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Kinetic and Structural Analyses of Hepatitis C Virus Polyprotein Processing

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Recombinant vaccinia viruses were used to study the processing of hepatitis C virus (HCV) nonstructural polyprotein precursor. HCV-specific proteins and cleavage products were identified by size and by immunoprecipitation with region-specific antisera. A polyprotein beginning with 20 amino acids derived from the carboxy terminus of NS2 and ending with the NS5B stop codon (amino acids 1007 to 3011) was cleaved at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites, whereas a polyprotein in which the putative active site serine residue was replaced by an alanine remained unprocessed, demonstrating that the NS3-encoded serine-type proteinase is essential for cleavage at these sites. Processing of the NS3′-5B polyprotein was complex and occurred rapidly. Discrete polypeptide species corresponding to various processing intermediates were detected. With the exception of NS4AB-5A/NS5A, no clear precursor-product relationships were detected. Using double infection of cells with vaccinia virus recombinants expressing either a proteolytically inactive NS3′-5B polyprotein or an active NS3 proteinase, we found that cleavage at the NS4A/4B, NS4B/5A, and NS5A/5B sites could be mediated in *trans*. Absence of *trans* cleavage at the NS3/4A junction together with the finding that processing at this site was insensitive to dilution of the enzyme suggested that cleavage at this site is an intramolecular reaction. The *trans*-cleavage assay was also used to show that (i) the first 211 amino acids of NS3 were sufficient for processing at all *trans* sites and (ii) small deletions from the amino terminus of NS3 selectively affected cleavage at the NS4B/5A site, whereas more extensive deletions also decreased processing efficiencies at the other sites. Using a series of amino-terminally truncated substrate polyproteins in the *trans*-cleavage assay, we found that NS4A is essential for cleavage at the NS4B/5A site and that processing at this site could be restored by NS4A provided in *cis* (i.e., together with the substrate) or in *trans* (i.e., together with the proteinase). These results suggest that in addition to the NS3 proteinase, NS4A sequences play an important role in HCV polyprotein processing.

Infection with hepatitis C virus (HCV) is considered to be the major cause of posttransfusion and sporadic, community-acquired non-A, non-B hepatitis (for a recent review, see reference 23). It can lead to various clinical manifestations, including acute hepatitis, chronic hepatitis, liver cirrhosis, or an asymptomatic carrier state (22). In addition, the high prevalence of anti-HCV antibodies in chronically infected anti-hepatitis B antigen-negative patients with hepatocellular carcinoma indicates a strong association between HCV infection and tumor development (9, 12, 15, 29, 35).

Since the initial cloning (10), a number of new HCV isolates have been characterized (for a summary, see references 4 and 31). As deduced from infectivity studies with chimpanzees and from comparative sequence analyses, HCV has been classified as a separate genus in the family *Flaviviridae* together with pestiviruses and flaviviruses (7, 11, 27). These viruses have in common a virion with a lipid envelope, a single-stranded RNA genome of positive polarity encoding one long open reading frame of ca. 3,000 to 4,000 codons, and a similar genomic organization. The structural proteins (in the case of HCV, core-envelope 1 [E1]-E2) are located in the amino-terminal region and are followed by the nonstructural (NS) proteins (NS2, NS3, NS4A/B, and NS5A/B for HCV).

Production of mature proteins from the polyprotein precursor is accomplished by a series of proteolytic cleavages. Recent studies have shown that HCV structural proteins are generated from the polyprotein by host cell signalases (18, 19, 24, 26, 32), whereas processing of the NS polyprotein requires at least two virus-encoded enzymes: the NS2-3 proteinase (16, 20) cleaving between NS2 and NS3 and the NS3 proteinase cleaving at all the sites further downstream (2, 13, 17, 20, 36). Biochemical and mutational analyses suggest that the NS2-3 proteinase is a zinc-dependent metalloproteinase (20), which is, to our knowledge, without precedent among flaviviruses and pestiviruses. In contrast, NS3 appears to be a serine-type proteinase as are flavivirus NS3 and pestivirus p80 proteinases (5, 6, 8, 14, 28, 37).

In this report, recombinant vaccinia viruses were used to further characterize HCV polyprotein processing and to define the activity of the NS3 proteinase more precisely. Our results strongly suggest that the carboxy terminus of NS3 is generated by an intramolecular reaction, whereas cleavage at all sites further downstream can be mediated in *trans* by a proteinase containing the amino-terminal 211 amino acids of NS3. Furthermore, we have found that sequences from the NS4 region play an important role in polyprotein processing, especially for cleavage at the NS4B/5A site.

MATERIALS AND METHODS

Construction of plasmids for homologous recombination. The basic plasmids pATA 1007-2234, pATA 1007-1830, and pBSK 1007-1912 containing HCV sequences inserted into the modified vaccinia virus recombination vector pATA-18 or into the T7 transcription plasmid pBSK (Stratagene, Zürich, Switzerland) have been described before (2) (numbers refer to the first and the last amino acids of the expressed HCV polypro-

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tein fragment). To obtain plasmid pATA 1007-3011 bearing either a wild-type or mutated NS3 proteinase, pATA 1007-2234/wild type (wt) and pATA 1007-2234/S→A (in which the putative active site serine residue was replaced by an alanine residue [2]) were restricted with *Spe*I at the multiple cloning site (MCS) and *Hpa*I at HCV nucleotide position 5969 (according to the nomenclature of Kato et al. [25]) and combined with *Hpa*I-*Bsu*36I (5969 to 8134) and *Bsu*36I-*Spe*I (8134 to MCS) HCV fragments. These fragments were isolated from plasmids in which HCV sequences cloned from the serum of a chronically HCV-infected patient had been inserted (2). Plasmids containing HCV sequences from NS4A to NS5B (amino acids 1658 to 3011 of the polyprotein), NS4B to NS5B (1712 to 3011), or NS5A to NS5B (1973 to 3011) were constructed by PCR with upstream primers containing at their 5′ ends an *Eco*RI restriction site and an ATG codon. After 10 cycles, DNA fragments were purified by preparative gel electrophoresis, restricted with *Eco*RI (HCV nucleotide position 6687), and inserted into the *Eco*RI-digested plasmid pATA 1007-3011. Plasmid pATA 1007-1647 was obtained by insertion of an *Eco*RI (sticky end)-*Nsi*I (blunt ended after treatment with the Klenow enzyme) HCV fragment into pATA-18 cut with *Eco*RI (sticky end) and *Bam*HI (blunt ended). To construct plasmid pATA 1007-1564 an *Eco*RI (MCS)-*Stu*I (nucleotide position 5020) fragment isolated from pBSK 1007-1912 was inserted in pATA-18 restricted with *Eco*RI and *Spe*I. Plasmid pATA 1007-1355 was obtained by insertion of an *Eco*RI (MCS)-*Bst*EII (nucleotide position 4391) fragment into the *Eco*RI-*Sma*I-restricted pATA-18. To obtain plasmid pATA 1007-1395, an *Eco*RI-*Xba*I fragment (MCS to 4513) was isolated from plasmid pBSK Δ4513-5050 (2) and inserted into pATA-18 restricted with *Eco*RI and *Spe*I. Plasmids pATA 1007-1269 and pATA 1007-1238 were constructed in the same way with pBSK Δ4133-4783 and pBSK Δ4040-4713 (2) to isolate the HCV DNA fragments. Construction of the basic plasmids carrying various 5′-deleted NS3 fragments has been described recently (2).

