predicted molecular masses are indicated on the right.

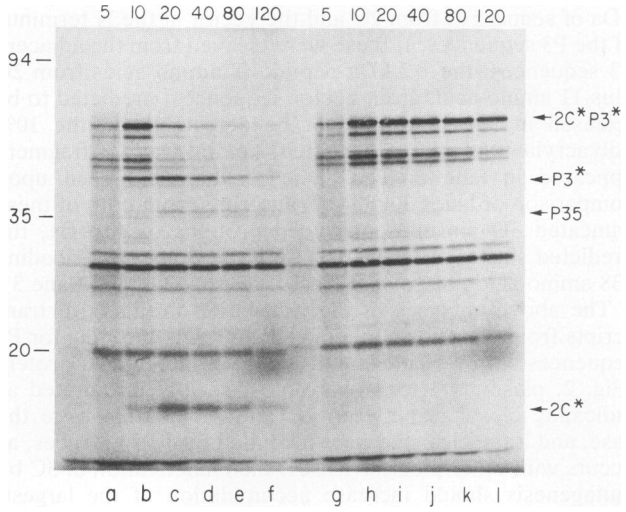


FIG. 4. Pulse-chase analysis of proteins translated from HAV RNAs. Transcripts of pTHAV/2C*P3* (lanes a to f) and pTHAV/2C*P3*-μ3C (lanes g to l) were incubated in translation reactions. After 12 min of incubation at 30°C, 12.5 μM edeine was added to inhibit further initiation. At the time indicated at the top of each lane, a 2-μl sample was diluted with SDS-PAGE sample buffer for analysis on an SDS–10% polyacrylamide gel. The molecular masses (kilodaltons) of brome mosaic virus proteins translated from brome mosaic virus RNA in the same lysate are indicated on the left. Putative HAV proteins are indicated on the right.

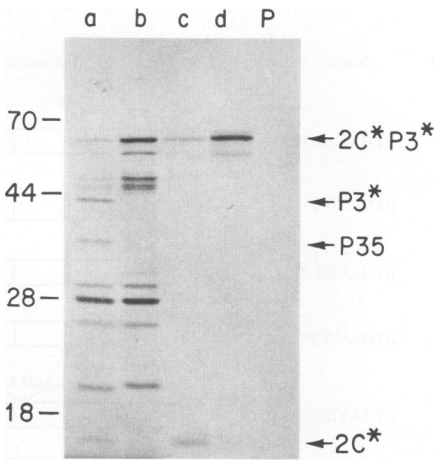


FIG. 5. Immunoprecipitation of translation products with anti-2C serum. Transcripts of pTHAV/2C*P3* (lanes a and c) and pTHAV/2C*P3*-μ3C (lanes b and d) were translated in rabbit reticulocyte lysate, and the products were analyzed before (lanes a and b) and after (lanes c and d) immunoprecipitation with anti-2C serum. Preimmune serum (P) was used to immunoprecipitate products from pTHAV/2C*P3* transcripts. The positions of prestained molecular mass markers (kilodaltons) are indicated on the left. HAV proteins are designated on the right.

nated the cleavage activity of the P3 translation products (4). The protease activity responsible for the cleavage between 2C and P3 sequences is dependent upon 3C sequences but independent of the length of the adjacent 3D sequences or the amount of the 2C sequence upstream of the cleavage site. No differences in the pattern of the numerous products smaller than P3 or P3*, which were aberrantly initiated at internal sites downstream of the 2C-P3 junction, were evident whether or not 3C was inactivated.

Pulse-chase analysis of HAV P3 translation products. Evidence for precursor-product relationships between the primary translation products and their proposed cleavage products was sought by a kinetic analysis of the polypeptides during a pulse-chase experiment. Translation reactions programmed by transcripts of pTHAV/2C*P3* and pTHAV/2C*P3*-μ3C were incubated for 12 min, and then 12.5 μM edeine, an inhibitor of translation initiation (12), was added. Samples were removed at various times from 5 to 120 min of incubation for analysis by SDS-PAGE (Fig. 4). When 3C function was inactivated, the amount of each protein increased between 5 and 10 min of translation (lanes g and h) and then remained stable during the 2-h chase with edeine (lanes g to l). In the presence of active 3C sequences, however, some processing at the 2C-P3 junction occurred rapidly, as is evident by the presence of P3* and 2C* by 10 min of translation; during the subsequent chase, all proteins larger than P3* decreased and the cleaved products P3* and 2C*, as well as a small amount of an additional band, labeled P35, appeared (lanes a to f). By 20 min, the amount of P3* protein exceeded the amount of 2C*P3*. After 30 to 40 min, both the P3* and 2C* products decreased. The reason for this instability is not known. The band labeled P35 seemed to appear slightly later than P3* and 2C* and was also dependent upon active 3C. It was considered likely to be a secondary cleavage product from P3*, either 3CD* (M_r, ~37,000) or 3ABC (M_r, ~35,000) (see below).

