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Substrate Determinants for Cleavage in *cis* and in *trans* by the Hepatitis C Virus NS3 Proteinase

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Processing of the hepatitis C virus polyprotein is accomplished by a series of cotranslational and posttranslational cleavages mediated by host cell signalases and two virally encoded proteinases. Of these the NS3 proteinase is essential for processing at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions. Processing between NS3 and NS4A occurs in *cis*, implying an intramolecular reaction mechanism, whereas cleavage at the other sites can also be mediated in *trans*. Sequence analysis of the amino termini of mature cleavage products and comparisons of amino acid residues around the scissile bonds of various hepatitis C virus isolates identified amino acid residues which might contribute to substrate specificity and processing efficiency: an acidic amino acid at the P6 position, a Thr or Cys at the P1 position, and a Ser or Ala at the P1′ position. To study the importance of these residues for NS3-mediated cleavage we have undertaken a mutational analysis using an NS3′-5B polyprotein expressed by recombinant vaccinia viruses in mammalian cells. For all NS3-dependent cleavage sites P1 substitutions had the most drastic effects on cleavage efficiency, showing that amino acid residues at this position are the most critical substrate determinants. Since less drastic effects were found for substitutions at the P1′ position, these residues appear to be less important for proper cleavage. For all cleavage sites the P6 acidic residue was dispensable, suggesting that it is not essential for substrate recognition and subsequent cleavage. Analysis of a series of mutations at the NS3/4A site revealed great flexibility for substitutions compared with more stringent requirements at the *trans* cleavage sites. On the basis of these results we propose a model in which processing in *cis* is determined primarily by polyprotein folding, whereas cleavage in *trans* is governed not only by the structure of the polyprotein but also by specific interactions between the proteinase and the polyprotein substrate at or around the scissile bond.

Hepatitis C virus (HCV) is the recently identified major etiologic agent of transfusion-associated non-A, non-B hepatitis, and it is implicated in a significant proportion of community-acquired hepatitis (7, 26, 27, 33, 36). Furthermore, epidemiologic data suggest a link between chronic infection with HCV and the development of hepatocellular carcinoma (10, 12, 15, 44, 48, 53).

Several different isolates have been molecularly cloned and sequenced and are now classified into a variety of different genotypes and subtypes (8, 49). On the basis of comparative sequence analyses (38) and infectivity studies employing the chimpanzee animal model (7), HCV has been classified as a separate genus in the *Flaviviridae* family, which includes the classic flaviviruses, like yellow fever virus and dengue virus (9), and the animal pathogenic pestiviruses, like bovine viral diarrhea virus (11). These viruses have in common a virion with a lipid envelope and a single-stranded nonsegmented RNA of positive polarity ranging in size between 9.5 kb (HCV) and 12.5 kb (bovine viral diarrhea virus) and encoding a single long open reading frame with similar genomic organizations in the different viruses. The structural proteins are encoded in the 5′-terminal quarter of the open reading frame, while the nonstructural (NS) proteins are expressed from the remainder. By using cell-free translation and expression studies with mammalian cells, a preliminary map of the gene order for HCV was established: 5′-core-envelope 1 (E1)-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ (19, 22, 24, 47, 54). Core, a basic RNA binding protein, is assumed to be the nucleocapsid protein (20, 28, 46), and E1 and E2 are assumed to be the viral envelope glycoproteins (37, 43, 50). NS2 may represent the catalytic domain of the NS2-3 proteinase, a zinc-dependent metalloproteinase cleaving at the NS2/3 junction (18, 23, 25). A second proteinase, essential for processing most of the NS proteins, is located in the amino-terminal domain of NS3 (2, 13, 17, 23, 54). The carboxy-terminal NS3 domain contains a nucleoside triphosphatase activity (52) consistent with its predicted function as an RNA helicase. NS4A appears to be an NS3 proteinase cofactor enhancing cleavage efficiency especially at the NS4B/5A site (3, 14). The functions of NS4B and NS5A are as yet unknown, as is the case for the analogous proteins in flavivirus and pestivirus polyproteins. Although experimental proof is lacking, the presence of a Gly-Asp-Asp motif characteristic of RNA-dependent RNA polymerases (29) in NS5B, as well as the location of this protein within the open reading frame, strongly suggests that NS5B is the virally encoded polymerase.

