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Nonstructural Protein 3 of the Hepatitis C Virus Encodes a Serine-Type Proteinase Required for Cleavage at the NS3/4 and NS4/5 Junctions

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We have studied processing of the nonstructural (NS) polyprotein of the hepatitis C virus. A series of cDNAs corresponding to predicted NS2/3/4 or NS3/4 regions were constructed, and processing of the polyproteins was studied in an in vitro transcription-translation system. We report that a catalytically active serine-type proteinase is encoded by the NS3 region. Substitution of the serine residue of the putative catalytic triad (H, D, and S) by alanine blocked cleavage at the NS3/4 junction, while processing between NS2 and NS3 was not affected. Thus, cleavage at the NS2/3 junction is mediated either by cellular enzymes or by an NS-2 inherent proteinase activity. Deletion analysis of an NS3/4 cDNA construct mapped the amino terminus of the enzymatically active proteinase between amino acids 1049 and 1065 of the polyprotein. As internal deletions of variable segments of the presumed helicase domain prevented processing at the NS314 junction, a continuous NS3 region appears to be required for processing at this site. To analyze hepatitis C virus polyprotein cleavage in vivo, recombinant vaccinia viruses expressing NS2/3/4 or NS3/4/5 proteins were generated. In agreement with the in vitro data, cleavage between NS2 and NS3 was independent of a catalytically active NS3 proteinase, whereas substitution of the active-site serine residue by the amino acid alanine completely blocked processing at the NS3/4 and NS4/5 junctions. These results demonstrate that NS3 encodes the viral proteinase essential for generating the amino termini of NS4 and NS5.

Hepatitis C virus (HCV) is the major etiologic agent of posttransfusion and sporadic, community-acquired non-A, non-B hepatitis (for a recent review, see reference 16). Characteristically, HCV causes a prolonged, persistent infection which eventually leads to cirrhosis or hepatocellular carcinoma, as indicated by the high prevalence of anti-HCV antibodies found in hepatitis B antigen-negative patients with hepatocellular carcinoma (8). By using recombinant DNA techniques, several different isolates of HCV have recently been cloned (7, 9, 17, 18, 21, 29). As deduced from sequence analysis, the HCV genome consists of a single positive-strand RNA, approximately 9,400 nucleotides in length, encoding a single viral polyprotein of about 3,010 amino acids. On the basis of limited nucleotide and amino acid sequence homologies, as well as similarities in the hydrophobicity profiles, HCV appears to be related to flavi- and pestiviruses (10, 19, 29).

The genomic organization of flavi- and pestiviruses has been determined (for recent reviews, see references 6 and 11). For flaviviruses, it is (from the 5′ to the 3′ end) nucleocapsid-premembrane-envelope-nonstructural region 1 (NS1)-NS2A/2B-NS3-NS4A/4B-NS5. This polyprotein is processed by the combined action of cellular proteinases and at least one virus-encoded serine proteinase (4, 5, 23, 31, 33, 34). While processing of the structural proteins is mediated by host cell signalases and, in the case of pestiviruses, in addition by a capsid-encoded serine proteinase (30, 32), nonstructural proteins are processed at most cleavage sites by a virus-encoded proteinase. The responsible enzyme has been located in the amino terminal region of NS3, designated p80 for pestiviruses. By analogy to these viruses, it is assumed that polyprotein processing of HCV is also dependent on both cellular and viral proteinases. In agreement with this view, Hijikata et al. (15) recently identified signalase cleavage sites in the structural region of the HCV genome required to generate the nucleocapsid protein and the envelope 1 protein. However, the mechanism underlying the processing of the nonstructural proteins remained unresolved.

To gain insight into HCV NS-protein cleavage, we studied the processing of various polyproteins corresponding to the presumed NS2/3/4 and part of the NS5 region. Using in vitro transcription-translation and recombinant vaccinia viruses, we show that NS3 encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions.

MATERIALS AND METHODS

Molecular cloning of the HCV genome. HCV cDNAs corresponding to positions 47 to 9363 of HCV J (18) were isolated from a chronic HCV carrier by nested RNA polymerase chain reaction (PCR) using oligonucleotides derived from sequence alignments of known HCV isolates. In brief, total nucleic acids were extracted from 350 μl of serum and used for cDNA synthesis with reverse transcriptase from Moloney murine leukemia virus (Superscript; BRL Life Technologies Inc., Gaithersburg, Md.) according to the instructions of the manufacturer. Following heat inactivation of the enzyme, the total reaction mixture was used for PCR (35 cycles) under standard conditions. One-tenth of the PCR mixture was used for a second PCR with a different antisense primer. HCV-specific PCR products were purified, phosphorylated, and inserted into the *Sma*I-linearized, dephosphorylated vector pBSK− (Stratagene, Zürich, Switzerland). For unknown reasons, in most cases inserted HCV sequences were oriented such that the 5′ end of the HCV plus strand was linked to the T7 promoter. Details of cloning and sequence analysis will be presented elsewhere (1a). (Since we have not yet determined the exact 5′ end of our isolate,

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which shows high homology to the HCV J isolate of Kato et al. [18], we adopted their numbering system, with the A residue of the ATG initiation codon of the polyprotein at nucleotide position 330.)

Combination of individual HCV genome fragments. Most recombinant plasmids were constructed by using standard methods (25). Five overlapping DNA fragments, spanning HCV nucleotide positions 2518 to 3259, 3234 to 4045, 3716 to 4908, 4530 to 6062, and 5650 to 7033, were combined to generate a continuous genome segment spanning the putative NS2/3/4 and part of the NS5 region (2518 to 7033) as follows. Fragments 2518–3259 and 3234–4045 were combined by PCR according to the method of No et al. (20) by using the exact homology of the HCV sequence at the overlapping region and T3 and T7 outside primers. The combined fragment (positions 2518 to 4045), containing sequences of the multiple cloning site (MCS) of pBSK− at its ends, was inserted into the same vector by using the *Eco*RI and *Spe*I restriction sites. Combination with fragment 3716–4908 was achieved by using a *Dra*III restriction site at position 3857 in the HCV genome and the *Spe*I restriction site in the MCS of pBSK−. To further extend HCV sequences, construct pBSK 4530–6062 was cut with *Aat*II (HCV position 4624) and *Spe*I (MCS) and inserted into the *Aat*II-*Spe*I-restricted plasmid pBSK 2518–4908, generating construct pBSK 2518–6062. To obtain plasmid pBSK 2518–7033, construct pBSK 5650–7033 was cut with *Hpa*I (HCV position 5969) and *Spe*I (MCS) and inserted into the *Hpa*I-*Spe*I-restricted construct pBSK 2518–6062.