Similar pulse-chase experiments were performed during translation of pTHAV/P3 and pTHAV/P3* transcripts. The results were similar to those shown in Fig. 4, except that no detectable 2C* was formed (data not shown).

Immunoprecipitation of P3 translation products. To confirm the identities of the various translation products, reaction mixtures were subjected to immunoprecipitation with several different anti-HAV protein sera. Figure 5 shows the proteins produced from translation of pTHAV/2C*P3* and the mutant pTHAV/2C*P3*-μ3C transcripts, immunoprecipitated with anti-2C serum (lanes c and d, respectively). As expected, only the largest primary translation products (2C*P3*) or other proteins larger than P3* immunoprecipitated, as well as 2C*. As seen before in Fig. 3, when 3C was inactivated, no 2C*, P3*, or cleavage product P35 was formed (compare lanes a and b) and precursor proteins larger than P3* accumulated (lane b). The smaller internal initiation products do not contain 2C* sequences. The smallest of these (~19 kDa) shows slightly reduced mobility in products from the transcript of the mutant 3C plasmid, likely because of the insertion of two extra amino acids from the mutation.

Other antisera were used for immunoprecipitation of translation products of pTHAV/2C*P3* and pTHAV/2C*P3 transcripts. The results are summarized in Table 1. The data confirm the identities of the largest translation products, as well as the products resulting from cleavage at the 2C-P3 junction. Products of the truncated 3D constructs (pTHAV/2C*P3*), as well as those containing the complete 3D sequence (pTHAV/2C*P3), were immunoprecipitated with anti-3D serum. Anti-2C3A serum precipitated both the primary translation products and their cleavage products, including those not detected by anti-2C antibodies alone. The additional anti-3A antibodies in this serum were therefore responsible for its reactivity with P3 and P3*. Most importantly, this panel of antisera allowed us to identify the product called P35 as 3ABC, since it was immunoprecipi-

TABLE 1. Immunoprecipitation of HAV RNA translation products

Protein	Immunoprecipitation with:			
	α2C	α2C3A	α3C	α3D
pTHAV/2C*P3 products				
2C*P3	+	+	+	+
P3	−	+	+	+
P35	−	+	+	−
2C	+	+	−	−
pTHAV/2C*P3* products				
2C*P3*	+	+	ND[a]	+
P3*	−	+	ND	+
P35	−	+	+	−
2C*	+	+	−	−

[a] ND, Not done.

tated with anti-2C3A and anti-3C but not with anti-2C or anti-3D sera. Therefore, subsequent to the cleavage at the 2C-P3 junction, some slower scission occurred at the 3C-3D junction; this reaction, however, was relatively inefficient in vitro.

***cis* versus *trans* cleavage at the 2C-P3 junction.** During picornavirus polyprotein processing, 3C-mediated cleavages at different sites occur either as autocatalytic reactions, in *cis*, or as bimolecular reactions, in *trans*. These reactions are catalyzed by 3C, 3CD, or possibly other, larger 3C-containing precursors (15). To see whether the observed cleavage at the 2C-P3 junction could occur in *trans*, we used the mutant 3C-containing translation products, which were unable to cleave themselves, as a substrate for potential *trans* cleavage by active 3C-containing products. A translation reaction programmed by transcripts of pTHAV/2C*P3*-μ3C in the presence of [^{35}S]methionine was mixed with an unlabeled translation reaction programmed by pTHAV/2C*P3*, and the mixture was incubated for 3 h at 30°C prior to analysis of the products by SDS-PAGE. Figure 6, lane a, shows radiolabeled translation products containing active 3C sequences prepared in parallel with the unlabeled material used as the source of a potential *trans*-acting enzyme. Lane b shows the translation products containing mutant 3C sequences used as the substrate for the *trans* cleavage test. Incubation of the two extracts together produced no change in the substrate pattern and no evidence of production of cleavage product 2C*, P3*, or P35 (lane c). Control incubation of the substrate with reticulocyte lysate programmed with no mRNA also showed no change in the product profile (lane d). In other experiments, the cleavage between 2C* and P3* was observed to be independent of the concentration of the precursor, suggesting a unimolecular rather than a bimolecular reaction (data not shown).