Expression of HCV-specific proteins in *Escherichia coli* and generation of antisera. Expression of NS3-, NS3/4-, and NS5A-specific polyprotein fragments has been described previously (2) (see Fig. 2A). To express the NS5B-specific protein, a DNA fragment from nucleotide positions 7587 to 8207 (amino acids 2419 to 2625 of the polyprotein) was amplified by PCR with oligonucleotides carrying *Bam*HI restriction sites at their 5′ ends and was inserted into the *Bam*HI-restricted vector pDS 561/RBSII 6xHis (33). After induction, the protein was purified by metal chelate affinity chromatography under denaturing conditions (33). Further purification of the protein and generation of antisera were done exactly as described previously (2). The antibody titer as determined by enzyme-linked immunosorbent assay was 1:512,000.

Generation of recombinant vaccinia viruses. Human tk^- 143 cells were infected with the temperature-sensitive vaccinia virus *vts*-7 (kindly provided by Hans-Jürgen Schlicht, University of Ulm, Ulm, Germany) at a multiplicity of infection of 1 for 1 h at room temperature in Dulbecco's modified minimal essential medium (DMEM). After removal of the inoculum, cells were incubated in medium supplemented with 10% fetal calf serum (FCS) for 2 h at 33°C. Medium was removed, cells were washed several times with phosphate-buffered saline (PBS), and the calcium phosphate precipitate, prepared as described previously (3) with 1 μg of vaccinia virus wild-type DNA and 0.5 to 2 μg of plasmid DNA in a total volume of 250 μl, was added dropwise. After 1 h at room temperature, DMEM without FCS was added, cells were incubated for 2 h at 40°C and washed four times with PBS, and FCS-containing medium was added. Cells were lysed after 48 h at 40°C in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and the lysate was used for infection of human tk^- 143 cells for 1 h at room temperature as described above. Finally, complete medium containing 100 μg of 5-bromo-2′-deoxyuridine (Sigma, Deisenhofen, Germany) per ml was added, and cells were incubated for 48 h at 37°C. Large-scale preparations of recombinant vaccinia viruses were grown on HeLa cells, and titers of infectious progeny were determined by plaque assay on human tk^- 143 cells.

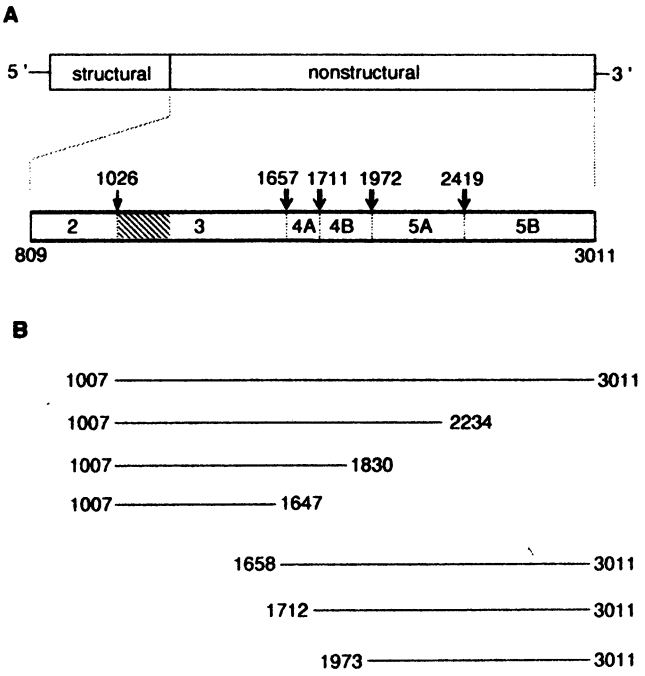

FIG. 1. HCV genome structure and expression constructs. (A) Diagram of the HCV genome encoding the structural proteins in the 5′-terminal quarter followed by the NS proteins 2 to 5B. The 5′ and 3′ nontranslated regions are indicated as thin lines. A detailed view of the NS protein region (amino acids 809 to 3011 of the polyprotein) is drawn below. Cleavage sites of the NS2-3 proteinase (↓) (16, 20) and the NS3 proteinase (⇓) (2, 13, 17, 20, 36) are given. Numbers above the arrows refer to the amino acids at the P1 positions of the scissile bonds. (B) HCV polyprotein expression constructs used in this study. Lines depict regions of the HCV genome expressed with recombinant vaccinia viruses and are drawn to scale and oriented with respect to the diagram in panel A. Numbers refer to the first and last amino acids of the HCV polyprotein expressed with the recombinant vaccinia viruses.

Metabolic labelling of infected cells and characterization of HCV-specific proteins. HeLa cells were infected at a multiplicity of infection of 5 to 10 as described above and 16 h later were incubated in methionine- and FCS-free medium for 1 h. After addition of the same medium supplemented with 100 μCi of [^{35}S]methionine (Amersham Life Science, Braunschweig, Germany) and incubation for various times, cells were lysed in TNE (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) with 1% Triton X-100. The lysate was clarified by a 15-min centrifugation at 15,000 × *g* at 4°C, and proteins in the supernatant were precipitated by the addition of 2% sodium dodecyl sulfate (SDS) and 5% trichloroacetic acid. After 10 to 30 min at 0°C, precipitated proteins were collected by a 5-min centrifugation at 6,000 × *g* and resolved in protein sample buffer (200 mM Tris-HCl [pH 8.8], 5 mM EDTA, 2% SDS, 1%