Construction of T7 transcription plasmids. To generate expression plasmids starting at HCV position 2755, a PCR fragment spanning positions 2755 to 3348 was inserted via *Nde*I (position 2856)-*Eco*RI (MCS) into the analogously restricted constructs pBSK 2518–4908, pBSK 2518–5650, and pBSK 2518–6062. Expression plasmids starting at HCV position 3348 were made with a PCR fragment from position 3348 to 4045 by using the *Dra*III (position 3857) and *Kpn*I (MCS) restriction sites for insertion of the HCV fragment into plasmid pBSK 2518–6062. To obtain plasmid pBSK 2755–5650, construct pBSK 2755–6062 was digested with *Aat*II (position 4624)-*Spe*I (MCS) and an HCV fragment isolated from pBSK 4530–5650 was inserted by using the same restriction sites. Site-directed mutagenesis (mutant S>A) was done with PCR (20) using two complementary HCV-specific oligonucleotides introducing a T-to-G nucleotide exchange at position 3822 and two pBSK− specific outside primers. 5′ deletions of the NS3 regions were generated by PCR using oligonucleotides starting at positions 3426, 3453, 3477, and 3525. HCV DNA fragments were inserted into pBSK 3348–6062 by using blunt-end ligation (for pBSK 3426–6062) or a 5′-introduced *Eco*RI site present also in the MCS of pBSK− and *Aat*II at position 4624. Internal deletions were made by generating PCR fragments starting at position 3348 and ending at positions 4513, 4133, and 4040 (5′ fragments) or starting at position 4783 or 5050 and ending at position 6062 (3′ fragments). Each 5′ fragment was combined with one of the 3′ fragments in a three-factor ligation by using the following restriction sites: *Eco*RI (MCS), *Xba*I (introduced by PCR [see Fig. 6A]), and *Spe*I (MCS). All PCR-derived DNA constructs were analyzed by nucleotide sequencing.

Construction of plasmids for homologous recombination. The basic vaccinia virus vector pATA-18 has been described previously (28). This vector was modified by insertion of two complementary oligonucleotides into the *Sph*I restriction site of the MCS, introducing a *Spe*I restriction site and three stop codons in three different reading frames. The NS4 region containing the 6xHis affinity tag at the carboxy terminus (see Fig. 7A) was obtained by PCR using an antisense oligonucleotide complementary to HCV positions 5800 to 5819 and containing six His codons and a *Kpn*I linker at the 5′ end. This fragment was combined with fragments containing HCV sequences from positions 2518 to 6062 or from positions 3348 to 6062 and inserted into the modified pATA-18 vector by a three-factor ligation by using the following restriction sites: *Eco*RI (MCS)-*Aat*II (position 4624)-*Kpn*I (MCS). To obtain plasmids for homologous recombination containing HCV sequences up to the NS5 region, construct pBSK 3348–7033 was cut with *Hpa*I and *Spe*I and the HCV fragment (position 5969 to 7033) was inserted into the analogously restricted plasmid pATA 3348–5819.

Expression of HCV fusion proteins and generation of antisera. DNA fragments from HCV positions 3348 to 4045, positions 5174 to 5650, and positions 6633 to 7034 were generated by PCR using oligonucleotides containing *Bam*HI restriction sites at their 5′ ends. The fragments were inserted into pDS561/RBSII 6xHis (27) by using the unique *Bam*HI site in the MCS. All HCV-specific proteins expressed in *Escherichia coli* were purified via metal chelate affinity chromatography under denaturing conditions as described previously (27). After elution from the column, proteins were dialyzed against 6 M urea and further purified by preparative gel electrophoresis. After electroelution, proteins were quantitated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining using a dilution series of a marker protein for comparison. Rabbit antisera were obtained by three or four injections of antigen with 200 μg for the first injection in combination with Freund's complete adjuvant and 100 μg for each subsequent injection together with incomplete adjuvant. Antibody titers as determined by enzyme-linked immunosorbent assays were 1:128,000 for anti-NS3A, 1:4,000 for anti-NS4A, and >1:256,000 for anti-NS5A.

In vitro transcription and translation. Plasmid DNAs purified by CsCl density gradient centrifugation were linearized with appropriate restriction enzymes. After phenol and chloroform extraction, DNAs were ethanol precipitated, dissolved in water, and used for in vitro transcription with T7 RNA polymerase (Promega, Madison, Wis.) according to the instructions of the manufacturer. After a 90-min incubation at 37°C, plasmids were digested with RNase-free DNase and RNA was extracted with phenol and chloroform. After ethanol precipitation, RNA was dissolved in water and one-seventh of the RNA was used for one in vitro translation reaction in a final volume of 25 μl according to the instructions of the manufacturer (Promega). After 20 or 60 min at 30°C, samples were quickly frozen and thawed twice and 2 μg of RNase A was added. After 5 min at 37°C, protein sample buffer (200 mM Tris-HCl, pH 8.0, 5 mM EDTA, 3.3% SDS, 2% 2-mercaptoethanol) was added and proteins in 5 μl of the translation reaction mixture were separated by electrophoresis through an 11% polyacrylamide gel. Proteins were detected by fluorography using Amplify (Amersham Life Science). Translations in the presence of dog pancreatic microsomal membranes were done exactly as recommended by the manufacturer (Promega).

Generation of recombinant vaccinia viruses, metabolic labelling, and isolation of HCV-specific proteins. The methods used for generation of recombinant vaccinia viruses, metabolic labelling, and isolation of HCV-specific proteins have been described in detail previously (2). In brief, HepG2 cells (1) were incubated with vaccinia virus at a multiplicity of infection of 10 in fetal calf serum-free medium for 1 h at room