Production of mature viral proteins is accomplished by a series of cotranslational and posttranslational proteolytic processing steps. Cleavages generating the amino termini of NS4A, NS4B, NS5A, and NS5B are mediated by the NS3-encoded serine-type proteinase. Processing at the NS3/4A junction appears to be a rapid intramolecular reaction, whereas processing at the other sites can be mediated intermolecularly (3, 54). By using amino-terminal sequence analysis of mature proteins expressed in cell culture, the NS3-dependent cleavage sites have been mapped (17, 41). On the basis of sequence comparison of these cleavage sites in available HCV

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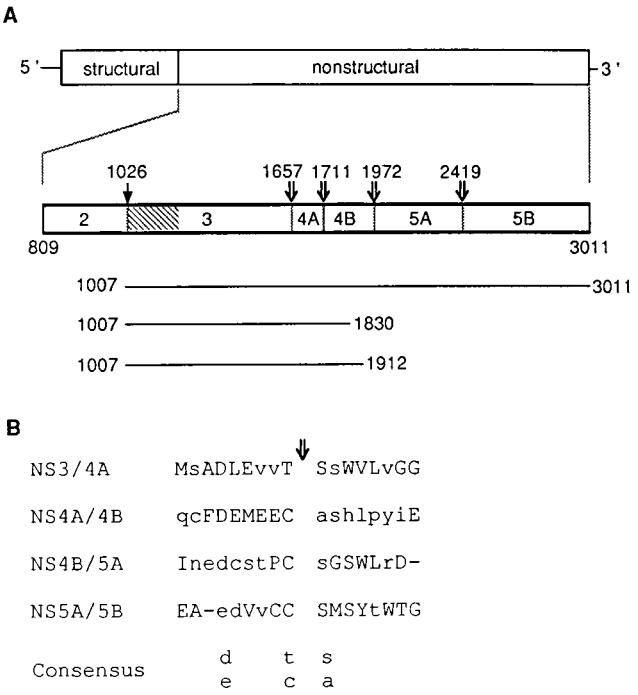

FIG. 1. (A) HCV genome structure and expression constructs. A schematic presentation of the HCV genome encoding the structural proteins in the 5′-terminal quarter followed by the nonstructural proteins 2 through 5B is shown. The 5′ and 3′ nontranslated regions are indicated as thin lines. A detailed view of the nonstructural protein region (amino acids 809 to 3011 of the polyprotein), including the cleavage sites for the NS2-3 proteinase (▾) and the NS3 proteinase (⇓), is drawn below. Numbers above the arrows refer to the amino acids at the P1 positions of the scissile bonds. The hatched box indicates the NS3 proteinase domain. Regions of the polyprotein expressed by recombinant vaccinia viruses (vv1007-3011 and vv1007-1830) or translated in the rabbit reticulocyte lysate (pBSK1007-1912) are drawn below. (B) HCV NS3 proteinase-dependent cleavage sites. Amino acids surrounding the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B cleavage sites of published HCV isolates, including our isolate, were aligned. Capital letters indicate residues identical among all isolates, small letters represent the dominant amino acids, and the dash indicates no conservation. The consensus sequence for all NS3-dependent cleavage sites is given below.

sequences, amino acids which may determine NS3 proteinase substrate specificity have been identified: an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (Fig. 1B) (according to the nomenclature of Berger and Schechter [6], the newly generated carboxy terminus, after cleavage of the peptide bond, is designated P1, and it is preceded by the P2 residue, etc.; the newly generated amino terminus is designated P1′, and it is followed by P2′, etc.).

Substrate specificity of a proteinase is determined primarily by the structural characteristics of the substrate binding pocket (SBP). For instance, the three-dimensional structure of trypsin, a serine-type proteinase cleaving after basic residues, shows that an aspartic acid resides at the base of the SBP. Consequently, lysine and especially arginine side chains at P1 are stabilized via an electrostatic bond with this S1 binding site (for a review, see reference 32). In agreement with this model a substitution of the Asp by Lys changes the selectivity of the enzyme (16). That the substrate P1 residue interacts with the S1 binding pocket is further exemplified by the flavivirus NS3 serine-type proteinases. Here an S1 aspartic acid is found at the proposed SBP (5) in accordance with the specificity of these enzymes for basic P1 residues (51).

Recently Pizzi and coworkers proposed a model for the SBP of the HCV NS3 serine-type proteinase (41). On the basis of the studies of serine proteinases described above, they suggest that amino acids at the P1 position play a crucial role in determining processing efficiency and cleavage specificity. However, no information on the roles of the conserved P1′ amino acid residues and the P6 residues is available. To analyze the importance of the P6, P1, and P1′ residues for NS3-mediated polyprotein processing, we have undertaken a mutational analysis of all NS3-dependent cleavage sites. The mutations were tested for their effects on polyprotein processing in *cis* (in the case of the NS3/4A site) or in *trans* (in the case of all the other sites). We found that the P1 amino acid is most critical in determining processing efficiency and that the P6 acidic residue is not essential for cleavage at any of the NS3-dependent sites. Furthermore, the high tolerance for substitutions at the NS3/4A site compared with more stringent sequence requirements at the other sites suggests that cleavage in *cis* is governed primarily by polyprotein structure, whereas more specific interactions between the proteinase and its substrate appear to dominate processing in *trans*.