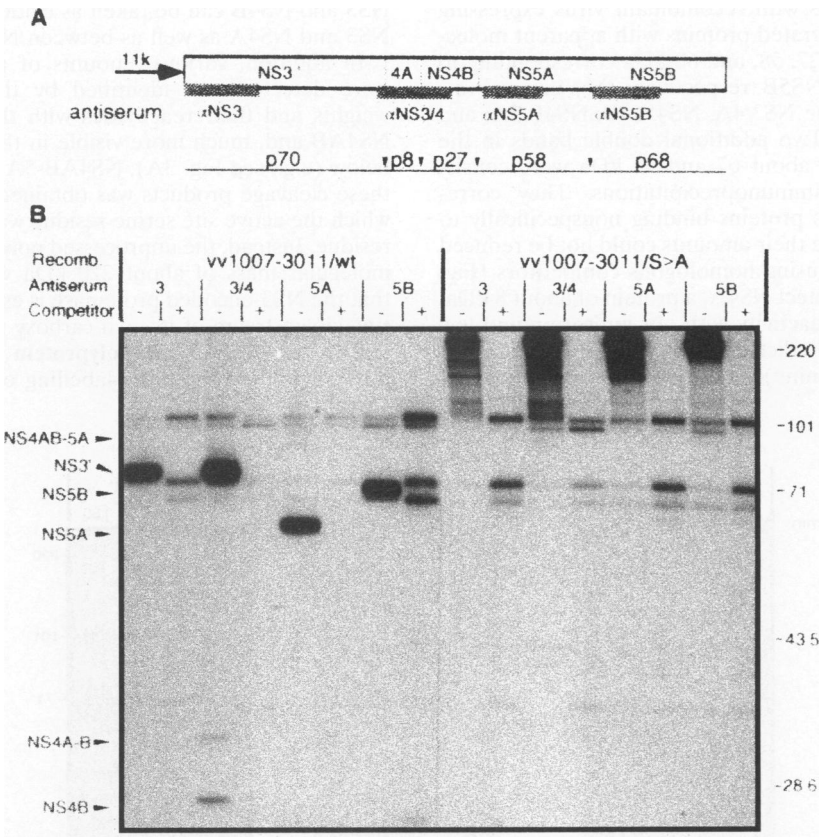

FIG. 2. Proteolytic processing of an NS3′-5B polyprotein expressed with recombinant vaccinia viruses. (A) Schematic representation of the HCV genome segment expressed under control of the vaccinia virus 11-kDa late promoter. Fragments of the polyprotein used to generate antisera are indicated as dotted bars. The apparent molecular masses (in kilodaltons) of individual processing products are given below. (B) Analysis of HCV-specific proteins isolated from HeLa cells infected with recombinants expressing an unaltered NS3′-5B polyprotein (vv1007-3011/wt) or an NS3′-5B with an enzymatically inactive NS3 proteinase (vv1007-3011/S→A). Cells were labelled for 1 h with [^{35}S]methionine, and proteins were isolated from the lysate by immunoprecipitation. To demonstrate the specificities of the detected proteins, immunoprecipitations were performed in the absence (−) or presence (+) of a homologous competitor. Numbers to the right refer to the sizes of marker proteins (in kilodaltons).

2-mercaptoethanol, 10% sucrose, and 0.1% bromophenol blue). After 5 min of boiling, samples were diluted to RIPA buffer by adding 20 volumes of RIPA buffer without SDS (PBS, 1% Triton X-100, 0.5% sodium deoxycholate) containing 1 mM phenylmethylsulfonyl fluoride (Sigma). Finally, 15 μl of packed protein A-Sepharose containing preadsorbed immunoglobulin (corresponding to 3 to 6 μl of antiserum) was added and the samples were incubated overnight at 4°C with agitation. After three washes of the immunocomplexes with RIPA buffer, protein sample buffer containing 3.3% SDS and 2% 2-mercaptoethanol was added, samples were boiled for 5 min, and half of the material was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Competitions were done by adding 10 μg of purified homologous antigen to the immunoprecipitation.

In vitro transcription and translation. A detailed description of these methods is given in reference 2. In brief, the linearized plasmid pBSK 1007-1912 was used for in vitro transcription, and after phenol extraction and ethanol precipitation, RNA was quantified by comparison of a serial dilution of the RNA with a concentration standard after electrophoresis through an agarose gel. RNA was used for in vitro translations in a rabbit reticulocyte lysate according to the instructions of the manufacturer (Promega, Heidelberg, Germany).

RESULTS

Processing activity of an HCV NS3′-5B polyprotein. The goal of our studies was an examination of HCV polyprotein processing mediated by the NS3 serine-type proteinase by using an NS3′-5B polyprotein expressed with recombinant vaccinia viruses (Fig. 1) (a prime indicates an HCV protein with a nonauthentic amino or carboxy terminus). This polyprotein was selected because it was expressed to much higher levels than the full-length polyprotein (1a) and because it is predicted to properly reflect all the cleavage reactions mediated by the NS3 proteinase for two reasons: (i) we (2) and others (13, 17, 20, 36) have shown that sequences amino terminal to NS3 are dispensable for all NS3-mediated cleavages, and (ii) mutational ablation of the NS2-3 proteinase does not affect processing at any of the NS3-dependent cleavage sites (16, 20). The NS3′-5B polyprotein includes the complete NS3-4AB-5AB open reading frame and initiates with 20 amino acids derived from the carboxy terminus of NS2 (Fig. 2, vv1007-3011/wt). To correlate the generation of individual processing products with NS3-encoded activity, a further recombinant expressing an NS3′-5B polyprotein in which the putative active site serine residue was replaced by an alanine residue (vv1007-3011/S→A) was made (Fig. 2).

Infection of HeLa cells with recombinant virus expressing the active proteinase generated proteins with apparent molecular masses of about 70, 27, 58, and 68 kDa corresponding to NS3′, NS4B, NS5A, and NS5B, respectively, thus demonstrating specific cleavage at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites (Fig. 2). Two additional double bands in the molecular mass range of about 67 and 95 kDa were consistently observed in all immunoprecipitations. They corresponded to vaccinia virus proteins binding nonspecifically to protein A-Sepharose since their amounts could not be reduced in immunoprecipitations using homologous competitors (Fig. 2, + lanes). We did not detect NS4A, a protein of about 8 kDa, possibly because of low reactivity with our antiserum and low labelling efficiency (the predicted NS4A of our isolate would contain only one methionine). However, the appearance of NS3 and NS4B can be taken as evidence of cleavage between NS3 and NS4A as well as between NS4A and NS4B.

In addition, various amounts of processing intermediates were detected and identified by their apparent molecular weights and their reactivities with the individual antisera as NS4AB and, much more visible in the experiments described below (e.g., see Fig. 3A), NS4AB-5A and NS4B-5A . None of these cleavage products was obtained with the polyprotein in which the active site serine residue was replaced by an alanine residue. Instead, the unprocessed polyprotein with an apparent molecular mass of about 220 kDa was detected, illustrating that the NS3-encoded proteinase is essential for cleavage at all sites downstream of its own carboxy terminus.

Kinetics of NS3′-5B polyprotein processing. To identify possible precursors, pulse-labelling experiments were carried