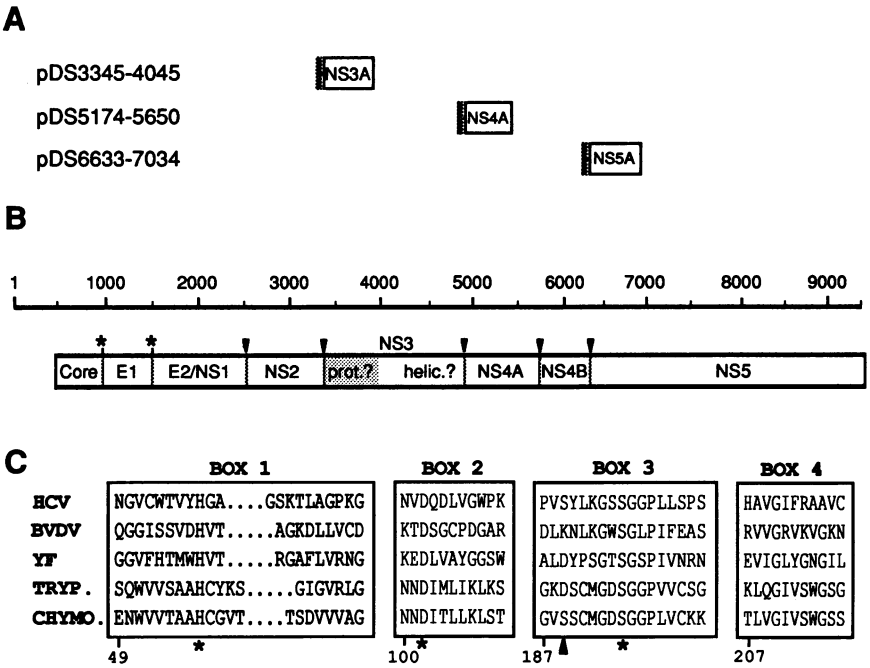

FIG. 1. (A) HCV genome fragments expressed in *E. coli*. The 6xHis affinity tag present at the amino termini of the proteins is indicated (stippled bar). (B) Deduced genetic organization of the HCV genome based on sequence comparison with flavi- and pestiviruses. Known proteolytic cleavage sites (15) (stars) and predicted cleavage sites (29) (arrowheads) are indicated. The NS3 region homologous with other serine proteinases (stippled prot.? box) is shown. The numbers correspond to the sequence of the HCV-2 isolate of Kato et al. (18), with the A residue of the ATG initiation codon of the polyprotein at position 330 (see Materials and Methods). helic., helicase. (C) Alignment of amino acid sequences in the amino-terminal regions of the NS3 proteins of HCV and yellow fever virus (YF) (24), p80 of bovine viral diarrhea virus (BVDV) strain NADL (12), trypsin (TRYP.), and chymotrypsin (CHYMO.). The three enzymatic domains (boxes 1 to 3) and the substrate-binding pocket (box 4), as proposed by Bazan and Fletterick (3), are shown. The catalytic triad of amino acids H, D, and S (stars) and the amino acid important for predicting cleavage specificity (arrowhead) are indicated. The numbers correspond to the amino acid sequence of chymotrypsin.

temperature and then for 16 h in fetal calf serum-containing medium at 37°C. After an additional 60-min incubation in fetal calf serum- and methionine-free medium, cells were labelled for 1 h by using the same medium supplemented with 100 μCi of [^{35}S]methionine (Amersham Life Science). After cell lysis, proteins were precipitated with 5% trichloroacetic acid and dissolved by boiling in protein sample buffer. After dilution in radioimmunoprecipitation assay buffer (phosphate buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), proteins were subjected to immunoprecipitation overnight at 4°C by using 4 μl of the anti-NS3A or anti-NS5A antiserum or 6 μl of anti-NS4A. Samples were washed several times with radioimmunoprecipitation assay buffer and finally analyzed as described above. For competition experiments, 5 μg of purified antigen was added to immunoglobulin, preadsorbed to protein A-Sepharose, and incubated for at least 2 h prior to addition of the sample.

RESULTS

In vitro processing of an HCV NS2/3/4′ polyprotein. Bazan and Fletterick (3) and Gorbalenya and coworkers (14) have recently proposed a model for prediction of serine-type proteinases, which is summarized in Fig. 1C and which includes the corresponding amino acid sequences from the amino-terminal NS3 region of our HCV isolate. On the basis of sequence alignments and structural-pattern analysis, they identified three similarity boxes (boxes 1 to 3) and a box containing amino acids that contribute to substrate binding (box 4). Despite low overall sequence identity, the characteristic feature of the first three boxes is the presence of three invariant amino acids forming the active site of the proteinase (the so-called catalytic triad [H, D, and S]), which are present in a characteristic spatial separation. This amino acid motif is found, for instance, in the amino-terminal region of flavivirus NS3 or pestivirus p80. In agreement with the prediction that this motif is crucial for proteinase function, it has been shown that mutational changes of amino acids of the catalytic triad of bovine viral diarrhea virus p80 or flavivirus NS3 lead to an inactivation of proteolytic activity (5, 31, 33).

A similar sequence motif can be found in the amino-terminal NS3 region of HCV (Fig. 1C), suggesting that it encodes a serine proteinase important for processing of the polyprotein precursor. To test this hypothesis, fragments of the HCV genome spanning NS2, NS3, and part of the NS4 region (NS4′ [for simplicity, subfragments will be labelled with a ′]) were cloned downstream of the promoter from bacteriophage T7 (Fig. 2A) and used for in vitro transcription-translation. To assay for the importance of membrane association of the viral proteins for cleavage efficiency and/or cleavage by signalases, translations were performed in the absence or presence of dog pancreatic microsomal membranes. The result of such an analysis is shown in Fig. 2B. Translation of an RNA spanning the NS2/3 cleavage site (positions 2755 to 4908) and containing most of the NS3 region revealed that a prominent protein of about 80 kDa was produced in the absence of membranes (Fig. 2B, lane 1). However, when membranes were included during transla-

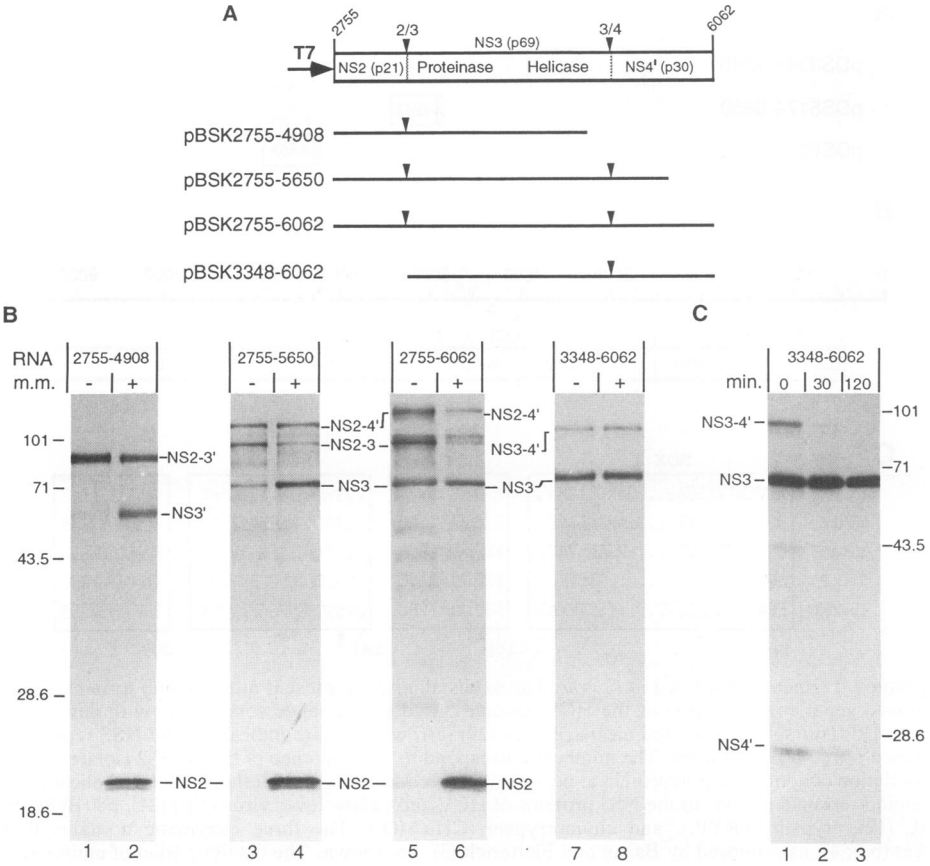

FIG. 2. Analysis of HCV polyprotein processing in an in vitro translation system. (A) Schematic drawing of the HCV genome segment used to characterize proteolytic processing. Individual RNAs used for in vitro translation reactions are shown below. Predicted cleavage sites are indicated (arrowheads). (B) Results of in vitro translations of the RNAs in the absence (−) or presence (+) of microsomal membranes (m.m.). The sizes (in kilodaltons) and positions of marker proteins are indicated on the left. (C) Pulse-chase analysis of RNA 3348–6062-programmed in vitro translations. Translations were labelled with [^{35}S]methionine for 15 min and chased for the indicated periods.

tion, two additional proteins of about 60 and 21 kDa were produced, demonstrating cleavage between NS2 and NS3 (Fig. 2B, lane 2). While the 60-kDa species corresponded to the NS3 cleavage product, the 21-kDa protein was derived from the NS2 region, since it was not present in translation reactions programmed with a 5′-truncated RNA (positions 3348 to 6062) (Fig. 2B, lanes 7 and 8).