The experiment shown in Fig. 6 tested the ability of 3ABCD* (P3*) or, possibly, low levels of 3ABC (P35) to cleave the 2C-P3 junction in *trans*. These sequences were sufficient to cleave in *cis* at this site. In the event that an intact 3D sequence might be required to form the active protease site for *trans* cleavage, an experiment similar to that whose results are shown in Fig. 6 was performed, in which unlabeled translation products of pTHAV/2C*P3 transcripts, which contain the complete P3-coding sequence, were incubated with labeled translation products from transcripts of pTHAV/2C*P3*-μ3C. Again no cleavage was detected (data not shown). Thus, unless the mutation in 3C alters the protein conformation so as to prevent its ability to serve as a substrate, all of the data indicate that the cleavage occurring at the 2C-P3 junction is likely an intramolecular reaction.

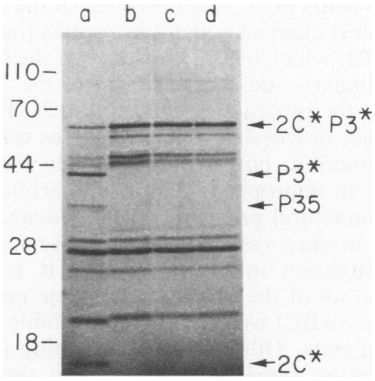


FIG. 6. *trans* cleavage assay of HAV 3C protease. Transcripts of pTHAV/2C*P3* and pTHAV/2C*P3*-μ3C were translated for 30 min in rabbit reticulocyte lysates, and their products were analyzed directly by SDS–10% PAGE (lanes a and b, respectively). Another translation reaction of pTHAV/2C*P3 transcripts was incubated in parallel in the absence of [^{35}S]methionine to produce unlabeled 3C-containing proteins. After 30 min, 3 μl of ^{35}S-labeled proteins from pTHAV/2C*P3*-μ3C transcripts was mixed with 6 μl of unlabeled products from pTHAV/2C*P3* transcripts, and the mixture was incubated at 30°C for 3 h in the presence of 1 mM cycloheximide to stop further translation. Samples of the incubated mixture were processed for SDS-PAGE analysis (lane c). As a control, the ^{35}S-labeled substrate was also incubated for 3 h with lysate without added RNA and processed similarly (lane d). The positions of prestained molecular mass markers (kilodaltons) are indicated on the left; HAV proteins are designated on the right.

DISCUSSION

The studies described in this report demonstrate an intrinsic proteolytic activity for HAV 3C sequences similar to that found in other picornavirus 3C proteins. Protease activity was demonstrated by cell-free translation of subgenomic transcripts of HAV cDNA from the P3 region and confirmed by the failure to obtain cleavage after introduction of mutations in the 3C coding region. We constructed plasmids whose transcripts produced different-sized protein cleavage products and identified the proteins by immunoprecipitation with specific antisera. The results showed that the first cleavage of the 2C*P3 protein occurs at the junction between 2C and P3, and this cleavage is most likely catalyzed by *cis* action. Although the order and rate of P3 autocatalytic cleavages in other picornavirus proteins have not been precisely determined, the observation of an intact P2-P3 protein is always rare, indicating that 2C-3A bonds are cleaved rapidly after synthesis (8, 16). From the kinetics of translation and processing of encephalomyocarditis virus RNA in vitro, it appears that cleavage at the P2-P3 junction occurs exclusively in *cis* (8). In the case of the poliovirus polyprotein, while cleavage must be able to occur in *cis*, processing in *trans* has also been observed (18). Although we have not demonstrated that the observed cleavage between 2C and P3 is between the precise predicted amino acid pair, the observation of rapid cleavage at this junction in HAV protein is consistent with the processing scheme seen in other picornaviruses.

The 2C sequences required for cleavage at the 2C-P3 junction are minimal. The pTHAV/P3 construct codes for

only 8 amino acids of 2C just upstream of the cleavage site, yet its protein is cleaved equally as well as that encoded by pTHAV/2C*P3, which has 138 residues of the C-terminal 2C sequence. Similarly, deletion of most of the 3D sequences did not affect the protease activity at the 2C-P3 junction.

Some further processing of P3 sequences was observed at the 3C-3D junction, but it was relatively inefficient. This could be due to improper folding of the artificial substrate, resulting in unnatural presentation of cleavage sites. Alternatively, the in vitro system used for these studies may not mimic the situation in infected cells. It is noteworthy, however, that all of the observed cleavage products (P3 or P3*, 2C*, and 3ABC) were relatively unstable during pulse-chase experiments. Other proteins resulting from aberrant internal initiation remained stable, even throughout prolonged incubations. When mutations were introduced into 3C so as to inactivate its cleavage activity, all translation products were stable. Thus, it appears that the properly cleaved HAV proteins expressed from the P3 region were inherently unstable in the reticulocyte lysate.