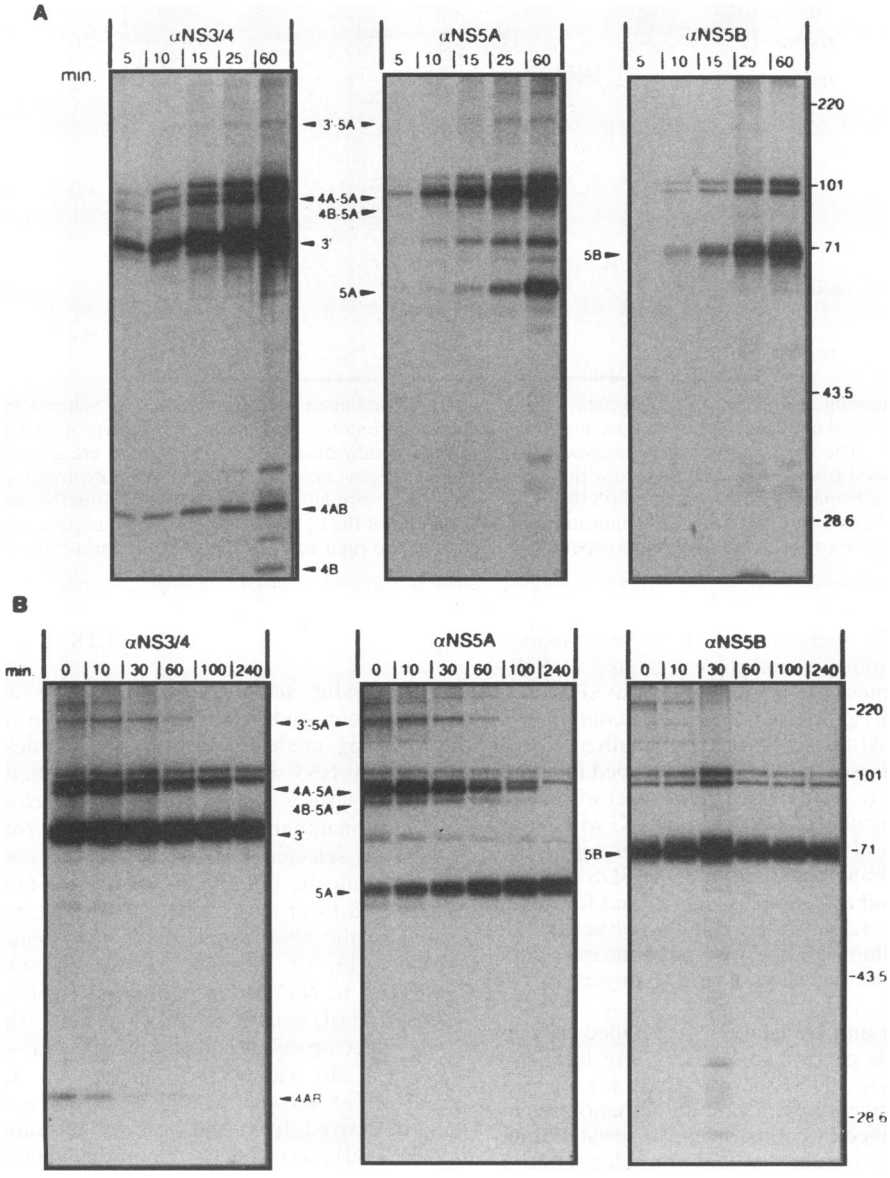

FIG. 3. Kinetic analyses of NS3′-5B polyprotein processing by continuous and pulse-chase labelling. (A) Cells were infected with the recombinant expressing NS3′-5B and 16 h later radiolabelled with [^{35}S]methionine for the indicated times. (B) Infected cells were labelled for 20 min and, after being washed, incubated in nonradioactive medium for various times. HCV-specific proteins were isolated from the cell lysate by immunoprecipitation with the indicated antisera.

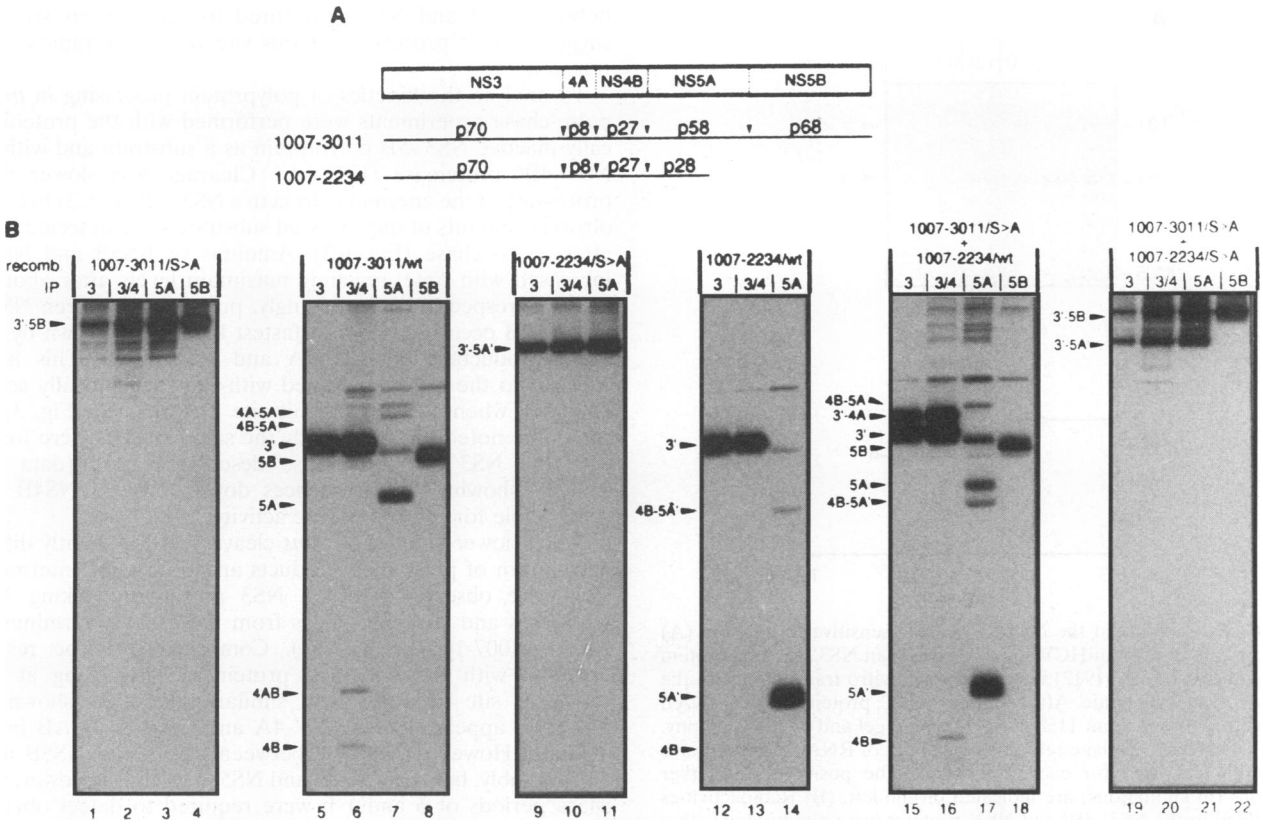

FIG. 4. Cleavage of an NS3′-5B polyprotein in *trans*. (A) Schematic representation of the HCV NS3′-5B and NS3′-5A′ polyproteins and the apparent molecular weights of processing products. (B) Cells were infected with either one or two recombinants (recomb.) expressing unaltered HCV polyproteins (wt) or polyproteins with an inactive NS3 proteinase (S→A). Sixteen hours after infection, cells were radiolabelled for 1 h and then underwent cell lysis and immunoprecipitation (IP).

out with HeLa cells infected with vaccinia virus recombinants expressing the enzymatically active NS3′-5B polyprotein. The processed NS3′ was already visible after 5 min of labelling (Fig. 3A, left panel); labelling times as short as 2 min still allowed clear detection of this protein (data not shown), suggesting that cleavage at the NS3/4A site is a rapid event. NS4B was detected after 60 min of continuous labelling (25 min with prolonged exposures), indicating delayed cleavage between NS4A and NS4B. In contrast, the NS4AB processing intermediate was generated very rapidly, being detectable after 5 min of labelling (Fig. 3A, left panel). NS5A and NS5B could be detected after 15 and 10 min of labelling, respectively. In addition to the mature processing products, several higher-molecular-mass proteins of about 85, 95, and 170 kDa were visible. Antiserum to NS3/4 and NS5A reacted with the 170 and 95 kDa proteins, whereas the 85 kDa protein was precipitated only by the NS5A-specific antiserum. None of these proteins reacted with the antiserum to NS5B. On the basis of these reactivities and deduced molecular masses, the 170-kDa protein can be designated NS3′-5A, the 95-kDa protein can be designated NS4AB-5A, and the 85-kDa protein can be designated NS4B-5A. No NS5B-specific processing intermediates were detected.