MATERIALS AND METHODS

Cell culture and growth of recombinant vaccinia viruses. HeLa cells were grown in RPMI medium supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum. Stocks of recombinant vaccinia viruses were grown in HeLa cell monolayers, and titers of infectious progeny were determined by plaque assay on human TK^- 143 cells.

Construction of expression plasmids. Standard methods were used for all plasmid constructions (45). Nucleotide positions are numbered according to the 5′ end of the RNA of our isolate. Since this 5′ end differed from the 5′ end of our previous reference isolate, described by Kato et al. in 1990 (30), by the presence of 12 additional nucleotides, the numbering system we had used so far was revised accordingly (2, 3). Amino acids are numbered relative to the methionine start codon of the open reading frame of our isolate.

Construction of the basic plasmid pATA1007-3011/wild type (wt), encoding a proteolytically active polyprotein, initiating with 20 amino acids derived from the carboxy terminus of NS2 and spanning the complete NS3-4A-4B-5A-5B open reading frame, was described recently (3). The derivative pATA1007-3011/S→A was generated by introducing a thymidine-to-guanosine nucleotide exchange at position 3834, leading to a substitution of the putative active site serine residue within the NS3 proteinase domain by alanine (2). Site-directed mutagenesis was done by PCR, and the subcloned fragments were analyzed by DNA sequencing. To generate mutations at the NS3/4A cleavage site, oligonucleotides containing the *Nsi*I restriction site of our isolate at position 5286 were used for PCR with an appropriate antisense primer. Finally, an *Nsi*I-*Hpa*I (position 5981) fragment was transferred into pATA1007-3011/wt. Mutations at the NS4A/4B site were obtained with antisense oligonucleotides containing the *Bsm*I restriction site of our isolate at position 5509 and an appropriate sense primer for PCR. After digestion with *Bsm*I and *Sal*I (position 4725) the fragment was inserted into the analogously restricted plasmid pATA1007-3011/S→A. To generate mutations at the NS4B/5A cleavage site and the NS5A/5B cleavage site, complementary oligonucleotides were used for PCR according to the method of No et al. (40). For the NS4B/5A site mutations, amplified fragments were restricted with *Avr*II and *Hpa*I (positions 5981 and 6486) and inserted into *Avr*II-*Hpa*I-digested pATA1007-3011/S→A. In the case of the NS5A/5B site mutations, the amplified fragment was restricted with *Pst*I and *Avr*II and combined in a three-factor ligation with an *Avr*II-*Sal*I HCV fragment and *Sal*I-*Pst*I-restricted pATA 1007-3011/S→A. Constructions of the plasmids pATA1007-1830, encoding an enzymatically active NS3′-4B′ proteinase, and pBSK1007-1912, used for in vitro transcription of an NS3′-4B′ RNA, have been described recently (2, 3).

Generation of recombinant vaccinia viruses. Recombinant vaccinia viruses were generated by homologous recombination into the thymidine kinase gene of wild-type vaccinia virus DNA, and recombinant viruses were selected on human TK^- 143 cells grown in 5-bromo-2′-deoxyuridine (Sigma, Heidelberg, Germany) containing medium as described in detail elsewhere (4).

Metabolic labelling of infected cells and characterization of HCV-specific proteins. HeLa cells (8×10^5 cells in a 35-mm-diameter dish) were infected at a multiplicity of infection of 5 to 10 and were incubated 16 h later in methionine- and fetal calf serum-free medium for 1 h. After the addition of the same medium supplemented with 100 μCi of [^{35}S]methionine (Amersham Life Science, Braunschweig, Germany) and incubation for 2 h, cells were lysed in TNE (10 mM Tris hydrochloride [Tris-HCl], pH 8.0, 100 mM NaCl, 1 mM EDTA) with 1% Triton X-100. The lysate was clarified by 15 min of centrifugation at 15,680 × *g* at 4°C in a microcentrifuge, and proteins in the supernatant were precipitated by the addition of 2% sodium dodecyl sulfate (SDS) and 5% trichloroacetic acid.

