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NS3 Is a Serine Protease Required for Processing of Hepatitis C Virus Polyprotein

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Hepatitis C virus (HCV) possesses a positive-sense RNA genome which encodes a large polyprotein of 3,010 amino acids. Previous data and sequence analysis have indicated that this polyprotein is processed by cellular proteases and possibly by a virally encoded serine protease localized in the N-terminal domain of nonstructural protein NS3. To characterize the molecular aspects of HCV protein biogenesis and to clearly identify the protein products derived from the HCV genome, we have examined HCV polyprotein expression by using the vaccinia virus T7 transient expression system in transfected cells and by cell-free translation studies. HCV proteins were identified by immunoprecipitation with region-specific antisera. Here we show that the amino-terminal region of the HCV polyprotein is processed in vitro by cellular proteases releasing three structural proteins: p21 (core), gp37 (E1), and gp61 (E2). Processing of the nonstructural region of HCV was evident in transfected cells. Two proteins of 24 and 68 kDa were immunoprecipitated with anti-NS2 and NS3 antisera, respectively. Antiserum against NS4 recognized three proteins of 6, 26, and 31 kDa, while antisera specific for NS5 immunoprecipitated two polypeptides of 56 and 65 kDa, indicating that each of these two genes encodes at least two different proteins. When the NS3 protease domain was inactivated by replacing the proposed catalytic Ser-1165 with Ala, processing at several sites was abolished. When Ser-1164 was mutated to Ala, no effect on the processing was observed. Cleavage activities at three of the four sites affected by NS3 were shown to occur in *trans*, while processing at the carboxy terminus of NS3 could not be mediated in *trans*. These results provide a detailed description of the protein products obtained from the processing of the HCV polyprotein. Furthermore, the data obtained implicate NS3 as a serine protease and demonstrate that a catalytically active NS3 is necessary for cleavage of the nonstructural region of HCV.

Hepatitis C virus (HCV) is considered to be the major etiologic agent of posttransfusion non-A, non-B hepatitis (4, 20). The enveloped virion consists of unknown species of structural proteins encoded by a positive-sense RNA genome. Sequence analysis has indicated that the viral genome is approximately 9,400 nucleotides long and includes a 5′ untranslated region of 341 nucleotides which precedes a single open reading frame (ORF) encoding a precursor polyprotein of 3,010 or 3,011 amino acids. This long ORF is followed by an untranslated region of 23 to 54 nucleotides located at the 3′ end (5, 18, 31). The genetic organization of the viral genome is similar to that of flaviviruses and pestiviruses, with the putative structural proteins located in the N-terminal region and a variety of nonstructural proteins located at the C terminus of the polyprotein (23). The putative structural region of HCV is shorter than that of flaviviruses and pestiviruses, and it lacks primary sequence similarity with these two virus families (6). However, it is organized in a similar fashion, with a basic N-terminal (p20) presumed nucleocapsid core protein (C), followed by two glycoproteins, gp35 and gp70. gp35 probably corresponds to the matrix/envelope glycoprotein in the virion (E1), whereas gp70 may correspond to an envelope glycoprotein equivalent to gp53/55 of pestiviruses (E2) or to the NS1 glycoprotein of flaviviruses (7, 25, 33). In vitro protein synthesis followed by amino acid sequence analysis of the products has demonstrated that these proteins are released from the precursor polyprotein by cellular proteases in association with membranes of the endoplasmic reticulum (14).

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The nonstructural region of the HCV genome has not been characterized in detail, but it is thought to be processed in a manner similar to that of flaviviruses and pestiviruses, releasing a series of proteins from the polyprotein precursor. The exact number of processed protein products derived from this region has not been identified. However, by analogy with the flaviviruses, the putative nonstructural polypeptides of HCV have been called NS2, NS3, NS4, and NS5. Although the amino acid sequence of the HCV polyprotein differs from that of the flavivirus polyprotein, the two polyproteins have similar hydropathy profiles. Tentative boundaries of the nonstructural proteins of HCV have been assigned on the basis of this similarity (5, 18, 31, 32). The NS2 and NS4 proteins are very hydrophobic, probably membrane bound, and of unknown function. Their predicted molecular sizes are 25 and 52 kDa, respectively (16). In flaviviruses, two proteins are encoded by each of the NS2 and NS4 genes (NS2a+b, and NS4a+b) (2). The NS5 gene of HCV is predicted to encode a polypeptide of 116 kDa, and it contains a GDD consensus sequence found in several viral RNA-dependent RNA polymerases, suggesting that it may be involved in viral replication (7, 31).

Cleavages generating the N termini of the flavivirus nonstructural proteins NS2b, NS3, NS4a, and NS5 follow dibasic amino acid residues, occur rapidly and efficiently in infected cells, and are mediated by a viral protease located in the cytoplasm (2, 24). The NS3 protein of pestiviruses and flaviviruses was found to be a component of the viral protease, as determined from sequence analysis and molecular modeling studies. The positions of three amino acid residues (His-53, Asp-77, and Ser-138) located within the N-terminal domain of NS3 are strictly conserved among

flaviviruses and are predicted to correspond spatially to the catalytic triad of trypsin-like serine proteases. Results from site-directed mutagenesis of these amino acid residues are consistent with the hypothesis that the catalytic activity resides in the NS3 domain and that these residues comprise the catalytic triad (1, 3, 34). Analysis of the amino acid sequence of the NS3 protein of HCV has suggested that this viral protein may encode a trypsin-like serine protease which could function in the processing of the viral polyprotein as in the case of flaviviruses and pestiviruses (23). Residues His-1083, Asp-1107, and Ser-1165, numbered according to their location within the HCV polyprotein, are found in the N-terminal domain of the NS3 protein and are highly conserved among all HCV strains sequenced so far (31). These residues are predicted to correspond spatially to the catalytic triad of the putative serine protease of HCV, suggesting that this protein may play a pivotal role in polyprotein processing. Furthermore, the NS3 polypeptide with a predicted molecular size of 69 kDa contains a nucleoside triphosphate-binding helicase domain that is presumably involved in unwinding of the RNA genome (3, 5).

In this study, we have examined HCV polyprotein expression and processing by cell-free protein synthesis studies and transient expression in transfected cells. We have identified specific protein products expressed by different regions of the viral genome by using region-specific antisera. We have obtained evidence that the structural components of HCV are processed in a fashion independent of NS3 synthesis, while a catalytically active NS3 protein is necessary for the correct maturation of the nonstructural proteins of HCV.

MATERIALS AND METHODS

Cells and virus. HeLa cells, originally obtained from the American Type Culture Collection, were grown in Dulbecco's modified Eagle's essential minimal medium (MEM) containing 10% fetal calf serum (FCS). Vaccinia virus vTF7-3 (10) was grown in RK-13 cells plated in Eagle's MEM containing 10% FCS.