To analyze also the cleavage between NS3 and NS4, an RNA covering NS2, NS3, and part of the NS4 region (RNA 2755–5650) (Fig. 2A) was used for in vitro translation in the same way. As shown in Fig. 2B (lane 3), translation in the absence of membranes produced proteins of about 110, 90, and 69 kDa, corresponding to the NS2-4′ precursor and processing products NS2-3 and NS3. The fact that no NS3/4 processing intermediate was detected indicated efficient processing between NS3 and NS4 in contrast to an inefficient cleavage at the NS2/3 junction. However, processing efficiency at this site was increased when translations were performed in the presence of membranes (Fig. 2B, lane 4), as shown by the reduction of the amount of the NS2-3 processing intermediate, concomitant with the increases in the amounts of NS3 and NS2. It should be mentioned at this point that no protein corresponding to NS4′ was detected under conditions used here for the in vitro translation.

An analogous result was obtained when a larger fragment of the NS4 region (RNA 2755–6062) was included. Cleavage at the NS3/4 junction was clearly observed in the absence of membranes (Fig. 2B, lane 5), while the amount of NS2-containing processing intermediates (NS2-4′ and NS2-3) was reduced in translations with membranes in parallel with an increase of the amount of NS2 (Fig. 2B, lane 6). These results suggested that membrane association of the NS2-containing proteins was essential for efficient cleavage at the NS2/3 junction, in contrast to cleavage between NS3 and NS4, which occurred efficiently also in the absence of membranes (RNA 3348–6062) (Fig. 2B, lanes 7 and 8).

With these in vitro translations performed under standard conditions (60 min at 30°C), we did not detect processing products from the NS4 region. We reasoned that this could be due to a very short half-life of the protein. To test this hypothesis, a pulse-chase experiment was performed. Translation of RNA 3348–6062 was allowed for only 20 min and was followed by the addition of an excess of nonradioactive methionine and cycloheximide. Aliquots of the reaction mix were withdrawn after 0, 30, and 120 min and analyzed by SDS-PAGE. As shown in Fig. 2C, the NS4 fragment could be detected after a 20-min translation reaction (lane 1). However, in contrast to NS3, the signal was significantly reduced after a 30-min chase (Fig. 2C, lane 2) and absent after 2 h (Fig. 2C, lane 3). Thus, each subsequent translation reaction was performed for 20 min to allow detection of the unstable NS4 processing products. It should be noted that,

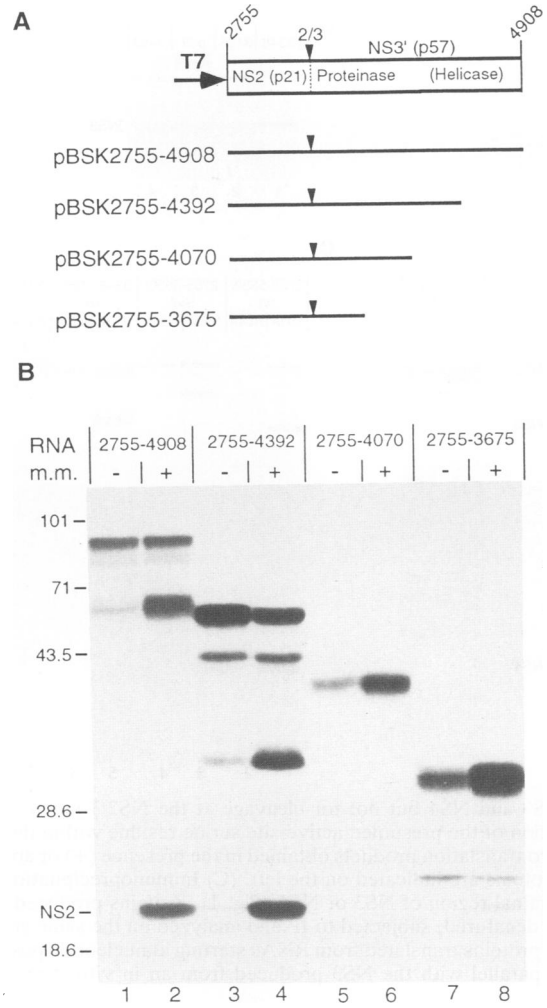

FIG. 3. The amino-terminal domain of NS3 is required for cleavage between NS2 and NS3. (A) Schematic representation of the RNAs used for in vitro translations (Fig. 2). (B) Results of translations in the presence or absence of microsomal membranes (m.m.). The positions of marker proteins and the NS2 cleavage product are indicated on the left.

even under these conditions, the smaller NS4′ fragment encoded from RNAs up to position 5650 could not be detected (see below).

In order to determine the minimal NS3 domain necessary for NS2/3 cleavage, plasmid pBSK 2755–4908 was digested with different restriction enzymes to generate a series of 3′-truncated runoff transcripts (Fig. 3A). RNAs were used for in vitro translations in the presence or absence of membranes, and proteins were analyzed as before. As shown in Fig. 3B, cleavage at this site occurred only when about 55% of NS3, encoding the predicted serine-type proteinase, was present (lanes 3 and 4) while no NS2 cleavage product was detected with further truncated polyproteins. This result suggested that the NS3-encoded proteinase domain is required for proteolytic cleavage at the NS2/3 junction.

NS3/4 cleavage requires an active NS3 proteinase. To evaluate whether cleavage sites NS2/3 and NS3/4 were processed by an NS3-encoded proteinase activity or whether NS3 was required as a structural component, the serine residue of the assumed catalytic triad was changed to an alanine and introduced into constructs encoding NS2-4′ or NS3-4′ polyproteins (Fig. 4A). RNAs derived from both the wild type (wt) and the mutant (S>A) constructs were used for in vitro translation in the presence or absence of membranes, and the resulting proteins were analyzed by SDS-PAGE. As shown in Fig. 4B, an unprocessed molecule of about 55 kDa was obtained when wt RNA 2755–4393 was translated in the absence of membranes (lane 1) while inclusion of membranes clearly led to cleavage between NS2 and NS3 (lane 2). The same result was found when the RNA containing the mutation of the catalytic triad was translated (Fig. 4B, lanes 3 and 4), demonstrating that the NS3-encoded proteinase activity was dispensable for cleavage at the NS2/3 junction.

To analyze processing between NS3 and NS4, in vitro translations were programmed with an RNA covering NS3 and part of the NS4 region. As described before (Fig. 2), cleavage at the NS3/4 junction occurred independently of membranes (Fig. 4B, lanes 9 and 10) (the lower signal of the NS4′ protein in lane 9 was due to a lower translation efficiency). However, a change of the active-site serine residue to alanine completely blocked processing at this site, demonstrating that a catalytically active NS3 proteinase is essential for cleavage between NS3 and NS4.