To analyze the stabilities of these proteins and to identify possible precursor-product relationships, pulse-chase experiments were performed (Fig. 3B). Results from immunoprecipitations with the NS3/4-specific antiserum revealed that NS3′ forms a stable peptide with a half-life of about 4 h. In contrast, the NS3′-5A and NS4AB intermediates were unstable and disappeared with half-lives of about 45 and 15 min, respectively. The only protein with a clear precursor-product relationship was NS5A, whose level increased over time concomitantly with a decrease of NS4AB-5A and NS4B-5A intermediates. Using longer pulse intervals, we could also detect NS4B but found it to be very unstable, with a half-life of only about 15 min (data not shown). The only protein detected with the NS5B-specific antiserum was NS5B (Fig. 3B), a stable protein decaying with a half-life of about 6 h.

Processing of an NS3′-5B polyprotein in *trans*. So far we had used a polyprotein in which the proteinase was part of the polyprotein and therefore directly linked with its substrate. To differentiate whether processing at the various sites was strictly an intramolecular reaction or could also occur intermolecularly, transcomplementation experiments were performed (Fig. 4). Cells were infected with a recombinant vaccinia virus expressing the proteolytically inactive NS3′-5B polyprotein along with a recombinant directing the expression of an NS3′-5A′ protein with an enzymatically active proteinase domain (Fig. 4A). As shown in Fig. 4B (lanes 15 to 18), this double infection yielded proteins corresponding to NS5A (lane 17) and NS5B (lane 18), both of which were not detected in cells expressing either inactive NS3′-5A′ or NS3′-5B protein (lanes 9 to 11 and 1 to 4, respectively) nor in cells expressing both defective polyproteins together (lanes 19 to 22). A novel processing product of 78 kDa which was not produced by the active NS3′-5B polyprotein (Fig. 4B, lanes 5 to 8) was observed

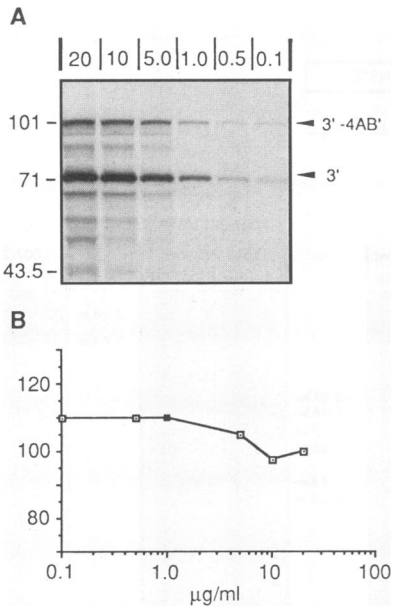

FIG. 5. Cleavage at the NS3/4A site is insensitive to dilution. (A) Various amounts of an HCV RNA encoding an NS3′-4B′ polyprotein (amino acids 1007 to 1912) were used for in vitro translation with the rabbit reticulocyte lysate. After 60 min at 30°C, proteins were analyzed by electrophoresis in an 11% polyacrylamide gel and by fluorography. Numbers above each lane refer to the amount of RNA (in micrograms per milliliter) used for each translation. The positions of marker proteins (in kilodaltons) are indicated on the left. (B) Radioactivities contained in the NS3′-4B′ and NS3′ proteins were quantitated with a phosphoimager. The precursor product ratio obtained with 20 μg/ml was set as 100.

reacting with the NS3/4-specific antiserum (compare lanes 6 and 16). The size and immunoreactivity of this protein, together with the fact that no NS4AB-5A was observed with this infection but only an NS4B-5A intermediate (Fig. 4B; compare lanes 7 and 17), suggested that this polypeptide corresponded to an unprocessed NS3′-4A fusion protein. It should be noted that the labelling procedure required to monitor efficient *trans* cleavage and the kinetics of processing in *trans* (see below) did not allow clear detection of the unstable NS4B (half-life of about 15 min). However, the appearance of NS3′-4A and NS5A is taken as evidence of processing at the NS4A/4B and NS4B/5A junctions. Taken together, we found that cleavage between NS4A and NS4B, NS4B and NS5A, and NS5A and NS5B could be mediated in *trans*, whereas processing at the NS3/4A site appeared to occur essentially in *cis*, i.e., intramolecularly.

If cleavage between NS3 and NS4A is an intramolecular reaction, it should follow first-order reaction kinetics and be concentration independent. Therefore, we examined the protein products obtained by in vitro translation of an NS3′-4AB′ RNA using different amounts of RNA to effectively change the concentration of the proteinase. As shown in Fig. 5A, translation of this RNA yielded unmistakable amounts of unprocessed precursor and the NS3′ cleavage product, while NS4AB′ could not be detected under these conditions (2). Quantitation of the radioactivities contained in these bands revealed no significant difference in the precursor-to-product ratio, irrespective of the RNA concentration (Fig. 5B). The same result was found with shorter and longer translation times (data not shown). These results together with the finding that cleavage between NS3 and NS4A appeared to occur in *cis* strongly suggested that processing at this site was an intramolecular reaction.

To analyze the kinetics of polyprotein processing in *trans*, pulse-chase experiments were performed with the proteolytically inactive NS3′-5B polyprotein as a substrate and with an NS3′-4B′ proteinase (Fig. 6A). Cleavage was slower than processing of the enzymatically active NS3′-5B (Fig. 3) because obvious amounts of unprocessed substrate were detected even after a 2-h chase (Fig. 6B). Amounts of NS5B and NS5A increased with time, reaching maximum levels after about 1 and 2 h, respectively. Surprisingly, processing between NS4A and NS4B occurred with the fastest kinetics as shown by the rapid production of NS3′-4A and NS4B-5AB. This is in contrast to the results obtained with the enzymatically active NS3′-5B, when cleavage at this site was delayed (Fig. 3). It should be noted that essentially the same kinetics were found with the NS3′-5A′ proteinase described above (data not shown), showing that sequences downstream of NS4B are dispensable for full proteolytic activity.

Much slower kinetics of *trans* cleavage and a slightly different pattern of processing products and processing intermediates were observed with an NS3 proteinase lacking NS4 sequences and 10 amino acids from the carboxy terminus of NS3 (vv1007-1647) (Fig. 6C). Compared with the results obtained with the NS3′-4B′ proteinase, processing at the NS4A/4B site occurred with similar kinetics as shown by the rapid appearance of NS3′-4A and the NS4B-5AB intermediate. However, cleavage between NS5A and NS5B and, most notably, between NS4B and NS5A was much slower, and chase periods of 6 and 1 h were required to detect obvious amounts of NS5A and NS5B, respectively. Furthermore, an unmistakable accumulation of the NS4B-5A processing intermediate during the 6-h chase was observed. Since essentially the same results were found with an NS3 proteinase with the authentic carboxy terminus (amino acid 1657 of the polyprotein) (data not shown), sequences of the NS4 region coexpressed with the proteinase enhance processing efficiencies at all *trans*-cleavage sites, particularly between NS4B and NS5A (see below).