Construction of recombinant plasmids. Plasmid pCD(38-9.4) encodes the HCV sequences from nucleotides 1 to 9416 downstream of a T7 promoter. The clone was constructed by joining individual cDNA fragments derived from plasmids BK146, BK144, BK112.1, BK112.5, and BK166 at overlapping restriction sites (31). The HCV cDNA clone was introduced into the plasmid vector pCDNA-1 (Invitrogen). The cDNA subclones were provided by Hiroto Okayama (Osaka University) and represent HCV clones isolated from Japanese patients.

pCITE(146) is derived from clone BK146 and contains HCV sequences from an *Msc*I site engineered at nucleotide 333 to an *Xba*I site introduced at nucleotide 4840. The cDNA fragment derived by polymerase chain reaction (PCR) amplification with sequence-specific primers lacks its own ATG and the untranslated region of HCV. The fragment was cloned downstream of a T7 promoter in the pCITE vector (Novagen) and inserted downstream of the 5′ untranslated region of encephalomyocarditis virus.

pSK(CORE) was derived from PCR amplification of nucleotides 330 to 938 (amino acid residues 1 to 200) with sequence-specific primers. The amplified DNA fragment derived from plasmid pCD(38-9.4) contains an *Xba*I site and an *Xho*I site engineered at the 5′ end and the 3′ end, respectively. The cDNA fragment was cloned downstream of a T7 promoter in the pBluescript vector SK II; it lacks the 5′ untranslated region of HCV and encodes the nucleocapsid protein core (11).

To construct plasmid pCITE(SX), clone pCD(38-9.4) was cleaved with *Sac*II and *Xba*I, and a DNA fragment containing nucleotides 3303 to 9416 (amino acid residues 991 to 3010) was purified. The DNA fragment was then inserted downstream of the T7 promoter into the *Bst*XI and *Xba*I sites of the expression vector pCITE.

pCITE(NS4-5) was obtained by cloning into the *Bst*XI and *Stu*I sites of pCITE a *Sph*I-*Kpn*I fragment derived from pCD(38-9.4) after treatment of both DNAs with Klenow polymerase. The construct encodes nucleotides 5281 to 9071 (amino acid residues 1651 to 2921) downstream of the T7 promoter and of the 5′ untranslated region of encephalomyocarditis virus.

pCITE(NS3) was derived from PCR amplification of nucleotides 3351 to 5171 (amino acid residues 1007 to 1616) with sequence-specific primers, using plasmid pCD(38-9.4) as the template. The amplified DNA fragment was cloned by blunt-end ligation into the expression vector pCITE which had been cleaved with *Nco*I and *Stu*I and blunted with Klenow polymerase.

Site-directed mutants in the NS3 catalytic serine, pCD(38-9.4:S_{1165}-A) and pCITE(SXS_{1165}-A), and the respective negative control mutants in the adjacent serine, pCD(38-9.4:S_{1164}-A) and pCITE(SXS_{1164}-A), were obtained by inserting the mutations in PCR primers that were then used to generate mutant DNA fragments according to the procedures of Higuchi et al. (13). The mutant DNA fragments were recloned into the parent plasmids by using restriction sites flanking the mutations and were subsequently sequenced. The triplet coding for serine 1165, TCG, was replaced by GCG, and that coding for serine 1164, TCT, was replaced by GCT. Both of these triplets code for alanine.

Constructs for the expression of TrpE fusion proteins with E1, E2/NS1, NS4, and NS5b sequences were made by using pATH plasmids (30). The NS2, NS3, and NS5a fusion proteins with glutathione *S*-transferase (GST) were made by using plasmid pGEX-3x (28). Cloning of the HCV fragments in the expression vectors was achieved by PCR amplification of the area of interest, using synthetic oligonucleotides containing appropriate restriction sites or by in-frame fusion of cDNA fragments by means of standard recombinant DNA protocols. Recombinant plasmids were transformed in *Escherichia coli* DH5α, with the exception of pCD(38-9.4), which was transformed in MC1061/P3.

Induction of expression plasmids and preparation of fusion proteins. TrpE fusion proteins were induced in *E. coli* DH5α cells harboring recombinant plasmids with of 3-β-hydroxy-indoleacrylic acid at a final concentration of 5 ng/ml. GST fusion proteins were expressed in *E. coli* DH5α cells upon induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The TrpE-E1, TrpE-E2/NS1, GST-NS2, TrpE-NS4, and TrpE-NS5b fusion proteins accumulated in the inclusion bodies of *E. coli* and were prepared by lysis of bacteria, DNase I digestion, and precipitation of the insoluble fraction as described previously (30).

The GST-NS3 and GST-NS5a fusion proteins were in the soluble fraction. These proteins were affinity purified on a glutathione-Sepharose CL4B column (Pharmacia), the HCV portion of the GST-NS3 protein was cleaved from the fusion protein by factor Xa (New England Biolabs) and purified as described previously (28).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel slices containing fusion proteins were ground in phosphate-buffered saline (PBS), emulsified in Freund's adjuvant, and used

to immunize rabbits (12). New Zealand White male and female rabbits were used for production of all antisera.

Immunoaffinity purification of anti-HCV antibodies from patient sera. Human antibodies against E1 and NS5a were immunopurified from patient sera. The TrpE-E1 protein was prepared in *E. coli* as described above, solubilized in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer, and run on a 10% polyacrylamide–SDS gel. The protein was transferred from the gel onto a nitrocellulose filter by electroblotting and used for immunoaffinity purification of anti-E1 antibodies as described previously (12).

Affinity-purified GST-NS5a fusion protein was cross-linked to an activated Affi-Gel 10 chromatography matrix (Bio-Rad). The affinity matrix thus obtained was then incubated for 1 h at 4°C with human sera and washed extensively with PBS, and the bound antibodies were eluted as described previously (12). In some preparations, anti-NS3 antibodies copurified with anti-NS5a immunoglobulins.

In vitro transcription and translation. Recombinant plasmids pCITE(146) and pSK(CORE) were linearized with restriction enzymes *Ssp*I and *Xho*I, respectively, and transcribed in vitro with T7 RNA polymerase as described previously (29). The transcripts were translated by using an mRNA-dependent rabbit reticulocyte lysate (Promega Biotec). All translation reactions were carried out in 50 μl in the presence or absence of canine pancreatic microsomal membranes (Promega Biotec). Translation mixtures were incubated at 30°C for 90 min with [^{35}S]methionine (Amersham) for labeling. Samples of translation mixtures were then immunoprecipitated with region-specific antisera, and the immunoprecipitated proteins were resolved by SDS-PAGE.