Principally the same result was obtained for a translation reaction with RNA 2755–5650/S>A. No cleavage was observed at the NS3/4 junction, while processing between NS2 and NS3 was unaffected by the NS3 mutation (Fig. 4B, lanes 7 and 8). The lower NS2 signal obtained with the mutant compared with that of the wt proteinase (Fig. 4B, compare lanes 6 and 8) probably was due to the nature of the construct, because no such difference was seen with translations programmed with RNA 2755–4393 containing either an unaltered or a mutated NS3 (Fig. 4B, lanes 2 and 4).

To characterize the processing products more precisely, proteins produced in the presence of membranes (Fig. 4B, lanes 6, 8, 10, and 12) were denatured and subjected to immunoprecipitation using antisera directed against the amino-terminal regions of NS3 or NS4 (Fig. 1A). As expected, all full-length proteins reacted with both antisera (Fig. 4C). In addition, the reactivity of the protein with an apparent molecular mass of about 85 kDa observed with mutant RNA 2755–5650/S>A confirmed that this protein was an NS3-4′ processing intermediate lacking the NS2 region (Fig. 4C, lanes 3 and 4). Finally, the reactivities of the smaller proteins of about 69, 72, and 26 kDa (Fig. 4C, lanes 1, 5, and 6, respectively) confirmed that they corresponded to processing products from the NS3 or NS4 region. From these results, we conclude that cleavage at the NS3/4 junction is dependent on a catalytically active NS3 proteinase whereas processing between NS2 and NS3 may be mediated by an NS2-inherent proteinase or by host cell enzymes.

For an approximate mapping of the NS2/3 cleavage site, a series of 5′-truncated RNAs starting at nucleotide positions 3348, 3426, and 3453 and ending at position 6062 were translated in parallel with RNA 2755–6062. Since the NS3 processing products differed only at their amino termini and were otherwise identical in sequence, a comparison of their apparent molecular weights should reveal the approximate position of the NS2/3 cleavage site. As shown in Fig. 4D, the naturally processed NS3 (p69) (lane 3) migrated very closely to the NS3 starting at position 3426 but was significantly faster than the NS3 starting at position 3348. In agreement with the idea of a host-cell-mediated cleavage at the NS2/3 junction, a putative signalase site, conserved between HCV-1 and HCV-2, is present around amino acid 1030 (LLAHITA/YSQQTR).

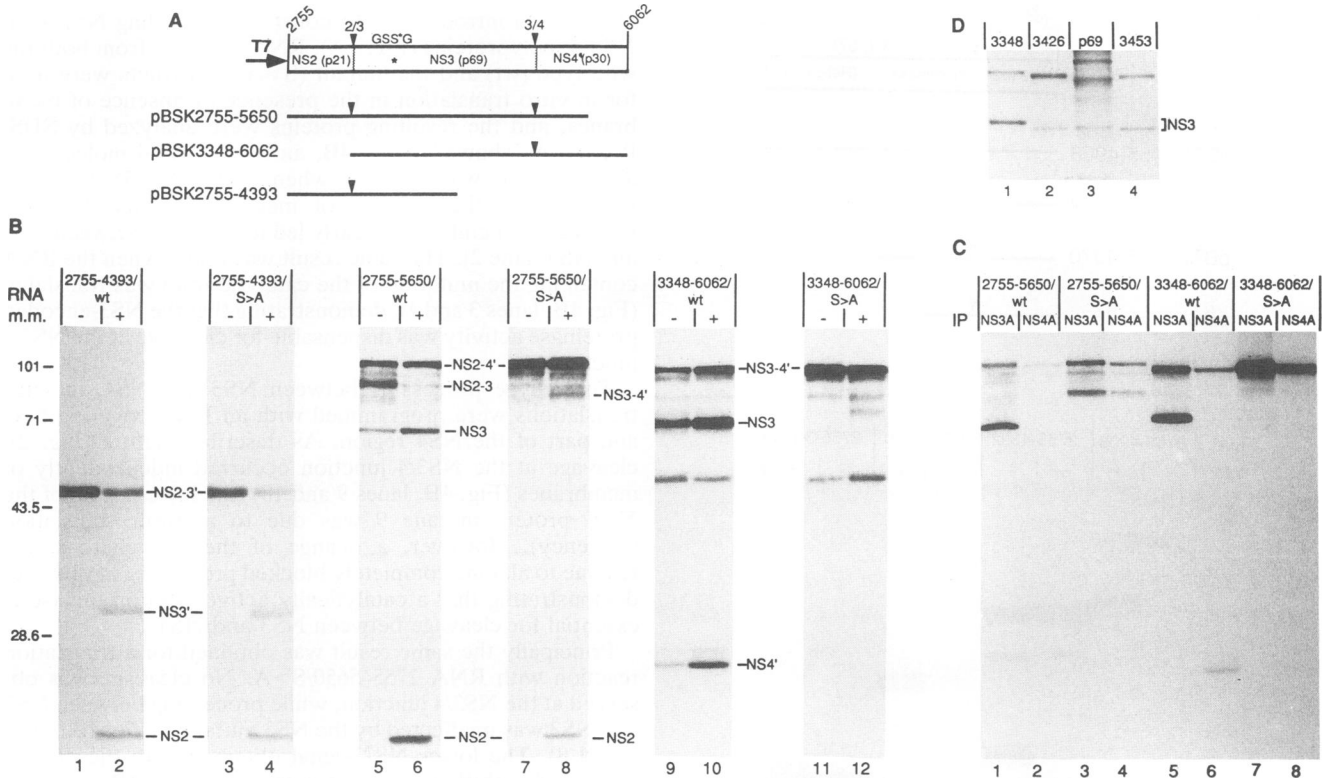

FIG. 4. The NS3-encoded proteinase is required for cleavage between NS3 and NS4 but not for cleavage at the NS2/3 junction. (A) Schematic representation of the RNAs used for in vitro translations. The position of the presumed active-site serine residue within the NS3 proteinase is indicated (star). For numbers, see Fig. 2. (B) Analysis of the in vitro translation products obtained in the presence (+) or absence (−) of microsomal membranes (m.m.). The sizes (in kilodaltons) of marker proteins are indicated on the left. (C) Immunoprecipitation (IP) of the HCV processing products using antisera directed against the amino-terminal region of NS3 or NS4 (Fig. 1). Proteins produced by in vitro translation in the presence of m.m. (panel B, lanes 6, 8, 10, and 12) were denatured, subjected to IP and analyzed on the same gel. (D) Approximate mapping of the cleavage site between NS2 and NS3. NS3-specific proteins translated from RNAs starting at nucleotide positions 3348, 3426, and 3453 and ending at position 6062 (Fig. 5) were analyzed in parallel with the NS3 produced from an in vitro translation programmed with RNA 2755–6062.