Mapping of the minimal NS3 domain required for proteolytic activity. Sequence comparisons between NS3 of HCV and other viral and nonviral proteinases suggest that the proteolytic activity is located in the amino-terminal domain of the molecule. Hence, to map the minimal NS3 domain required for polyprotein processing, a series of recombinant vaccinia viruses was generated, directing the expression of various NS3 proteins beginning at amino acid 1007 of the polyprotein and ending at different positions in the NS3 region. These recombinants were used for double infections along with the recombinant expressing the inactive NS3′-5B substrate polyprotein. Because of the slow reaction kinetics observed with proteinases lacking NS4 sequences, in this and all subsequent experiments, cells were radiolabelled for 1 h and then chased for 6 h. To exclude possible cleavage products generated by cellular- or vaccinia virus-encoded enzymes, infections with wild-type vaccinia virus were included. As shown by the production of NS3′-4A, NS5A, and NS5B (Fig. 7A, B, and C, respectively), all variant NS3 proteinases could process the substrate at all *trans*-cleavage sites. In independent experiments, no significant differences with respect to processing efficiencies and kinetics were found between these NS3 truncations and the full-length NS3 proteinase (data not shown). Variations in the amounts of processing products were due to variations in the expression levels of the NS3 proteins and to their different stabilities. Most of them had half-lives much shorter than those observed

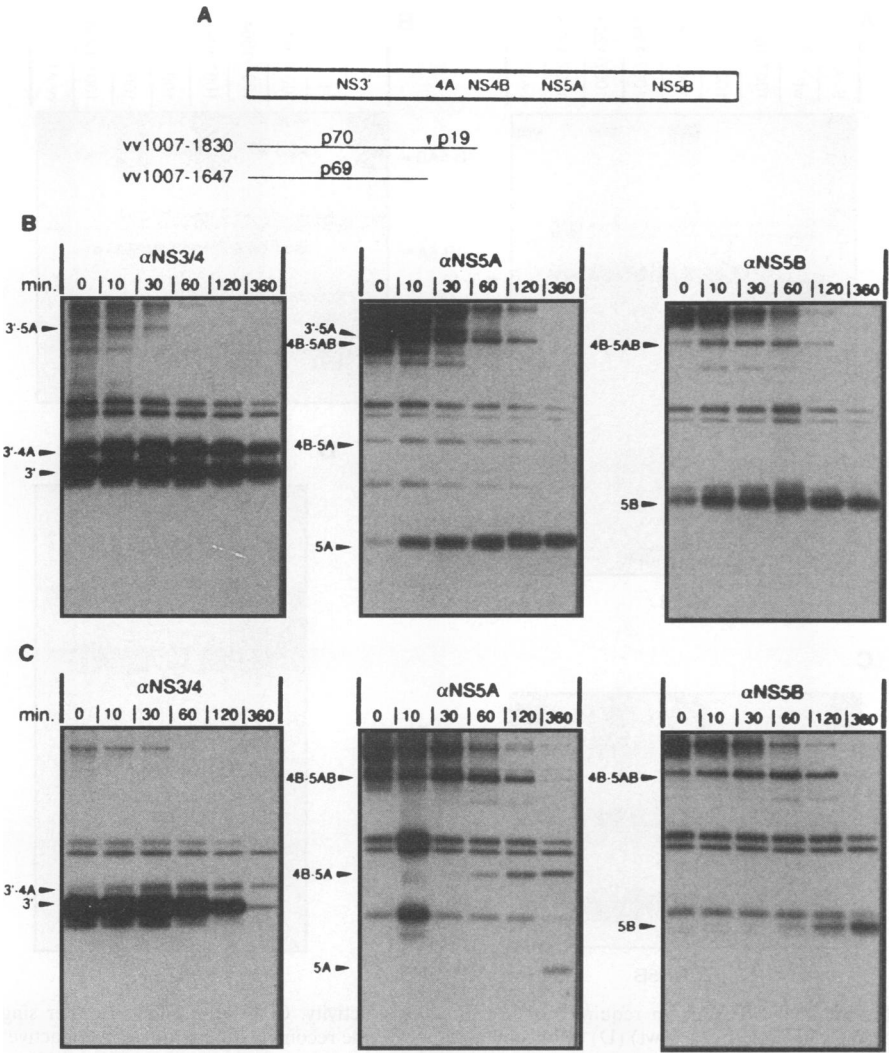

FIG. 6. Pulse-chase analysis of NS3′-5B polyprotein processing in *trans* by the NS3 proteinase in the presence and absence of NS4 sequences. (A) Schematic representation of the enzymatically inactive NS3′-5B polyprotein substrate and two variant proteinases. (B) Cells were infected with a combination of the recombinant expressing the inactive polyprotein and a recombinant expressing an enzymatically active NS3′-4B′ proteinase (amino acids 1007 to 1830 of the polyprotein). Sixteen hours after infection, proteins were radiolabelled metabolically for 20 min and then incubated in nonradioactive medium for the indicated times. (C) Results from an analogous experiment with the same NS3′-5B substrate and an almost complete NS3 proteinase lacking NS4 sequences (amino acids 1007 to 1647 of the polyprotein).

for full-length and nearly full-length NS3 molecules and could be detected only in cell lysates prepared directly after the pulse-labelling (Fig. 7D). In summary, these results demonstrate that the first 211 amino acids of NS3 (expressed from recombinant vv1007-1238) are sufficient for processing at all *trans*-cleavage sites.

To determine the borders of the minimal NS3 proteinase domain more precisely, the smallest protein was used to introduce a series of amino-terminal deletions which were tested in the same way. Removal of only 7 amino acids from the amino terminus of the NS3 protein abolished cleavage at the NS4B/5A site (vv1034-1238) (Fig. 8B) without affecting processing at the other sites (Fig. 8A and C). A similar pattern was found when 23 amino-terminal amino acids were deleted (vv1050-1238). Deletion of an additional 16 amino acids from the NS3 amino terminus also abolished cleavage at the NS5A/5B site, whereas processing between NS4A and NS4B still occurred, albeit with lower efficiency (vv1066-1238). None of the cleavage products was obtained with an inactive NS3 proteinase (vv1050-1238/S→A), excluding the possibility that vaccinia virus or cellular enzymes could substitute for an active NS3 proteinase. These results show that deletions in the NS3 domain have differential effects on processing at the various cleavage sites and that cleavage at the NS4B/5A site is the most sensitive to amino-terminal deletions in the proteinase domain, whereas *trans* cleavage between NS4A and NS4B can be mediated by a proteinase as short as 172 amino acids from the amino-terminal region of NS3.

Further attempts to narrow down the minimal proteinase domain were complicated by the fact that these proteins probably did not react or reacted very poorly with our NS3-specific antiserum; therefore, the expression of these proteins could not be determined.