Preparation of labeled cell extracts. HeLa cells seeded at a density of 6×10^5 cells per plate were infected with vaccinia virus vTF7-3 at a multiplicity of 5 PFU per cell (19). After adsorption for 30 min at 37°C, 3 ml of Dulbecco's modified Eagle's MEM supplemented with 10% FCS was added. Cells were incubated an additional 30 min at 37°C. Twenty micrograms of recombinant plasmid DNA was precipitated in calcium phosphate as described previously (26) and added directly to each plate in a 500-μl volume. In the cotransfection experiments, 10 μg of each plasmid was precipitated in calcium phosphate. At 4 h posttransfection, the medium was replaced with MEM lacking methionine (GIBCO), and the cells were starved for 1 h at 37°C. Cells were then radiolabeled for 3 h with 400 μCi of ^{35}S label (ICN) in 2 ml of MEM lacking methionine and supplemented with 2% dialyzed FCS. Cells were harvested and prepared for immunoprecipitation in IPB_{150} (20 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1% Triton) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM dithiothreitol.

Immunoprecipitation. Prior to immunoprecipitation, SDS and dithiothreitol were added to the cell lysates to final concentrations of 2% and 10 mM, respectively. The lysates were then incubated at room temperature for 1 h and heated at 95°C for 10 min. Ten-microliter samples of antisera used in the immunoprecipitation reactions were preadsorbed for 1 h at 4°C in a 400-μl volume of IPB_{150} with vT7F3-infected HeLa cell extracts spotted on nitrocellulose filters. The antibody suspension was then incubated with 60 μl of protein A (PA)-Sepharose for 1 h at 4°C. The PA-Sepharose beads were pelleted by centrifugation, washed three times in 1 ml of IPB_{150}, resuspended in 400 μl of IPB_{150}, and incubated for an additional hour at 4°C with 20 μl of cell lysate. All reactions were performed with constant mixing on an end-over-end rotator. The PA-Sepharose suspension was then layered on 0.9 ml of 0.5× NDET (0.5% Nonidet P-40, 0.2% sodium deoxycholate, 33 mM EDTA, 10 mM Tris-Cl [pH 7.4]) containing 30% sucrose and pelleted by centrifugation in a microcentrifuge for 10 min at room temperature. The pellet was resuspended in 300 μl of NDET–0.3% SDS and then washed twice with the same buffer and once with water (27). The sample was resuspended in 20 μl of sample buffer and heated at 95°C, and the supernatant was then subjected to SDS-PAGE.

TABLE 1. Region-specific antisera to E1, E2/NS1, NS2, NS3, NS4, and NS5

Target protein	Predicted boundaries (amino acids)[a]	HCV cDNA fragment (nucleotides)[b]	Antiserum specificity
E1	193–383	990–1350 (220–340)	E1[c]
E2/NS1	384–729	1501–2525 (392–733)	E2/NS1
NS2	731–1007	3018–3301 (896–990)	NS2
NS3	1008–1616	3890–4716 (1187–1496)	NS3
NS4	1617–2013	5184–6210 (1618–1960)	NS4a, NS4b
NS5	2014–3011	6940–7467 (2204–2379)	NS5a[c]
		8079–8926 (2585–2880)	NS5b

[a] Boundaries of E1 and E2/NS1 on the HCV polyprotein were assigned as described by Hijikata et al. (14). The limits of the nonstructural proteins were assigned as described by Takamizawa et al. (31).

[b] cDNA fragments were cloned in pATH and pGEX-3x expression vectors as described in Materials and Methods. Amino acid positions are given in parentheses.

[c] Antibodies specific for this protein were purified from HCV-seropositive patients as described in Materials and Methods.

RESULTS

Production of fusion proteins and region-specific antisera. To monitor the expression and processing of the HCV polyprotein, several region-specific antisera were prepared in rabbits against HCV fusion proteins expressed in bacteria. Human polyclonal antibodies specific for the N-terminal half of NS5 and for the E1 protein were purified from patient sera by immunoaffinity. Table 1 describes the cDNA fragments used for the construction of pATH/HCV and pGEX/HCV plasmids containing the relevant regions of the HCV genome. The cDNA fragments were chosen on the basis of putative boundaries of each HCV viral protein. These boundaries were established by comparing the HCV polyprotein sequence with that of flaviviruses and identifying the putative processing sites which could be responsible for the release of HCV proteins from the polyprotein precursor (14, 31).

In vitro processing of the structural proteins of HCV. Cell-free protein synthesis experiments were performed with truncated cDNA clones to examine the processing of HCV structural proteins. Figure 1 shows a diagram of all constructs used in this study. The results of in vitro processing assays using clones pCITE(146) and pSK(CORE) are shown in Fig. 2. The translation product of an RNA derived from plasmid pCITE(146) linearized at the *Ssp*I site at position 2873 was processed into three major proteins of 21, 37, and 61 kDa (Fig. 2, lane 1). The pattern of translation was significantly different when the reaction was carried out in the absence of microsomal membranes, as shown by the lack of the processed protein bands and by the presence of a large precursor which ran close to the origin of the gel (Fig. 2, lane 2). The 61-kDa protein was immunoprecipitated with a region-specific antiserum directed against the putative E2/NS1 region (Fig. 2, lane 3). The p21 polypeptide originates from the amino-terminal region of the polyprotein, and it was