Mapping of the minimal NS3 proteinase domain. To map the minimal proteinase domain required for cleavage at the NS3/4 junction, a series of 5′-truncated NS3-4′ constructs were generated and used for in vitro transcription-translation (Fig. 5A). As a control, RNAs covering HCV sequences from position 3348 to 6062 and encoding either a wt or a mutated NS3 proteinase were included. As shown in Fig. 5B, translation of the wt control RNA gave rise to the unprocessed NS3-4′ protein as well as the NS3 and NS4′ processing products (lane 1). As before, the latter species were not obtained when the active site serine residue was changed to an alanine (Fig. 5B, lane 2). Translation of the truncated RNAs revealed that deletion of 27, 36, and 44 amino acids from the amino terminus did not affect NS3/4 cleavage (Fig. 5B, lanes 3 to 5, respectively). However, removal of 60 N-terminal amino acids abolished processing at this site, demonstrating that the amino terminus of a catalytically active NS3 proteinase lies between amino acids 1049 and 1065.

For a location of the 3′ boundary of the minimal proteinase domain, we considered that, in analogy to pestiviruses, cleavage between NS3 and NS4 could be mediated in *cis*. In agreement with this assumption, we were unable to detect processing at this site when mixing lysates containing a catalytically active proteinase and a mutant substrate (e.g., from RNA 3348–6062/S>A) (data not shown). In addition, cleavage at the NS3/4 junction was insensitive to dilution (data not shown), suggesting that it was indeed mediated in *cis*. Thus, to map the 3′ boundary of the proteinase, a panel of internal NS3 deletions lacking sequences predicted to encode the helicase activity was created (Fig. 6A). Again, wt and mutant RNA 3348–6062 were included as controls. As shown in Fig. 6B, all deletion constructs produced clearly visible amounts of precursor protein. However, in no case could processing products be detected either with longer exposures or in immunoprecipitations (e.g., with the anti-NS4A antiserum) (data not shown). Currently, we cannot decide whether the deletions affected the proteinase activity or accessibility of the cleavage site or both, but the result suggests that a complete NS3 region is required for processing at the NS3/4 junction.

In vivo processing of HCV polyprotein fragments expressed with recombinant vaccinia viruses. To correlate the results of in vitro processing of the HCV polyprotein with processing in vivo, two different genome fragments, encoding a wt or mutated NS2/3/4′ or NS3/4′ protein, were inserted downstream of the vaccinia virus 11K late promoter and used to generate recombinant viruses (Fig. 7A). Following infection of a human hepatoma cell line (HepG2) (1) with the various recombinants, proteins were metabolically labelled with [^{35}S]methionine and subjected to immunoprecipitation with the anti-NS3A or anti-NS4A antiserum. Since some vaccinia

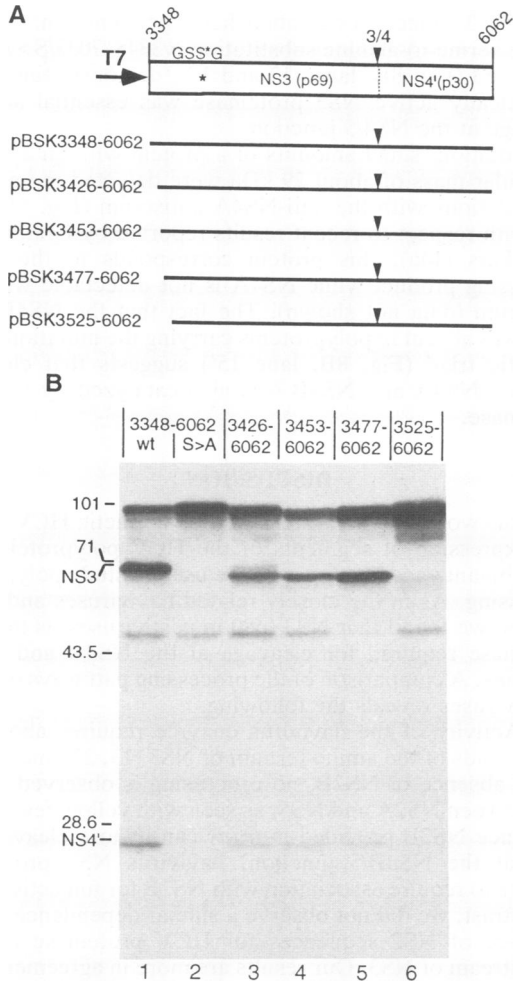

FIG. 5. Determination of the amino-terminal boundary of an enzymatically active NS3 proteinase. (A) Schematic representation of 5′-truncated RNAs used for in vitro translation. For numbers, see Fig. 2. (B) Results of translation reactions programmed with HCV-specific RNAs. The sizes (in kilodaltons) of marker proteins and positions of the NS3 and NS4′ cleavage products are indicated on the left.

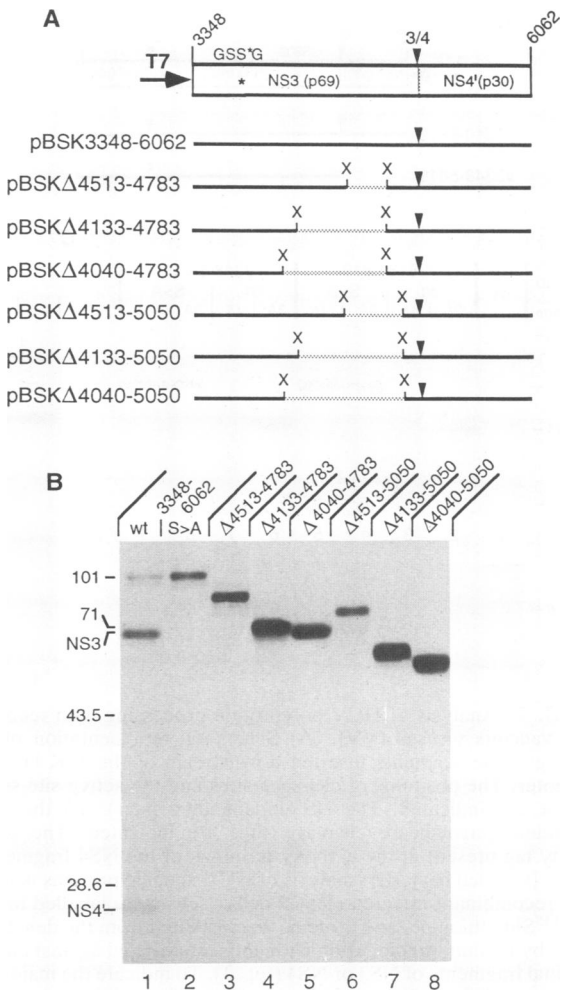

FIG. 6. Analysis of the effects of internal deletions in NS3 on cleavage between NS3 and NS4. (A) Schematic summary of the deletion mutants. Deleted regions are shown as dotted lines. The positions of the *Xba*I (X) restriction sites used to combine PCR fragments (see Materials and Methods) are indicated. Numbers in the designations of the mutants refer to the first and last deleted nucleotides. (B) Analysis of HCV-specific proteins obtained after in vitro translation of the RNAs shown in panel A. The sizes (in kilodaltons) of marker proteins and the position of the NS4′ processing product are indicated on the left.

virus proteins tend to bind nonspecifically to protein A, infections with wt vaccinia virus were always included as a control. As shown in Fig. 7B, an anti-NS3A-reactive protein with an apparent molecular mass of about 69 kDa could be detected in v2518–5819/wt infected cells (lane 2), indicating that the precursor was cleaved at both the NS2/3 and the NS3/4 junctions. While the NS3-specific protein was clearly visible, only very small amounts of the NS4′ cleavage product, not visible on the photograph (Fig. 7B, lane 3, lower panel), could be detected. This was due to the instability of the molecule. Using pulse-chase experiments, we determined that the NS4′ protein had a half-life of only about 15 min in contrast to a half-life of about 6 h for NS3 (data not shown).