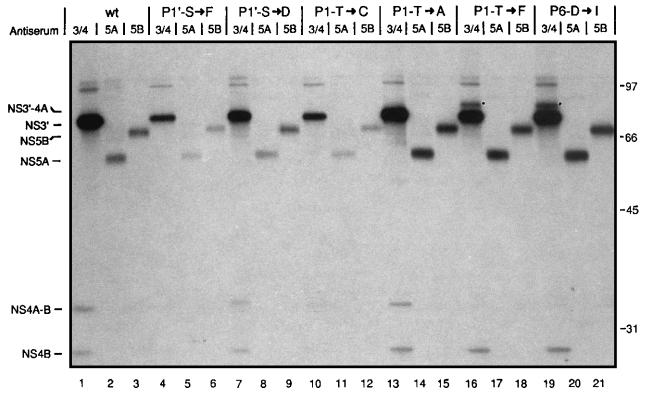

FIG. 2. Mutations at the NS3/4A cleavage site and their effects on polyprotein processing. HeLa cells infected with recombinant vaccinia viruses expressing the parental NS3′-5B (wt) or NS3′-5B polyproteins bearing various mutations around the NS3/4A cleavage site were labelled with [^{35}S]methionine for 2 h, and cells were lysed as described in Materials and Methods. HCV-specific proteins were isolated by immunoprecipitation using antisera directed against an antigen covering the 20 carboxy-terminal amino acids of NS3 and NS4AB (3/4), against NS5A (5A), or against NS5B (5B) sequences and analyzed by electrophoresis on an SDS–11% polyacrylamide gel. The identities of HCV proteins are given on the left, and the positions of protein molecular mass markers (in kilodaltons) are given on the right. The band corresponding to the NS3′-4A protein is marked with a dot.

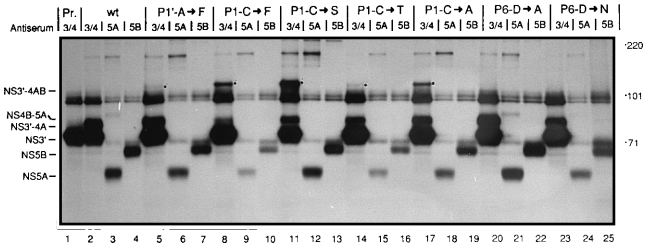

FIG. 3. *trans* cleavage of NS4A/4B cleavage site mutants. HeLa cells were infected with the recombinant vaccinia virus expressing the NS3′-4B′ proteinase (Pr.; lane 1) or doubly infected with this recombinant virus and one of the recombinants expressing the proteolytically inactive NS3′-5B polyprotein mutated at the NS4A/4B cleavage site. For comparison of cleavage efficiency, a double infection with the recombinant vaccinia virus expressing the parental polyprotein substrate (wt) was included. Cells were labelled for 2 h with [^{35}S]methionine. Lysis and immunoprecipitations were done as described in Materials and Methods. HCV-specific proteins and the positions of protein molecular mass standards (in kilodaltons) are given on the left and the right, respectively. The NS3′-4AB processing intermediate is labelled with a dot.

After at least 10 min at 0°C, precipitated proteins were collected by 5 min of centrifugation at 3,340 × *g* and dissolved in protein sample buffer (200 mM Tris-HCl, pH 8.8, 5 mM EDTA, 2% SDS, 1% 2-mercaptoethanol, 10% sucrose, 0.1% bromophenol blue). After 5 min of boiling, samples were diluted by adding 20 volumes of RIPA I buffer (phosphate-buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma). Finally, 15 μl of packed protein A-Sepharose containing preadsorbed immunoglobulin (corresponding to 5 to 8 μl of antiserum) was added and the samples were incubated overnight at 4°C with agitation. After washing of the immunocomplexes three times with RIPA II buffer (RIPA I buffer containing 0.1% SDS), protein sample buffer containing 3.3% SDS and 2% 2-mercaptoethanol was added, and samples were boiled for 5 min and analyzed by electrophoresis on an SDS–11% polyacrylamide gel and by fluorography.

In vitro transcription and translation. Plasmids were linearized with *Spe*I downstream of the HCV sequence, and after phenol and chloroform extraction DNAs were ethanol precipitated and dissolved in water. A 2-μg portion of DNA was used for a 50-μl in vitro transcription reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM each ribonucleotide, 50 U of RNasin, and 15 U of T7 RNA polymerase (Promega, Madison, Wis.). After 90 min at 37°C, plasmids were digested with 2 U RNase-free DNase (Promega) and RNA was extracted with phenol and chloroform and ethanol precipitated. Pellets were dissolved in water, and RNA was analyzed by electrophoresis through a nondenaturing agarose gel. A 1- to 2-μg portion of RNA was used for one in vitro translation reaction in a final volume of 12.5 μl with a rabbit reticulocyte lysate (Promega) according to the instructions of the manufacturer. After 15 or 60 min samples were treated with 1 μg of RNase A for 5 min at 30°C. Finally, protein sample buffer was added and proteins contained in one-half of the reaction mixture were analyzed by electrophoresis on an SDS–11% polyacrylamide gel and by fluorography.