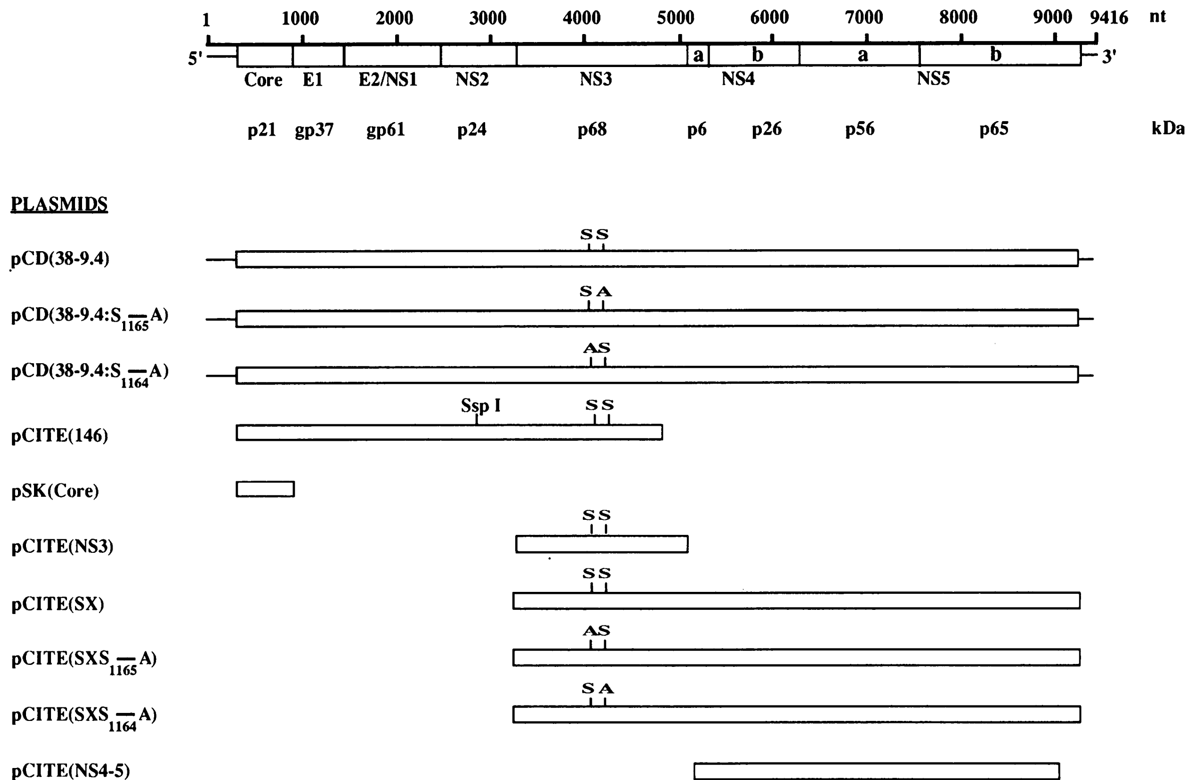

FIG. 1. Schematic representation of the HCV genome, predicted protein-coding domains, and recombinant expression plasmids used in this study. The viral genome is shown at the top (thin line, untranslated regions; open box, ORF); the molecular sizes of the viral proteins are indicated. The constructs expressing specific regions of the HCV genome are shown below. The name of each plasmid is shown on the left. The *Ssp*I site at nucleotide (nt) 2873 used to linearize plasmid pCITE(146) in the in vitro transcription-translation experiments is indicated. The letter designations outside the ORF box of the recombinant plasmids indicate the serine (S) residues at amino acid 1164 and 1165 which have been individually changed to an alanine (A) residue in several constructs.

identified as the core protein since it comigrated with the translation product derived from plasmid pSK(CORE) (Fig. 2, lane 4). The 37-kDa protein is derived from the middle portion of pCITE(146) and probably corresponds to the structural protein E1. Thus, the results are in agreement with the published data indicating that in vitro processing of the structural region of HCV polyprotein is dependent of the presence of microsomal membranes in the translation reaction, suggesting that it is mediated by cellular signal peptidases (14).

Transient expression of HCV cDNA encoding the entire polyprotein. Attempts to use cell-free protein synthesis to examine processing of the nonstructural region of HCV polyprotein were undermined by complex patterns of translation products, making it difficult to obtain conclusive results (data not shown). To examine the processing of the nonstructural region, we used the vaccinia virus T7 transient expression system (10). This system is based on the transfection of mammalian cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase. The T7 RNA polymerase produced by the recombinant vaccinia virus drives the expression of the transfected plasmid. Plasmid pCD(38-9.4) was constructed with the entire HCV ORF positioned downstream of bacteriophage T7 RNA polymerase promoter and was used for transfection experiments in HeLa cells. The transfected cells were labeled with [^{35}S]methionine, and the cell lysates were denatured in SDS and then immunoprecipitated with region-

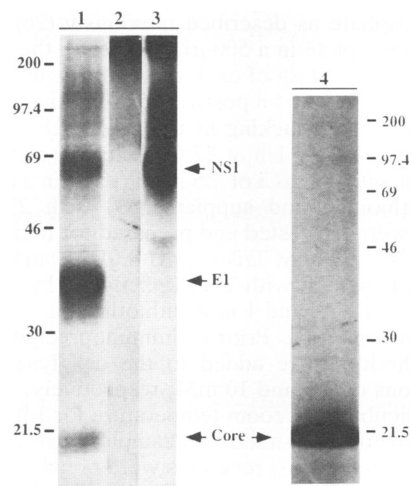

FIG. 2. Autoradiogram of SDS-PAGE analysis of the in vitro translation products. The transcripts of pCITE(146) (lanes 1 to 3) and of pSK(Core) (lane 4) were translated in vitro with a rabbit reticulocyte lysate in the presence (lanes 1, 3, and 4) or absence (lane 2) of microsomal membranes. Translation products labeled with [^{35}S]methionine were analyzed on an SDS–10% polyacrylamide gel directly (lanes 1, 2, and 4) or after immunoprecipitation with an anti-E2/NS1 antiserum (lane 3). Positions of the relevant translation products and of molecular weight standards (in kilodaltons) are indicated.

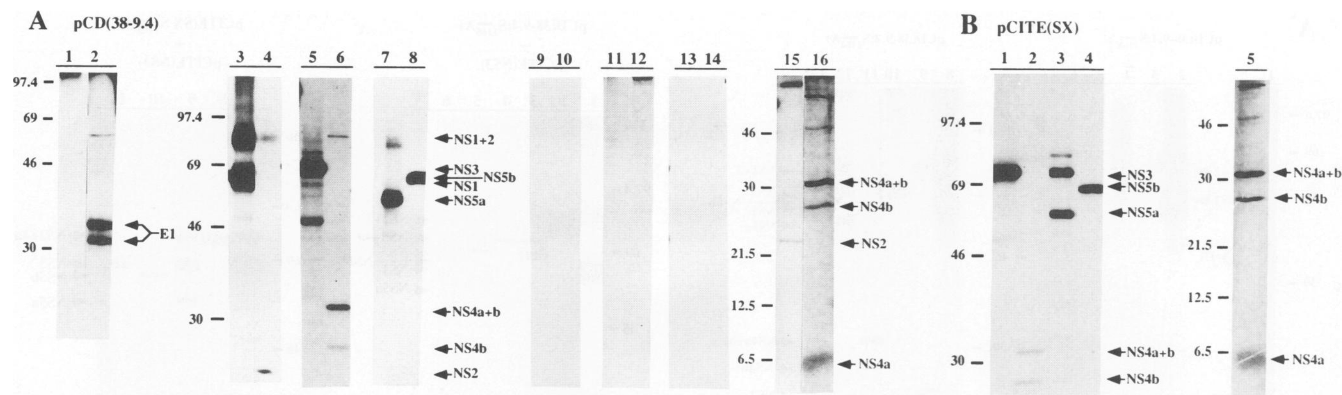

FIG. 3. Transient expression of plasmids pCD(38-9.4) and pCITE(SX) in mammalian cells. Recombinant plasmids pCD(38-9.4) and pCITE(SX) were transfected into vaccinia virus vT7F-3-infected HeLa cells as described in Materials and Methods. Cells were labeled with [^{35}S]methionine for 3 h, and SDS-denatured lysates were immunoprecipitated with antisera to specific regions of HCV ORF. Positions of the relevant HCV proteins immunoprecipitated with anti-HCV antibodies are indicated. Sizes of molecular weight standards are indicated in kilodaltons. (A) Lysates from cells transfected with pCD(38-9.4) were immunoprecipitated with anti-E1 (lane 2), anti-E2/NS1 (lane 3), anti-NS2 (lanes 4 and 15), anti-NS3 (lane 5), anti-NS4 (lanes 6 and 16), anti-NS5a (lane 7), and anti-NS5b (lane 8) antibodies. The immunoprecipitated products were resolved on an SDS–10% (lanes 1 to 14) or SDS–15% (lanes 15 and 16) polyacrylamide gel. Mock-transfected cell lysate was also immunoprecipitated with anti-HCV antibodies (lane 1, anti-E1; lane 9, anti-E2/NS1; lane 10, anti-NS2; lane 11, anti-NS3; lane 12, anti-NS4; lane 13, anti-NS5a; lane 14, anti-NS5b). (B) Lysates from cells transfected with pCITE(SX) were immunoprecipitated with anti-NS3 (lane 1), anti-NS4 (lanes 2 and 5), anti-NS5a (lane 3), and anti-NS5b antibodies (lane 4). Immunoprecipitated products were resolved on an SDS–10% (lanes 1 to 4) or SDS–15% (lane 5) polyacrylamide gel.