As expected, when cells were infected with the recombinant expressing a mutated proteinase (v2518–5819/S>A) no cleavage between NS3 and NS4 was observed (Fig. 7B, lanes 4 and 5). In agreement with the results of the in vitro experiments, the majority of the molecules lacked the NS2 portion and only small amounts of unprocessed precursor could be detected as a minor band of about 120 kDa. Thus, processing at the NS3/4 junction was mediated by the NS3-encoded proteinase, while cleavage at the NS2/3 site appeared to be catalyzed by host cell enzymes.

Analogous results were obtained with recombinant viruses expressing the NS3/4′ protein. Efficient cleavage was observed with the unaltered NS3 proteinase (Fig. 7B, lanes 6 and 7), while processing was abolished by the serine-to-alanine substitution (Fig. 7B, lanes 8 and 9).

To analyze whether cleavage at the NS3/4 junction in vivo and in vitro occurred at about the same position, proteins obtained from in vitro translations with RNA 3348–6062 were analyzed in parallel with the NS3 produced from recombinant v3348–5819. As shown in Fig. 7C, the protein produced in the reticulocyte lysate (lane 2) comigrated with the protein isolated from infected HepG2 cells by immunoprecipitation with the NS3-specific antiserum (lane 1), indicating that NS3/4 cleavage in each system occurred at about the same position.

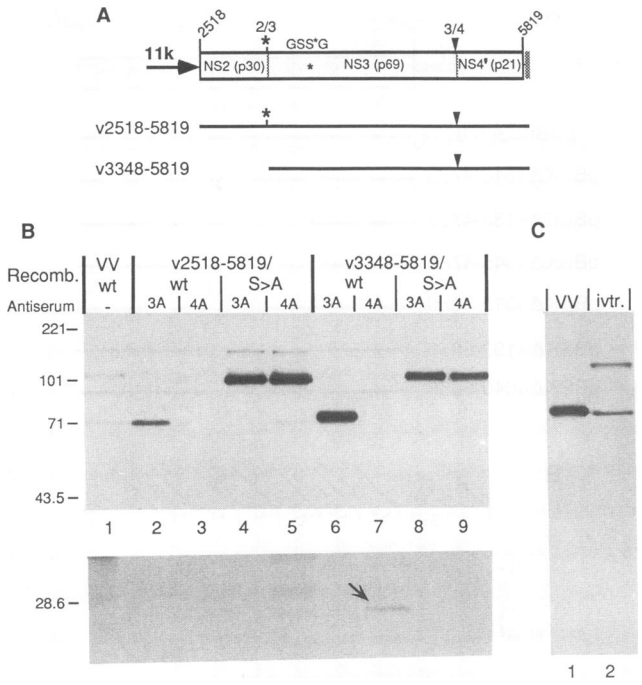

FIG. 7. Analysis of HCV polyprotein processing with recombinant vaccinia viruses (VV). (A) Schematic representation of the HCV genome segments inserted downstream of the 11K late VV promoter. The positions of cleavage sites and the active-site serine residue are indicated. The NS3-independent (star) and the NS3-dependent (arrowhead) cleavage sites are indicated. The 6xHis affinity tag present at the carboxy terminus of the NS4 fragment is shown (stippled bar). (B) Analysis of HCV-specific proteins isolated from recombinant-infected HepG2 cells. Cells were labelled for 1 h with [^{35}S]methionine, and proteins were isolated from the denatured lysate by immunoprecipitation with antisera directed against amino-terminal fragments of NS3 or NS4 (Fig. 1). To indicate the major VV proteins, 1/200 of the crude lysate from VV wt-infected cells is shown in lane 1. To detect the low signal of the NS4′ fragment (lanes 3 and 7), a 10-fold longer exposure of the lower part of the gel is shown in the lower panel, with the arrow indicating the position of the NS4 cleavage product. (C) Comparison of the apparent molecular weights of NS3 obtained from v3348–5819-infected HepG2 cells and that from RNA 3348–6062-programmed in vitro translation (ivtr.).

As shown for flaviviruses and pestiviruses, the NS5 region located at the carboxy terminus of the polyprotein encodes the RNA-dependent RNA polymerase which is cleaved from the precursor by the NS3 (p80)-encoded proteinase (5, 33). To test whether the same holds true for HCV, two recombinants encoding an NS3/4/5′ polyprotein with an unaltered or mutated NS3 proteinase were generated (Fig. 8A). Recombinants were used for infection of HepG2 cells, and HCV-specific proteins were analyzed by immunoprecipitation using antisera directed against subfragments of NS3, NS4, and NS5 (Fig. 1A). The specificities of the immunologic reactivities were demonstrated by adding an excess of nonradioactive, homologous antigen as a competitor (see Materials and Methods). In agreement with the result described above, an NS3-specific protein with an apparent molecular mass of about 72 kDa could be detected in v3348–7033/wt-infected cells (Fig. 8B, lane 7). In addition, an anti-NS5A-reactive protein of about 30 kDa was observed (Fig. 8B, lane 11), suggesting that cleavage at the NS4/5 junction had occurred. However, processing at this site and at the NS3/4 junction was abolished when a mutant containing the serine-to-alanine substitution (v3348–7033/S>A) was expressed (Fig. 8B, lanes 13 and 17), demonstrating that a catalytically active NS3 proteinase was essential also for cleavage at the NS4/5 junction.

In addition, small amounts of a protein with an apparent molecular mass of about 29 kDa were detected in immunoprecipitations with the anti-NS4A antiserum (Fig. 8B, lane 9′). With respect to recent results reported by Grakoui and coworkers (14a), this protein corresponds to the NS4B processing product while NS4A is not detectable with our antiserum (data not shown). The fact that the NS4B fragment was absent in polyproteins carrying the mutation of the catalytic triad (Fig. 8B, lane 15′) suggests that cleavage between NS4A and NS4B was also catalyzed by the viral proteinase.

DISCUSSION

In this work, in vitro translation of synthetic HCV RNAs and expression of segments of the HCV polyprotein with recombinant vaccinia viruses were used to study polyprotein processing. As in the closely related flaviviruses and pestiviruses, we found that NS3 (p80 in pestiviruses) is the viral proteinase required for cleavage at the NS3/4 and NS4/5 junctions. A comparison of the processing pathways of these RNA viruses reveals the following.