RESULTS

Proteolytic processing of HCV polyproteins mutated at the *cis* cleavage site. To analyze the importance of conserved amino acids at the P6, P1, and P1′ positions of the NS3-dependent cleavage sites (Fig. 1B), single amino acid substitutions were introduced into an NS3′-5B polyprotein and expressed by recombinant vaccinia viruses in HeLa cells (vv1007-3011; Fig. 1A). This polyprotein, which encodes 20 amino acids derived from the carboxy terminus of NS2 and the complete NS3-5B ORF, was shown previously to undergo all proteolytic cleavages mediated by the NS3 proteinase (3).

Kinetic and genetic analyses have shown that processing at the NS3/4A site is an intramolecular reaction, whereas cleavage at the other sites can be mediated in *trans* (3, 54). Thus, to analyze the substrate determinants of cleavage specificity in *cis* and in *trans*, mutations at the NS3/4A (*cis* cleavage) site were introduced into an NS3′-5B polyprotein containing an enzymatically active NS3 proteinase, whereas mutations at the *trans* cleavage sites were analyzed in the context of an enzymatically inactive NS3′-5B polyprotein substrate which was coexpressed with an NS3′-4B′ proteinase (vv1007-3011/S→A and vv1007-1830, respectively; Fig. 1A). This proteinase was chosen because it includes the NS4A cofactor required to increase processing efficiency (3, 14). Effects on processing of the NS3′-5B polyprotein caused by the introduced mutation were tested by analysis of HCV-specific proteins isolated from infected HeLa cells by immunoprecipitation with region-specific antisera directed against NS3 and NS4 sequences (anti-NS3/4), against NS5A (anti NS5A), or against NS5B (anti-NS5B). Since this NS3/4-specific antiserum did not detect mature NS4A, cleavage at the NS3/4A site and the NS4A/4B site was monitored by the production of NS3 and NS4B. Given the complex nature of processing intermediates and the low labelling efficiency of some cleavage products, quantitations were not attempted. However, reproducible results were obtained in independent experiments.

Figure 2 shows the processing patterns of the parental NS3′-5B polyprotein and the various NS3/4A cleavage site mutants obtained after immunoprecipitation with the NS3/4-, NS5A-, and NS5B-specific antisera. Mature NS3′, NS4B, NS5A, and NS5B as well as an NS4AB processing intermediate were detected for the parental NS3′-5B polyprotein (Fig. 2, lanes 1 through 3). Indistinguishable patterns were obtained for two substitutions each at the P1′ (S→F and S→D) and P1 (T→C and T→A) positions. In contrast, substitution of a Phe for the Thr at P1 (P1 T→F) reduced cleavage efficiency, as indicated by the presence of an NS3′-4A processing intermediate and the reduced amount of NS4AB (compare lanes 1 and 16). The same pattern was found for a nonconservative substitution at the P6 position (D→I). None of these mutations influenced production of mature NS4B, NS5A, and NS5B, indicating that a reduction of cleavage efficiency at the NS3/4A site does not impair processing at the unaltered sites.

Substitutions at the NS4A/4B, NS4B/5A, and NS5A/5B sites and their effects on processing in *trans*. To examine the effects of substitutions at the P6, P1, and P1′ positions on processing at the *trans* cleavage sites, cells were doubly infected with a recombinant vaccinia virus expressing a proteolytically inactive

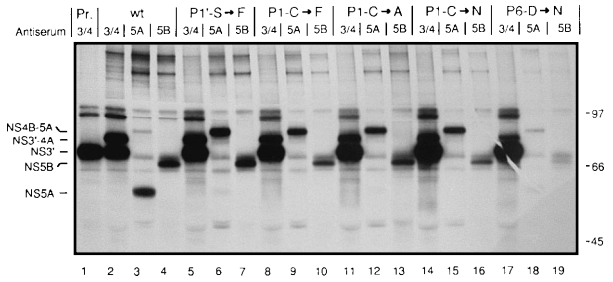

FIG. 4. Mutations at the NS4B/5A cleavage site and their effects on polyprotein processing. Cells priorly infected with the recombinant expressing the NS3′-4B′ proteinase (Pr.) or a combination of this recombinant vaccinia virus and a virus directing the expression of the polyprotein substrate with an unaltered (wt) or a mutated NS4B/5A cleavage site were labelled with [^{35}S]methionine, and HCV-specific proteins were analyzed by immunoprecipitation and electrophoresis on an SDS–11% polyacrylamide gel. The identities of HCV proteins are given on the left, and the positions of protein molecular mass markers (in kilodaltons) are given on the right.