specific antisera. As shown in Fig. 3A, transfection of pCD(38-9.4) generated proteins that reacted specifically with anti-HCV antibodies. Two bands of 34 and 37 kDa were immunoprecipitated with the anti-E1 antiserum, indicating that these two polypeptides are derived from the E1 region (Fig. 3A, lane 2). The multiple bands of the expressed E1 protein may be due to incomplete processing or glycosylation in this experimental system. Anti-E2/NS1 antiserum immunoprecipitated a 61-kDa protein as observed in the in vitro translation studies; this protein presumably represents the mature form of E2/NS1. A second band of 77 kDa was recognized by anti-E2/NS1 and anti-NS2 antisera, suggesting that it may represent an uncleaved E2/NS1-NS2 precursor (Fig. 3A, lanes 3 and 4). A 24-kDa band was immunoprecipitated with the NS2-specific antiserum, consistent with the predicted molecular weight (Fig. 3A, lane 4). An NS3-related protein of 68 kDa was produced, as expected from the predicted molecular weight of this protein. A minor 47-kDa band was also consistently observed in the immunoprecipitation reactions, which could be due to an alternate cleavage of NS3 (Fig. 3A, lane 5). NS4-related proteins of 6, 26, and 31 kDa were immunoprecipitated with the region-specific antiserum, indicating that this region of the HCV polyprotein is cleaved into distinct protein products (Fig. 3A, lane 6). The NS2- and NS4-related proteins were clearly visible on an SDS–15% polyacrylamide gel (Fig. 3A, lanes 15 and 16). The 6-kDa protein was designated NS4a, and the 26-kDa protein species was called NS4b; the 31-kDa band probably represents the NS4a-NS4b precursor (see below).

Two proteins of 56 and 65 kDa were immunoprecipitated with the antisera specific for the N and C halves of the NS5 gene, respectively, indicating that the predicted 116-kDa protein is cleaved into at least two smaller products (Fig. 3A, lanes 7 and 8). The N-terminal protein was designated NS5a, whereas the C-terminal protein was called NS5b. In addition, the antisera specific for NS4 and NS5 recognized higher-molecular-weight bands which probably represent uncleaved precursors. These data suggest that apparently authentic processing of the entire HCV polyprotein can occur in this test system and that the function of NS3 as a viral protease can be determined.

In view of the observation that cleavage of the nonstructural region of the flavivirus polyprotein is dependent not only on the NS3 protease but also on the NS2b protein product, which may act as a cofactor for functional activity (3, 9), we determined whether the HCV polyprotein region encoding proteins NS3, NS4, and NS5 could be expressed and processed correctly despite the absence of the structural proteins and of NS2. To this end, a plasmid encompassing nucleotides 3303 to 9416 was constructed. This clone, named pCITE(SX), expresses all of NS3, NS4, and NS5 in addition to a few amino acid residues of NS2. As shown in Fig. 3B, transfection of pCITE(SX) generated proteins consistent in size with processed NS4a, NS4b, NS5a, and NS5b. The NS3 protein band immunoprecipitated with the anti-NS3 antiserum was slightly larger than that expressed by the full-length clone pCD(38-9.4), with an apparent molecular size of 74 kDa (Fig. 3B, lane 1). Furthermore, NS3 coprecipitated with the NS5a protein in several immunoprecipitation experiments because of the presence of anti-NS3 antibodies in some of the human anti-NS5a immunoglobulin preparations used in these studies (Fig. 3B, lane 3). These data indicate that processing of the N termini of NS4a, NS4b, NS5a, and NS5b is independent of the NS2 protein, while cleavage at the N terminus of NS3 may be affected by NS2 sequences.

The NS3 catalytic domain is necessary for processing of the HCV polyprotein. Site-directed mutagenesis was used to test the role of the putative NS3 protease domain in processing of the HCV polyprotein. Mutagenesis of this protein was accomplished by using synthetic oligonucleotides and PCR as described in Materials and Methods. Nucleotide 3825, T, was converted to a G, resulting in an amino acid change at position 1165. This mutation substitutes the catalytic serine residue with alanine. The mutant plasmid was designated pCD(38-9.4:S_{1165}-A). As a comparison, Ser-1164, which is not part of the putative catalytic triad, was changed to Ala by