(i) Activity of the flavivirus enzyme requires about 185 amino acids of the amino termini of NS3 (13, 23) and NS2B. In the absence of NS2B, no processing is observed at any site between NS2A and NS5, as seen with yellow fever virus (5). Since NS2B provided in *trans* can restore cleavage, at least at the NS4B/5 junction, flavivirus NS3 proteinase appears to require association with NS2B for full activity (5). In contrast, we did not observe a similar dependence on the presence of NS2 sequences for HCV proteinase activity downstream of NS3. Our results are more in agreement with observations described for bovine viral diarrhea virus, a pestivirus, showing that cleavage at all sites downstream of p80 occurred independently of the presence of an NS2-like sequence (p54) (33).

(ii) For bovine viral diarrhea virus, Wiskerchen and Collett (33) reported that a catalytically active proteinase (p80) is required for cleavage between p54 and p80, although with respect to recent results reported by the same group this question still is not clearly answered (22). Similarly, cleavage between NS2B and NS3 in flavivirus polyproteins also depends on an active NS3 proteinase (13). For HCV, we found that mutational inactivation of the NS3 proteinase did not affect cleavage at the NS2/3 junction. Although we cannot formally rule out that NS2 itself encodes a proteinase activity, this hypothesis seems unlikely because we did not observe cleavage of a polyprotein containing NS2 and about 40% of NS3 (RNA 2755–4070) (Fig. 3) and no sequence homology to known proteinases can be found. Thus, it appears more likely that cleavage at the NS2/3 junction is mediated by host cell enzymes. Whatever the origin of the proteinase, since addition of membranes to the translation reaction mixture clearly increased cleavage efficiency at the NS2/3 junction, membrane association of the NS2-containing protein, in particular via the hydrophobic NS2 portion, appears to be required for processing at this site.

(iii) In flavi- and pestiviruses, NS3 and p80 act as bifunctional molecules containing a proteinase at the amino terminus and a helicase at the carboxy terminus. These functions are not separated physically by a proteolytic cleavage. This

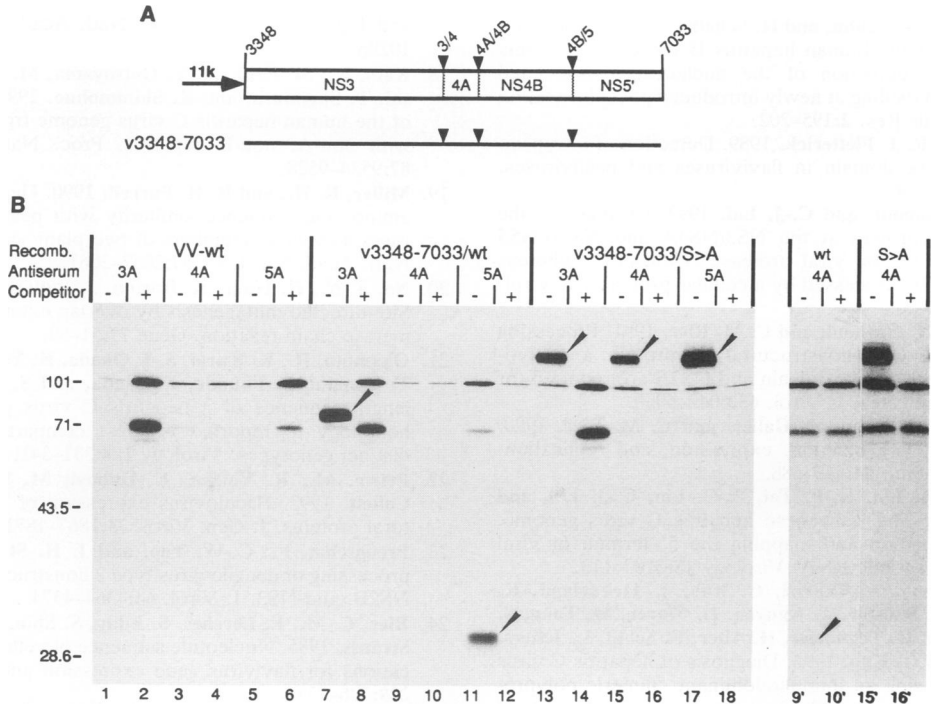

FIG. 8. A functional NS3 proteinase is required for cleavage at the NS3/4 and NS4/5 junctions. (A) Schematic representation of HCV sequences inserted into recombinant vaccinia viruses. Cleavage sites are indicated (arrowheads). For numbers, see Fig. 2. (B) Cells were labelled with [^{35}S]methionine, and HCV-specific proteins were isolated by immunoprecipitation in the absence (−) or presence (+) of a homologous competitor. The sizes (in kilodaltons) of marker proteins are indicated on the left. HCV-specific proteins are indicated (arrowheads). To detect the small amounts of the NS4-specific processing product, a fivefold longer exposure of proteins in lanes 9, 10, 15, and 16 is shown on the right (lanes 9', 10', 15', and 16', respectively).

feature seems to be conserved in HCV, as we were not able to detect NS3-specific subfragments either in in vitro translation experiments or with polyproteins expressed from recombinant vaccinia viruses.

(iv) Kinetic analyses of flavivirus polyprotein processing in vitro suggest that intramolecular cleavages occur at the NS2A/2B and NS2B/3 junctions (23) while processing, at least between NS4B and NS5, can be obtained in *trans* (4, 5). Similarly, cleavages at the amino and the carboxy termini of the pestivirus bovine viral diarrhea virus p80 appear to be mediated in *cis*, while processing at sites downstream of the carboxy terminus of p80 can occur in *trans* (33). Using in vitro-translated HCV wt and mutated polyproteins, we were unable to demonstrate cleavage at the NS3/4 junction in *trans*. In addition, processing at this site was insensitive to dilution (data not shown). As for flavi- and pestiviruses, we speculate that an intramolecular cleavage at this site occurs ahead of *trans* cleavages downstream of NS3.

(v) As deduced from sequence comparison of known serine proteinases, the amino acid at position −6 with respect to the active-site serine residue can be used to predict substrate specificity. For flaviviruses, the presence of an aspartic acid residue (Fig. 1) indicates a cleavage specificity similar to trypsin-like enzymes, i.e., cleavage behind basic residues. By introducing a negatively charged aspartic acid residue at the bottom of the binding pocket, the positively charged amino acids at the processing site can be positioned for proper cleavage. In agreement with this prediction, flavivirus proteinase was found to cleave behind two basic amino acids (26). In the case of pestiviruses, cleavage specificity has not been determined but the presence of a lysine residue at the −6 position argues against a trypsin-like activity. In HCV, this position is occupied by a serine residue, as it has been found in chymotrypsin and elastase (Fig. 1), suggesting that NS3 of HCV is a chymotrypsin-like proteinase. However, we were not able to significantly inhibit processing of the in vitro-translated polyprotein with specific chymotrypsin or elastase inhibitors (data not shown). Whether this reflects a different specificity of the proteinase or is due to our experimental system remains to be examined.

In conclusion, our experimental results show that the virus-encoded serine proteinase of HCV has a very specific and pivotal role in polyprotein processing and may therefore represent a novel target for antiviral drug development. With the high prevalence of chronic HCV carriers, the need for a specific therapy is obvious.

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