Information about HAV protein synthesis and processing is needed to understand the differences between the biological properties of this virus and other members of the picornavirus family. In vitro translation of the full-length HAV RNA was relatively uninformative about processing activities and cleavage sites, predominantly because of the high frequency of utilization of aberrant internal sites for initiation of translation in reticulocyte lysates (9). In the present study, translation of subgenomic RNAs allowed us to detect 3C-mediated cleavage events. Additional approaches may be required to elucidate the complete processing pathway of HAV structural and nonstructural proteins.

ACKNOWLEDGMENTS

We thank Verena Gauss-Müller for sharing anti-2C3A and anti-3C sera with us and Michael Tesar and Wanda Updike in this laboratory for providing anti-2C and anti-3D sera.

This work was supported by the U.S. Army Medical Research and Development Command under contract DAMD17-88-C-8122 and by Public Health Service grant AI26350 from the National Institutes of Health.

ADDENDUM IN PROOF

While this work was in progress, Gauss-Müller et al. expressed HAV 3C in *E. coli* as part of a β-galactosidase fusion protein, with short flanking sequences from 3B and 3D (V. Gauss-Müller, D. Jürgensen, and R. Deutzmann, Virology, in press). Autocatalytic cleavages mediated by 3C sequences at the 3B-3C and likely the 3C-3D junctions were observed. These data also demonstrate proteolytic activity of HAV 3C.

REFERENCES

1. **Cho, M., and E. Ehrenfeld.** 1991. Rapid completion of the replication cycle of hepatitis A virus subsequent to reversal of guanidine inhibition. Virology **180**:770–780.
2. **Cohen, J. I., J. R. Ticehurst, R. H. Purcell, A. Buckler-White, and B. M. Baroudy.** 1987. Complete nucleotide sequences of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. J. Virol. **61**:50–59.
3. **Diamond, D. C., E. Wimmer, K. von der Helm, and F. Deinhardt.** 1986. The genomic map of hepatitis A virus: an alternate analysis. Microb. Pathog. **1**:217–219.
4. **Harmon, S. A., and X.-Y. Jia.** Unpublished data.
5. **Harmon, S. A., O. C. Richards, D. F. Summers, and E. Ehrenfeld.** 1991. The 5′-terminal nucleotides of hepatitis A virus RNA, but not poliovirus RNA, are required for infectivity. J. Virol. **65**:2757–2760.
6. **Harmon, S. A., D. F. Summers, and E. Ehrenfeld.** 1989. Detection of hepatitis A virus RNA and capsid antigen in individual cells. Virus Res. **12**:361–370.
7. **Ivanoff, L. A., T. Towatari, J. Ray, B. D. Koront, and S. R. Pettaway, Jr.** 1986. Expression and site-specific mutagenesis of the poliovirus 3C in E. coli. Proc. Natl. Acad. Sci. USA **83**:5392–5396.
8. **Jackson, R. J.** 1986. A detailed kinetic analysis of the in vitro synthesis and processing of encephalomyocarditis virus products. Virology **149**:114–127.
9. **Jia, X., G. Scheper, D. Brown, W. Updike, S. Harmon, O. Richards, D. Summers, and E. Ehrenfeld.** Translation of hepatitis A virus RNA in vitro: evidence for aberrant initation of translation. Virology, in press.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lawson, M. A., and B. L. Semler.** 1990. Picornavirus processing—enzymes, substrates and genetic regulation. Curr. Top. Microbiol. Immunol. **161**:49–87.
12. **Legon, S.** 1976. Characterization of the ribosome-protected region of ^{125}I-labeled rabbit globin messenger RNA. J. Mol. Biol. **106**:37–53.
13. **Linemeyer, D. L., J. G. Menke, A. Martin-Gallardo, J. V. Hughes, A. Young, and S. W. Mitra.** 1985. Molecular cloning and partial sequencing of hepatitis A viral cDNA. J. Virol. **54**:247–255.
14. **Palmenberg, A. C.** 1990. Proteolytic processing of picornaviral polyprotein. Annu. Rev. Microbiol. **44**:603–624.
15. **Parks, G. D., J. C. Baker, and A. C. Palmenberg.** 1989. Proteolytic cleavage of encephalomyocarditis virus capsid region substrates by precursors to the 3C enzyme. J. Virol. **63**:1054–1058.
16. **Shih, D. S., C. T. Shih, O. Zimmern, R. R. Rueckert, and P. Kaesberg.** 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. **30**:472–480.
17. **Ticehurst, J., J. I. Cohen, S. M. Feinstone, R. H. Purcell, R. W. Jansen, and S. M. Lemon.** 1989. Replication of hepatitis A virus: new ideas from studies with cloned cDNA, p. 27–50. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
18. **Ypma-Wong, M. F., and B. L. Semler.** 1987. In vitro molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase. Nucleic Acids Res. **15**:2069–2088.