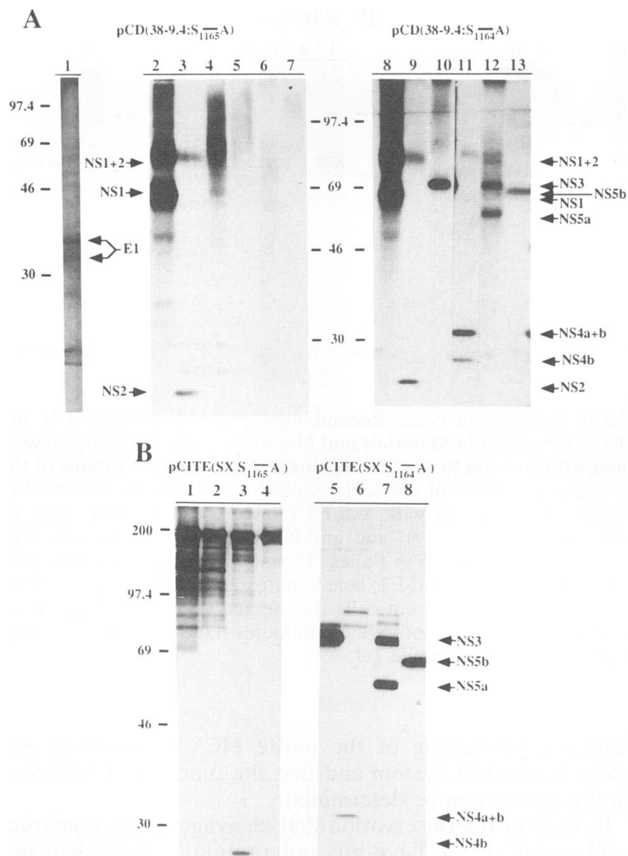

FIG. 4. Processing of the HCV ORF containing altered residues in NS3. Constructs containing a Ser-to-Ala substitution at the putative catalytic Ser-1165 or at Ser-1164 were transfected into vTF7-3-infected HeLa cells. Cell lysates were immunoprecipitated with anti-HCV antibodies, and HCV proteins were resolved on an SDS–10% polyacrylamide gel. Positions of relevant HCV proteins and of molecular weight standards (in kilodaltons) are indicated. (A) Lysates from cells transfected with plasmid pCD(38-9.4:S_{1165}-A) or pCD(38-9.4:S_{1164}-A) were immunoprecipitated with anti-E1 (lane 1), anti-E2/NS1 (lanes 2 and 8), anti-NS2 (lanes 3 and 9), anti-NS3 (lanes 4 and 10), anti-NS4 (lanes 5 and 11), anti-NS5a (lanes 6 and 12), and anti-NS5b (lanes 7 and 13) antibodies. (B) Lysates from cells transfected with plasmid pCITE(SXS_{1165}-A) or pCITE (SXS_{1164}-A) were immunoprecipitated with anti-NS3 (lanes 1 and 5), anti-NS4 (lanes 2 and 6), anti-NS5a (lanes 3 and 7), and anti-NS5b (lanes 4 and 8) antibodies.

substituting nucleotide 3822, T, with a G. This construct was designated pCD(38-9.4:S_{1164}-A). Mutagenesis was confirmed by sequencing the region of the mutation in both parent and mutant plasmids.

The transfection of plasmid pCD(38-9.4:S_{1165}-A) in HeLa cells resulted in the correct synthesis of the putative structural proteins, as indicated by the immunoprecipitation of E1 and E2/NS1 (Fig. 4A, lanes 1 and 2). NS2 was also released from the polyprotein precursor and immunoprecipitated with the region-specific antiserum (Fig. 4A, lane 3). The mature products of NS3, NS4a, NS4b, NS5a, and NS5b were not detectable in the transfected cell extracts, suggesting that the mutation of the putative catalytic Ser residue had specifically interfered with the cleavage of these proteins (Fig. 4A, lanes 4 to 7). A similar pattern of immunoprecipitations of uncleaved precursors was observed when the Ser-to-Ala substitution was introduced at position 1165 in construct pCITE(SX) (Fig. 4B, lanes 1 to 4). In contrast, the Ser-to-Ala substitution at residue 1164 did not have any effect on the processing profile of the HCV protein products, as shown by the transfection of plasmids pCD(38-9.4:S_{1164}-A) (Fig. 4A, lanes 8 to 13) and pCITE(SXS_{1164}-A) (Fig. 4B, lanes 5 to 8). Both the structural and nonstructural proteins were detected in their processed form. Thus, these results are consistent with the hypothesis that the NS3 protein is indeed a serine protease and that residue 1165 is the catalytic serine of this enzyme. Furthermore, they demonstrate that a functional NS3 protease domain is required for efficient cleavage at the NS3-NS4a, NS4a-NS4b, NS4b-NS5a, and NS5a-NS5b sites.

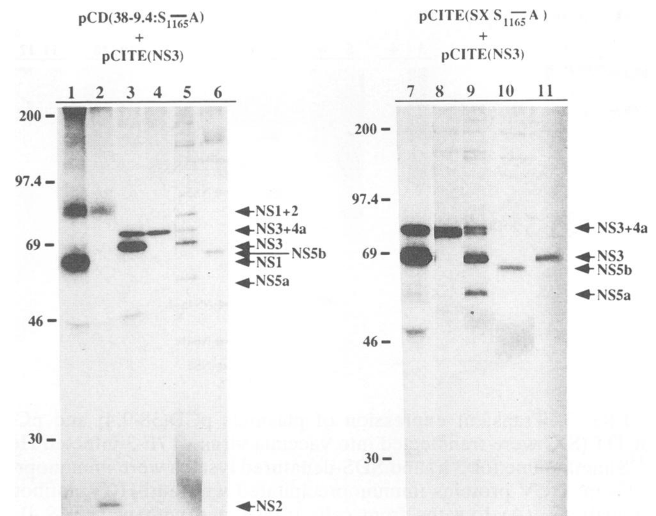

FIG. 5. Cotransfection experiments to examine *trans* cleavage of a catalytically inactive HCV ORF substrate. Plasmid pCITE(NS3) was cotransfected with pCD(38-9.4:S_{1165}-A) or pCITE(SXS_{1165}-A) as described in the legend to Fig. 3 and in Materials and Methods. Cell lysates were immunoprecipitated with anti-E2/NS1 (lane 1), anti-NS2 (lane 2), anti-NS3 (lanes 3 and 7), anti-NS4 (lanes 4 and 8), anti-NS5a (lanes 5 and 9), and anti-NS5b (lanes 6 and 10) antibodies and loaded on an SDS–10% polyacrylamide gel. NS3 protein immunoprecipitated from lysate of cells transfected with pCITE(NS3) alone is shown in lane 11. Positions of relevant HCV proteins and of molecular weight standards (in kilodaltons) are indicated.

***trans*-cleavage activity of NS3.** The data presented above established that NS3 is required for the proper biogenesis of mature HCV proteins. To determine whether processing at the NS3-dependent sites could be mediated in *trans*, plasmid pCD(38-9.4:S_{1165}-A) was cotransfected with pCITE(NS3), a clone expressing amino acids 1007 to 1615 encompassing the entire NS3 protease domain. Figure 5 illustrates the results of the cotransfection experiments. As expected, coexpression of the NS3 protein with the entire polyprotein lacking a functional NS3 did not change the processing of the structural region and of NS2, since the cleavage of these proteins does not require NS3 (Fig. 5, lanes 1 and 2). The wild-type expression of NS5a and NS5b was restored, indicating that cotransfection of the NS3 clone with the mutated plasmid had abolished abnormal processing of these proteins (Fig. 5, lanes 5, 6, 10, and 11). In contrast, the NS4-specific antiserum immunoprecipitated a 78-kDa band not detected in cells transfected with pCD(38-9.4:S_{1165}-A) or pCITE (SXS_{1165}-A) alone. This protein, in addition to the NS3

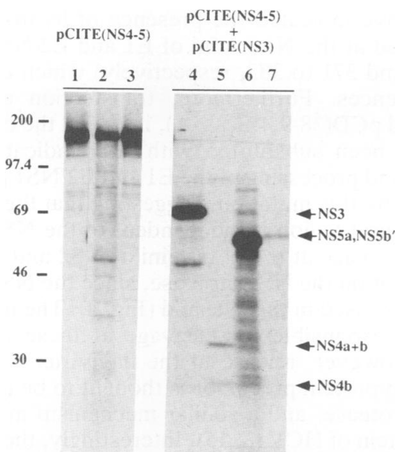

FIG. 6. Processing of HCV polyprotein substrate in *trans*. Plasmid pCITE(NS4-5) was transfected alone (lanes 1 to 3) or with clone pCITE(NS3) (lanes 4 to 7). Cell lysates were immunoprecipitated with anti-NS3 (lane 4), anti-NS4 (lanes 1 and 5), anti-NS5a (lanes 2 and 6), and anti-NS5b (lanes 3 and 7) antibodies. The truncated form of NS5b is indicated as NS5b′. Proteins were resolved on an SDS–10% polyacrylamide gel. Positions of molecular weight standards (in kilodaltons) and of relevant HCV products are indicated.