NS4 sequences are essential for cleavage at the NS4B/5A site. In all the *trans*-cleavage assays described so far, we used the NS3′-5B polyprotein as a substrate. To analyze whether

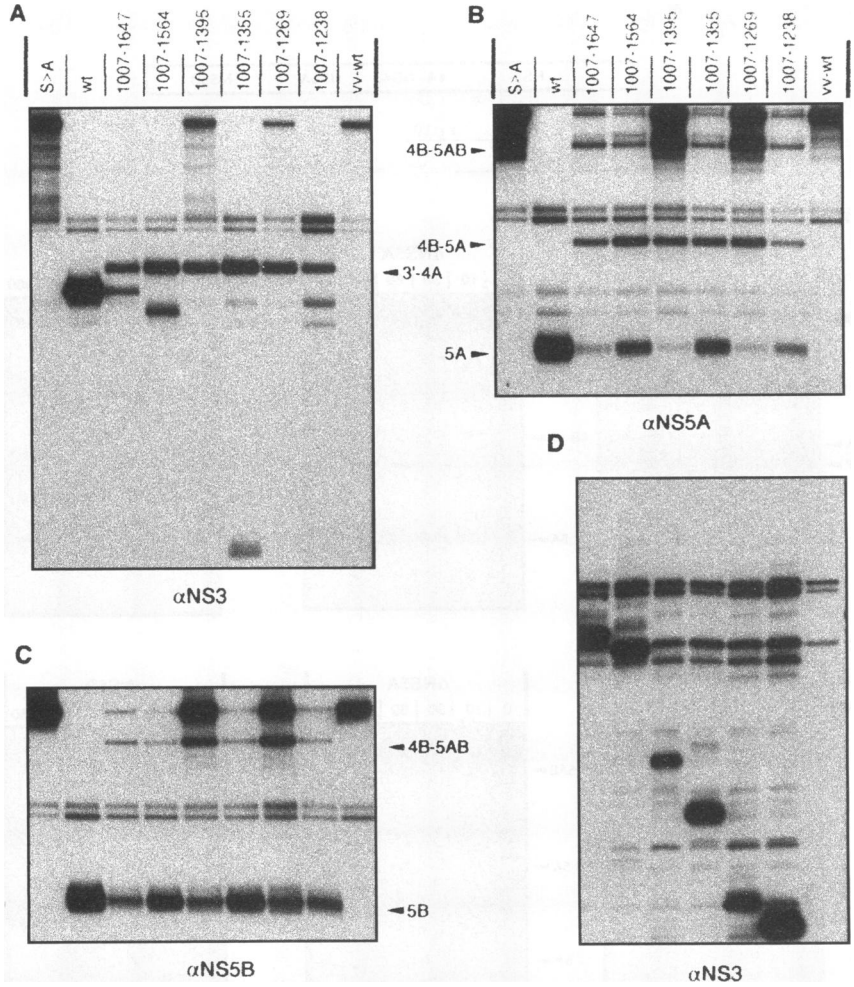

FIG. 7. Mapping of the minimal NS3 domain required for full proteolytic activity. Cells were infected either singly with one of the NS3 recombinants or with vaccinia wild-type virus (vv-wt) (D) or in combination with the recombinant expressing the inactive NS3′-5B substrate (A to C). Sixteen hours later, cells were labelled for 1 h and lysed directly (D) or after a 6-h chase (A to C). HCV-specific proteins were analyzed by immunoprecipitation with antisera specific for NS3 (A and D), NS5A (B), or NS5B (C). As a control, the results obtained with cells expressing an unaltered (wt) or mutated (S→A) NS3′-5B polyprotein are shown in the two lanes on the far left. Descriptions of the individual lanes in panels A and B also refer to the lanes directly below in panels C and D, respectively.

sequences of the substrate have an influence on cleavage at the various sites, recombinant vaccinia viruses expressing NS4A-5B, NS4B-5B, and NS5A-5B polyproteins were made, and we determined their processing patterns as generated by an NS3′-4B′ proteinase (Fig. 9, vv1007-1830, II) or the NS3′ proteinase lacking NS4 sequences and 10 amino acids from the NS3 carboxy terminus (Fig. 9, vv1007-1647, I).

trans cleavage of the NS4A-5B substrate with either of the proteinases gave rise to mature NS5A and NS5B, demonstrating cleavage at the NS4B/5A and NS5A/5B sites (Fig. 9A). Furthermore, small amounts of NS4B indicative of processing between NS4A and NS4B were detected (data not shown). Cleavage of the NS5A-5B polyprotein substrate by either of the proteinases occurred with similar efficiency (Fig. 9C). However, complete processing of the NS4B-5B substrate was observed only with the NS3′-4B′ proteinase (II) (Fig. 9B), whereas in the case of the pure NS3 proteinase, cleavage occurred only at the NS5A/5B junction and no processing between NS4B and NS5A was detected (I) (Fig. 9B). The same result was found when we increased the amount of the NS3′ proteinase by raising the multiplicity of infection of the vv1007-1647 recombinant. These results strongly suggest that NS4, particularly NS4A, is essential for processing between NS4B and NS5A and that NS4A provided by either the substrate (i.e., in *cis*) or the proteinase (i.e., in *trans*) can restore cleavage at this site.

DISCUSSION

The present study examined processing of the HCV NS proteins with the help of recombinant vaccinia viruses expressing an NS3′-5B polyprotein. This protein was chosen because it contains all the sequences required for NS3-mediated polyprotein processing and has considerably higher expression levels than the complete polyprotein (1a).

Results from kinetic studies revealed a complex pattern of NS polyprotein processing. The kinetics of processing between NS3 and NS4A were compatible with the view that cleavage at this site occurs first. However, in addition, small amounts of an NS3′-5A intermediate were detected, suggesting that alterna-

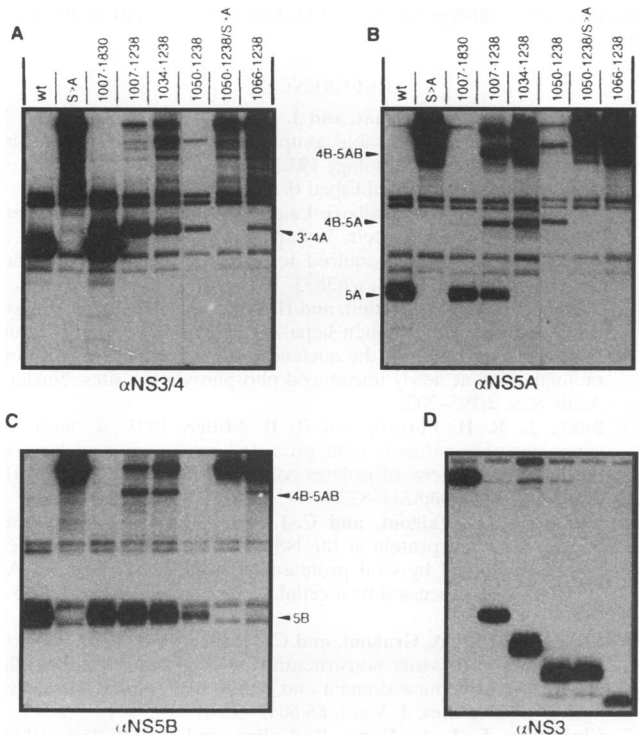

FIG. 8. Fine mapping of the minimal NS3 proteinase domain. Cells were doubly infected with the recombinant expressing the inactive NS3′-5B polyprotein substrate and one of several recombinants expressing various NS3 truncations. Following radiolabelling for 1 h, cells were lysed either directly (D) or after a 6-h incubation in nonradioactive medium (A to C). HCV-specific proteins were isolated by immunoprecipitation with the given antisera. Results obtained with cells expressing an unaltered (wt) or mutated (S→A) NS3′-5B polyprotein are shown in the two lanes on the far left. (D) To determine the expression of various NS3 truncations, cells were infected with the corresponding recombinants, labelled for 1 h, and analyzed by immunoprecipitation with the NS3-specific antiserum. Descriptions of the individual lanes in panels A and B also refer to the lanes directly below in panels C and D, respectively.