NS3′-5B polyprotein substrate into which single amino acid exchanges had been introduced and a second recombinant directing the expression of an NS3′-4B′ proteinase (vv1007-1830; Fig. 1A). The effects of substitutions at the NS4A/4B site as determined in this assay are shown in Fig. 3. *trans* cleavage of the polyprotein substrate with the natural cleavage site resulted in the production of NS3′-4A, NS5A, and NS5B (NS4B could not be detected under these experimental conditions) and small amounts of processing intermediates (Fig. 3, lanes 2 through 4). The same pattern was found for the nonconservative substitutions at the P6 position (D→A and D→N), whereas changes at the P1′ position (A→F) and the P1 position (C→F, C→S, C→T, and C→A) reduced cleavage at the NS4A/4B site to various extents, as indicated by smaller amounts of NS3′-4A and the appearance of an NS3′-4AB intermediate not detected with the parental polyprotein substrate (compare, e.g., lane 2 with lane 8). No influence on production of mature NS5A and NS5B was found.

Substrate determinants at the NS4B/5A site were examined by using various substitutions at the P1′, P1, and P6 positions, as depicted in Fig. 4. All mutations severely reduced cleavage at this site, as indicated by the reduction of the amount of mature NS5A and an increase of the amount of NS4B-5A in the immunoprecipitations with the NS5A-specific antiserum (Fig. 4; compare lane 3 with lanes 6, 9, 12, 15, and 18 [for unknown reasons the level of expression of the polyprotein mutated at the P6 position was reproducibly low]). Small amounts of mature NS5A were detected for the P6 D→N, P1′ S→F, and P1 C→A substitutions (barely visible in the photograph), whereas the P1 C→F and P1 C→N substitutions reduced cleavage to below the limit of detection. No influence on production of NS3′-4A and NS5B was found.

To analyze amino acid residues important for efficient cleavage at the NS5A/5B site, the following substitutions were generated: P1′ S→F and S→A; P1 C→F, C→N, and C→T; P2 C→A; P5 D→N; and P6 E→N. Figure 5 shows the results of *trans* cleavage of the parental NS3′-5B polyprotein and the various substitutions obtained during 2 h of continuous labelling. As deduced from the absence of mature NS5A and NS5B and the presence of uncleaved NS5AB, the C→N and C→F substitutions at the P1 position reduced cleavage to below the limit of detection. Less drastic effects were found for the P1 C→T and P1′ S→F substitutions, whereas the mutations at the P2 and P5 positions as well as the drastic change at the P6 position had no influence on *trans* cleavage at this site. In all cases, processing between NS4A and NS4B was unchanged, as seen by the appearance of NS3′-4A. Depending on the extent of cleavage reduction, various amounts of an anti-NS5A- and anti-NS5B-reactive protein with an apparent molecular mass of about 90 kDa were detected. The nature of this protein was not investigated, but its absence in *trans* cleavage of the parental NS3′-5B polyprotein suggests that it is generated by aberrant processing perhaps due to the prolonged presence of uncleaved NS5AB.

High tolerance for amino acid substitutions at the NS3/4A *cis* cleavage site. The observed tolerance for substitutions at the NS3/4A site compared with the more stringent requirements at the *trans* cleavage sites (Table 1) suggested that *cis* cleavage is dominated primarily by the local folding of the polyprotein, bringing the scissile bond into close proximity to the catalytic center of the enzyme. We anticipated that as long as the polyprotein structure was not grossly disturbed, cleavage activity would be rather insensitive to the amino acids close to or at the scissile bond. To experimentally test this assumption, a series of substitutions at the NS3/4A site was constructed. To simplify the analysis, these mutations were introduced into an NS3′-4B′ polyprotein which efficiently undergoes *cis* cleavage at the NS3/4A junction upon expression in a cell-free translation system (2). In vitro translations were performed for various times to analyze possible effects on cleavage efficiency more precisely. To compare the results from the cell-free system with those obtained by polyprotein expression in cell