product derived from pCITE(NS3), was recognized by the NS3-specific antiserum (Fig. 5, lanes 3, 4, 7, and 8). The 78-kDa protein probably represents the uncleaved NS3-NS4a precursor. The processed NS4a and NS4b products were not detected in the cotransfected cells, suggesting that expression of the NS4-related proteins had not been restored to normal by the cotransfection of pCITE(NS3) and pCD(38-9.4:S_{1165}-A) (Fig. 5, lanes 4 and 8).

To further examine processing of the NS4 and NS5 regions, cotransfection experiments were performed with pCITE(NS3) and pCITE(NS4-5). The latter plasmid contains nucleotides 5281 to 9071 encoding amino acids 1651 to 2921 which represent NS4 and part of NS5. Transfection of pCITE(NS4-5) alone resulted in the synthesis of uncleaved precursors which were immunoprecipitated by both NS4- and NS5-specific antisera (Fig. 6, lanes 1 to 3). In contrast, cotransfection with pCITE(NS3) resulted in the synthesis of mature forms of NS4a, NS4b, and NS5a and of the truncated form of NS5b, NS5b′ (Fig. 6, lanes 4 to 7). Thus, cleavage at the N termini of NS4b, NS5a, and NS5b can be mediated in *trans* by NS3 whereas processing at the N terminus of NS4a cannot, suggesting that cleavage occurs as an intramolecular event (in *cis*).

To characterize in detail the NS4-related proteins, we compared the molecular weights of the NS4 polypeptides derived from the cotransfection of pCITE(NS3) and pCITE(NS4-5) with those of the NS4 products obtained from the transfection of pCD(38-9.4). As shown in Fig. 7, NS4a protein derived from pCD(38-9.4) is slightly smaller than that obtained from pCITE(NS4-5). This difference in molecular weight is probably due to the presence of NS3-related residues at the N terminus of NS4 derived from pCITE(NS4-5) which cannot be removed in *trans* by the HCV protease. This observation suggests that the actual border between NS3 and NS4 is located downstream of amino acid residue 1651. Furthermore, the observation that the difference in molecular weight is also observed for the putative NS4a+b precursor but not for the NS4b protein

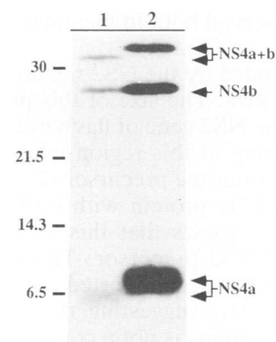

FIG. 7. Identification of NS4 precursor and mature products. Lysates from cells transfected with plasmid pCD(38-9.4) (lane 1) or pCITE(NS4-5) (lane 2) were immunoprecipitated with anti-NS4 antibodies. HCV proteins were resolved on an SDS–15% polyacrylamide gel. Positions of molecular weight standards (in kilodaltons) and of relevant HCV proteins are indicated.

supports our interpretation of the 31-kDa polypeptide as the NS4a+b precursor; as observed for the NS4a polypeptide, the NS4a+b protein derived from plasmid pCITE(NS4-5) contains additional residues originating from NS3 and therefore is larger than the same protein derived from the wild-type plasmid.

DISCUSSION

We have analyzed HCV protein biogenesis by in vitro translation studies and by transient expression in mammalian cells to characterize the sizes of the proteins expressed by specific regions of the HCV ORF and to determine the role of cellular protease and of the putative viral protease in HCV polyprotein processing. The results obtained confirm the previously reported observation that the structural region of HCV is processed by cellular proteases independently of the presence of the nonstructural proteins (14). Furthermore, they demonstrate that the NS3 protein is a serine protease and that a catalytically active NS3 is required for the proper cleavage of most of the nonstructural proteins.

A more detailed description of the genetic order and size of the proteins encoded by the HCV genome can now be provided on the basis of immunoprecipitation of virus-specific proteins with antisera directed against precise regions of the HCV ORF. In addition to the putative structural components of HCV, which have already been mapped to the N terminus of HCV ORF, other proteins can now be identified. Therefore, the HCV genetic order is the following: NH_2-p21-gp37-gp61-p24-p68-p6-p26-p56-p65-COOH. p21, gp37, and gp61 correspond to the structural proteins C, E1, and E2/NS1, respectively, as shown by the in vitro translation of the most N-terminal 850 amino acids (Fig. 2) and as reported by Hijikata et al. (14). Interestingly, the expression of E1 in transfected cells resulted in the synthesis of two proteins of 34 and 37 kDa which were both immunoprecipitated with an anti-E1 antiserum (Fig. 3). The pattern of expression of the E1 protein in transfected cells is therefore different from that observed in in vitro translation studies, in which a single protein product of 37 kDa is observed (Fig. 2). The two proteins corresponding to E1 expressed in transfected cells are probably due to incomplete glycosylation or processing of the viral glycoprotein

and have been observed both in mammalian and insect cells (19, 22).

The protein encoded by the NS2 region of HCV ORF is a polypeptide of 24 kDa. The size of this protein differs from that encoded by the NS2 gene of flaviviruses, indicating that no further processing of this region takes place other than the release of p24 from the precursor (2). Immunoprecipitation of a large 78-kDa protein with both anti-E2/NS1 and anti-NS2 antisera suggests that this protein represents an uncleaved E2/NS1-NS2 precursor. This observation is not unique to HCV but has been reported also for the processing of flaviviruses (2, 21), suggesting that the maturation of E2/NS1 and NS2 proteins is not a cotranslational event, but that it may occur at later stages of the biogenesis of these two polypeptides. Alternatively, expression of the HCV ORF in this particular system is higher than normal physiological levels such that the cleavage between E2/NS1 and NS2 may be a limiting event due to the shortage of specific cellular factors required for proper processing at this site.

NS3 antiserum recognizes a 68-kDa protein, in agreement with the predicted molecular weight of NS3. Interestingly, a minor 47-kDa band is also consistently recognized by anti-NS3 antibodies in immunoprecipitation experiments using denatured antigens (Fig. 3) as well as in Western immunoblots (data not shown). The 47-kDa protein therefore very probably does not represent a cellular product associated with the viral protease but rather is a further cleavage or degradation product derived from the NS3 region. The functional significance of this 47-kDa protein in HCV processing is unknown.