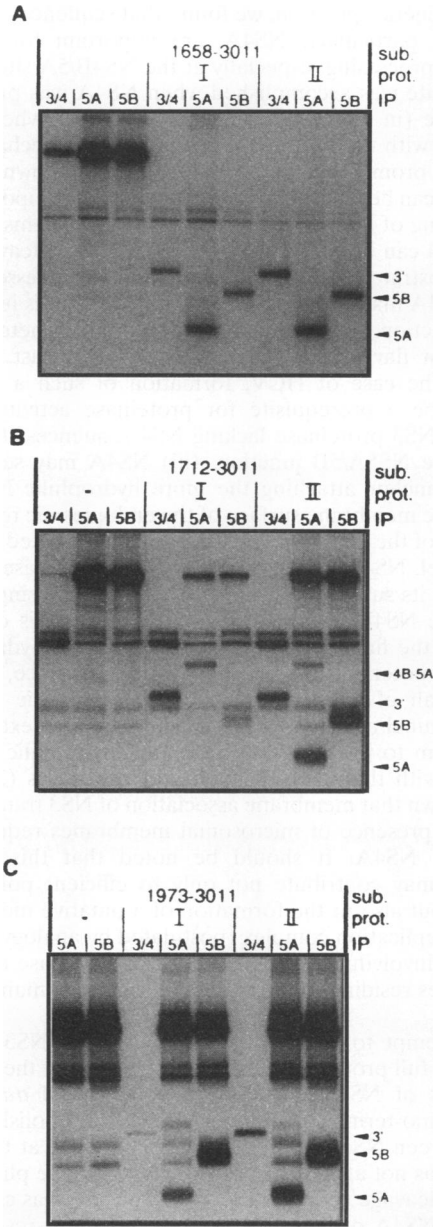

FIG. 9. Processing of NS4A-5B (A), NS4B-5B (B), and NS5A-5B (C) polyproteins (amino acids 1658 to 3011, 1712 to 3011, and 1973 to 3011 of the HCV polyprotein, respectively) in *trans* with recombinant vaccinia viruses expressing an almost complete NS3 proteinase (vv1007-1647) (I) or an NS3′-4B′ proteinase (vv1007-1830) (II). For details, see the legend to Fig. 7.

tive cleavages such as initial processing at the NS5A/5B site exist. Cleavage between NS4A and NS4B appeared to be delayed as shown by the presence of the NS4A/B intermediate and the slow production of NS4B, which was detected only after 60 min of labelling compared with 2 min in case of NS3.

At least two cleavage pathways seem to operate at the NS4B/5A site: (i) rapid cleavage as indicated by rapid production of the NS4AB intermediate and (ii) slow cleavage as indicated by the presence of the relatively stable NS4AB-5A intermediate (half-life of ca. 1 h), for which a clear precursor-product relationship was found. In contrast, processing between NS5A and NS5B appears to be rather efficient because no precursor of NS5B could be detected. Thus, NS5B seems to be generated either cotranslationally from an unprocessed polyprotein by an intramolecular reaction or from an unstable processing intermediate which is cleaved very rapidly intermolecularly.

Different kinetics were observed in the *trans*-cleavage reaction. Processing at the NS3/4A site could not be detected, and cleavage was fastest at the NS4A/4B site. A possible explanation for this apparent difference is that in the case of the enzymatically active NS3′-5B polyprotein, a transient intramolecular complex between the NS3 proteinase domain and NS4 sequences, particularly NS4A, forms, which brings the cleavage site in close proximity to the active site of the enzyme to ensure rapid processing between NS3 and NS4A. Consistent with this idea, we obtained strong evidence that cleavage at this site occurs in *cis*. However, in the case of the inactive NS3′-5B polyprotein, the proteinase domain would remain stably associated with NS4, making the cleavage site inaccessible to proteinase molecules provided in *trans*. Hence, one might expect processing at this site to occur in *trans* with a polyprotein lacking the NS3 proteinase domain. We are currently testing this hypothesis.

Using a genetic approach, we found that sequences from the NS4 region, particularly NS4A, are important for efficient polyprotein processing, especially at the NS4B/5A site. Cleavage at this site was accomplished when NS4A was present in the substrate (in *cis*) and even more efficiently when it was coexpressed with the proteinase (in *trans*). The mechanism by which NS4A promotes cleavage is currently not known. Several possibilities can be envisaged. (i) NS4A might be important for proper folding of the substrate. This possibility seems unlikely because NS4 can be provided in *trans* to restore cleavage and the NS5 substrate lacking NS4 sequences is processed properly. (ii) NS4A may form a complex with NS3 that is important for NS3 function and analogous to the NS2B/3 heterodimer described for flaviviruses (1). However, in contrast to those viruses, in the case of HCV, formation of such a complex would not be a prerequisite for proteinase activity per se because an NS3 proteinase lacking NS4 sequences still could cleave at the NS5A/5B junction. (iii) NS4A may serve as a membrane anchor attaching the more hydrophilic NS3 proteinase to the membrane surface of the endoplasmic reticulum, where most of the HCV proteins appear to be located (21, 30). In this model, NS4A would bring the NS3 proteinase in close proximity to its substrate, allowing efficient processing, particularly at the NS4B/5A junction. Sequence analysis of NS4A reveals that the first half of the molecule is very hydrophobic and has the potential to span the lipid bilayer once, whereas the other half of the molecule is very hydrophilic (5 of 10 carboxy-terminal amino acids are acidic) and may extend into the cytoplasm to interact with the more hydrophilic NS3. In agreement with this idea, Hijikata and coworkers (21) have recently shown that membrane association of NS3 translated in vitro in the presence of microsomal membranes requires the presence of NS4A. It should be noted that this kind of interaction may contribute not only to efficient polyprotein processing but also to the formation of a putative membrane-associated replication complex (postulated by analogy to flaviviruses [7]) involving the nucleoside triphosphatase and helicase activities residing in the carboxy-terminal domain of NS3 (34).

In an attempt to narrow down the minimal NS3 domain required for full proteolytic activity, we found that the first 211 amino acids of NS3 suffice for cleavage at all *trans* sites. Further amino-terminal deletions selectively abolished processing between NS4B and NS5A while cleavage at the other *trans* sites was not affected. Interestingly, the same phenotype, the loss of cleavage ability at the NS4B/5A site, was caused by the lack of NS4A protein. On the basis of these results, one might postulate specific interactions between the NS3 amino terminus and the NS4A protein. However, the existence and nature of such interactions remain to be determined.

For many viruses, proteolytic processing mediated by virus-encoded proteinases plays an important role for virus replication. Recently, it was shown that mutational ablation of the NS3-encoded proteinase of yellow fever virus severely reduced virus replication (8). Thus, it seems likely that the analogous proteinase of HCV plays an equally important role for the viral life cycle. Although we are still far away from a complete understanding of the pathways governing HCV polyprotein processing, our results are a first step in this direction and may provide a basis for the rational design of an antiviral drug.

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