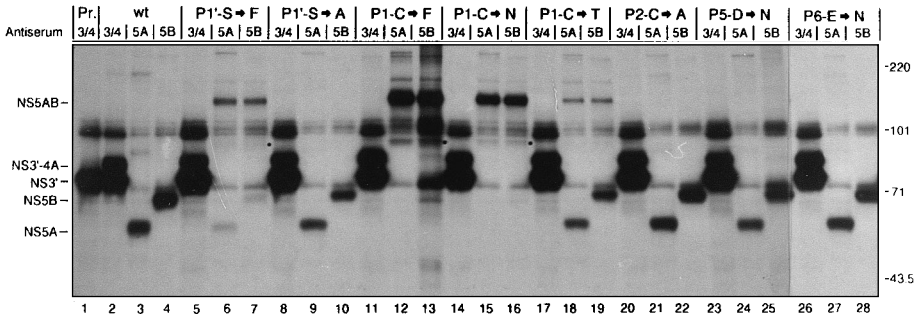

FIG. 5. *trans* cleavage of NS polyprotein substrates mutated at the NS5A-5B cleavage site. Cells were infected with the recombinant expressing the proteinase (Pr.; lane 1) alone or in combination with one of the recombinant vaccinia viruses directing the expression of an NS3′-5B polyprotein substrate containing the parental (wt) or a mutated NS5A/5B cleavage site. Cell lysates and immunoprecipitations were done as described in Materials and Methods. HCV-specific proteins and the positions of protein molecular mass standards (in kilodaltons) are given on the left and the right, respectively. The extra cleavage product observed with some of the mutants is marked with a dot.

TABLE 1. Mutations at the NS3-dependent cleavage sites and their effects on processing efficiencies at the altered sites

Mutated sites	Sequence and residue(s)	Cleavage efficiency[a]
wt (NS3/4A)	ADLEVVT⇓S	++
P1′	D	++
	F	++
	I	+
	N	++
	A	++
P1	I	±
	N	++
	M	++
	C	++
	F	+
	V	++
	A	++
P2	A	++
P3	A	++
P4	A	++
P5	A	++
P6	A	++
	K	++
	I	+
	N	++
P6/5	AD	±
P7/6	DA	++
wt (NS4A/4B)	FDEMEEC⇓A	++
P1′	F	+
P1	F	±
	S	±
	T	+
	A	±
P6	A	++
	N	++
wt (NS4B/5A)	EDCSTPC⇓S	++
P1′	F	±
P1	F	−
	A	±
	N	−
P6	N	+
wt (NS5A/5B)	GEDVVCC⇓S	++
P1′	F	±
	A	++
P1	F	−
	N	−
	T	+
P2	A	++
P5	N	++
P6	N	++

[a] Mean values from at least three independent experiments obtained from estimations of the precursor/product ratios. ++, efficiency comparable to the wild-type level; +, <50% reduction of cleavage at the mutated site; ±, >50% reduction; −, no detectable cleavage.

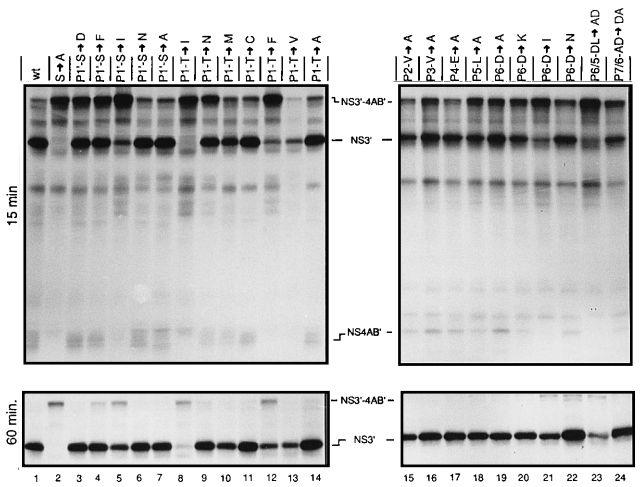

FIG. 6. Mutations at the NS3/4A cleavage site and their effects on processing. Uncapped RNA transcripts encoding an NS3′-4B′ polyprotein (amino acids 1007 to 1912) mutated at the NS3/4A cleavage site were translated in a rabbit reticulocyte lysate for 15 min (upper panels) or for 60 min (lower panels). As controls, translations of RNAs with an unaltered NS3/4A cleavage site (wt) or with an enzymatically inactive NS3 proteinase (S→A) were included. Proteins were analyzed by electrophoresis on an SDS–11% polyacrylamide gel. The positions of the uncleaved NS3′-4AB′ precursor and the NS3′ as well as NS4AB′ processing products are given.