The NS4 region of the HCV ORF is cleaved into two distinct products, NS4a and NS4b, which are both recognized by the rabbit polyclonal antiserum. This observation is consistent with the processing profile of flavivirus nonstructural region (2). Although we do not have antisera directed against selected regions of NS4, it is very likely that the NS4a protein of 6 kDa is the most N-terminal part of the NS4 gene, whereas the NS4b protein of 24 kDa is encoded by the C-terminal region of the NS4 gene. This conclusion is in part supported by complementation studies of the mutated clones lacking a catalytically active NS3, which showed that the NS3-NS4 uncleaved product has the molecular weight which corresponds to the sum of the molecular weight of NS3 and NS4a (Fig. 5). Furthermore, we have consistently observed the presence of an NS4-related 31-kDa protein in our immunoprecipitation experiments, which we interpret as being the NS4a+b precursor.

The NS5 region of the HCV polyprotein is cleaved into two smaller products of 47 and 65 kDa; the processing of this region therefore differs from that of flavivirus NS5, which is released from the polyprotein precursor as a single protein of 110 kDa. The GDD consensus sequence characteristic of RNA-dependent RNA polymerases is located in NS5b (residues 2736 to 2738), indicating that this protein may act as a viral RNA replicase during HCV-specific RNA synthesis (17). However, NS5a could also have a function in the replication of the viral genome, acting as a component of the replication complex involved in the reaction.

Processing of C, E1, and E2/NS1 is mediated by signal peptidases located in the endosplasmic reticulum lumen of the host cell as in the case of the flavivirus structural proteins. This conclusion is based on in vitro translation studies, in which mature forms of the putative structural components of HCV are observed when the translation reaction is carried out in the presence of microsomal membranes. A similar result has been obtained by Hijikata et al. (14), who have indicated the presence of hydrophobic segments located at the N termini of E1 and E2/NS1 (residues 174 to 191 and 371 to 383, respectively) which could act as signal sequences. Furthermore, transfection experiments with plasmid pCD(38-9.4:S_{1165}-A), in which the catalytic Ser residue has been substituted with Ala, indicates that the expression and processing of the E1 and E2/NS1 proteins are not affected by this mutation, suggesting that the processing of the structural region is independent of the NS3 protease. Similarly, cleavage at the N termini of NS2 and NS3 is also not dependent on the NS3 protease, since the NS2 protein is properly processed in this plasmid (Fig. 4). The nature of the protease(s) responsible for cleavage at these two sites is unknown; however, release of the flavivirus NS2a protein from the polyprotein precursor is thought to be mediated by a cellular protease, and a similar mechanism may generate the NS2 protein of HCV (8, 15). Interestingly, there seems to be a requirement for NS2 in the processing of the N terminus of NS3, as shown by the transfection of pCITE(SX) (Fig. 3B). In this construct, most of NS2 has been removed, with a concomitant impairment of the cleavage at the NS2-NS3 site demonstrated by the larger size of the viral protease (Fig. 3B). In view of the hydrophobic nature of the NS2 polypeptide, it is possible that the NS2 protein is required for correct localization of the NS2-NS3 protein in the cytoplasm, which renders the precursor available to cellular protease(s) for proper cleavage. Recently, the cellular localization of the dengue type 2 virus NS3-NS4a-NS4b-NS5 precursor protein was shown to be distinctly different from the perinuclear localization of the mature NS5 protein, and the distribution of these proteins within the cytoplasm has been suggested to be determined, at least in part, by the NS2b polypeptide (35).

The data reported here indicate that the NS3 protease activity is required for processing of the nonstructural proteins NS3, NS4, and NS5. The NS3 protease acts in *cis* at its C terminus to release itself from the polyprotein precursor in a fashion similar to that of the viral protease of flaviviruses and pestiviruses. This conclusion is based on the observation that cleavage at the N terminus of NS4a cannot be complemented in *trans*, whereas cleavage activity at the N termini of NS4b, NS5a, and NS5b can be demonstrated in *trans* (Fig. 5 and 6). The N termini of NS5a and NS5b are processed when pCITE(NS3) is cotransfected with the full-length clone pCD(38-9.4:S_{1165}-A) or with clones pCITE (SXS_{1165}-A) and pCITE(NS4-5), whereas the processed NS4b product is detected only when pCITE(NS3) is cotransfected with plasmid pCITE(NS4-5) (Fig. 6). A possible explanation of the difference in protein profile in these cotransfection experiments may be that detection of the NS4b protein in transfected cells depends on the presence of processed NS4a. Possibly, NS4a and NS4b form a complex which determines the stability of both polypeptides in transfected cells. However, the lack of release of NS4a from the polyprotein precursor may undermine this interaction, compromising the stability of NS4b and therefore preventing its detection in the transfected cells. Alternatively, the cleavage at the N terminus of NS4b may not be complemented in constructs pCD(38-9.4:S_{1165}-A) and pCITE(SXS_{1165}-A), resulting in the lack of detection of the processed NS4b. This latter possibility is less probable since an uncleaved precursor with a molecular size corresponding to the sum of the sizes of NS3, NS4a, and NS4b (approximately 100 kDa) is not detected in the transfected cells (Fig. 5).

The data presented here are in complete agreement with sequence alignment studies which had predicted that NS3 is

a serine protease important for HCV polyprotein processing and therefore assigned a pivotal role to this protein in the biogenesis of mature HCV polypeptides. The exact location of the protease domain on the amino acid sequence of NS3 is not yet available but is predicted to be near the N terminus of this protein (23). Although no deletion studies have been presented, the identification of Ser-1165 as the catalytic residue clearly shows that the catalytic site is located close to the putative N terminus of NS3.

Although the N terminus of each protein has been roughly positioned on the HCV polyprotein on the basis of similarity to flaviviruses, no information is available concerning the exact sequence at the cleavage sites with the exception of the data provided by Hijikata et al., who have identified the N termini of E1 and E2/NS1 (14). Efforts directed toward obtaining direct amino-terminal sequence data should be highly rewarding because such data will allow a better definition of HCV polyprotein map and provide useful information on the sequence requirement for the cleavage activity of NS3.

The results described here provide an important description of the genetic order of the HCV proteins on the viral polyprotein and of the processing events required for the biogenesis of the HCV polypeptides. This information reinforces the genetic similarity between HCV and flaviviruses and pestiviruses, substantiating the common genetic origin which places these viruses in the same family. It is clear, however, that the results obtained with this transient expression system may not faithfully reproduce the proteolytic events which take place during HCV infection. It is possible that the level of protein expression obtained in this system may be much higher than normal, affecting important equilibria between precursors and proteases, which in turn may regulate HCV replication and protein synthesis. It is also impossible at this time to correlate the proteolytic activity of NS3 with virus replication. This type of consideration awaits the development of an in vitro infection system or of an infectious cDNA clone with which it should be possible to examine more closely the intracellular events that regulate HCV replication and protein biogenesis.

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