culture, in the initial set of experiments the NS3/4A site mutations analyzed with recombinant vaccinia viruses were transferred into pBSK 1007-1912 (Fig. 1A) and in vitro transcripts were translated in a rabbit reticulocyte lysate. As controls, NS3′-4B′ polyproteins with the natural cleavage site and an enzymatically active NS3 proteinase or with an inactive NS3 proteinase were translated in parallel (Fig. 6, lanes 1 and 2, respectively). In agreement with the results shown in Fig. 2, both the P1 T→F substitution and the P6 D→I substitution reduced the cleavage rate, as indicated by increased amounts of unprocessed precursor and smaller amounts of NS3′ and NS4AB′ processing products observed during the 15-min translation (lanes 12 and 21), whereas no effect was found for the other substitutions (lanes 3, 4, 11, and 14; in most experiments NS4AB′ appeared as a diffuse set of bands, most likely because of its rapid degradation, which also explains why it could not be detected in the 60-min translation).

Having shown that processing of the in vitro-translated cleavage site mutants properly reflects the results obtained with the mutated polyproteins expressed in HeLa cells, we next introduced a series of conservative and nonconservative amino acid substitutions at the P1′, P1, and P6 positions. In addition, amino acids at the P2, P3, P4, and P5 positions were replaced by alanine to determine their possible influence on cleavage at this site. As shown in Fig. 6 and summarized in Table 1, substitutions at the P1 position had more-drastic effects than did changes at the P1′ and P6 positions. In general, large hydrophobic (Ile) or aromatic (Phe) P1 residues were only poorly tolerated, whereas small hydrophobic residues (Ala and Val) or polar residues (Cys and Asn) did not affect cleavage. In the case of the P1′ substitutions, only Ile reduced processing, whereas a P1′ Phe had no effect. None of the P2 to P5 substitutions influenced cleavage, demonstrating that the primary sequence of these residues is not important for processing between NS3 and NS4A. Interestingly, the P6/5 DL→AD double mutation shifting the P6 Asp to the P5 position severely reduced cleavage, whereas the double mutant in which the acidic P6 residue was shifted to the P7 position was unaffected (Fig. 6, lanes 23 and 24). This reduction cannot be ascribed to the substitution of the P6 Asp by Ala, as shown by the wild-type phenotype of the analogous single mutant (lane 19). Rather, a steric disturbance rendering the cleavage site poorly accessible for the enzyme seems to be responsible. None of these mutations completely blocked cleavage at the NS3/4A site, demonstrating a great flexibility of the substrate at this site for cleavage by the NS3 proteinase.

DISCUSSION

Sequence comparison of amino acid residues flanking the scissile bonds cleaved by the NS3 proteinases of various HCV isolates reveals that only the residues at positions P6, P1, and P1′ are conserved (17, 41). Acidic amino acids (primarily Asp) are found at the P6 position. At the P1 position at the NS3/4A (*cis*) cleavage site a Thr is found, whereas a Cys is present at the P1 position of the other (*trans*) cleavage sites. Amino acids with small polar or hydrophobic side chains are found at the P1′ position (Ser or, in the case of the NS4A/4B site, Ala). On the basis of this sequence homology the consensus sequence defining an NS3-dependent cleavage site of HCV would be D/E-X_4-T/C⇓S/A (Fig. 1).

To examine the potential role of the conserved amino acid residues in determining cleavage site specificity and processing efficiency, we have analyzed various substitutions around the scissile bonds for their effects on polyprotein cleavage. By using an NS3′-5B polyprotein expressed by recombinant vaccinia viruses in mammalian cells, the mutations were analyzed for their effects on cleavage at the four NS3-dependent sites. Table 1 summarizes the results and includes the substitutions at the NS3/4A site tested in the cell-free system. Although only a limited number of mutations were made at the *trans* cleavage sites, our results suggest that amino acids at the P1 position are major determinants of cleavage efficiency, whereas residues at the P1′ position play a subordinate role. Furthermore, the P6 acidic amino acid is not essential for processing at any of the NS3-dependent cleavage sites, suggesting that it is not involved directly in substrate recognition and subsequent cleavage. However, it should be noted that an additional acidic residue is always present in close proximity at the P side of the scissile bond (at the P4 position in the case of the NS3/4A site; at the P2, P3, and P5 positions in the case of the NS4A/4B site; at the P7 position in the case of the NS4B/5A site; and at the P5 position in the case of the NS5A/5B site [Fig. 1B]). Whether these residues can functionally substitute for an altered P6 residue remains to be determined.

On the basis of known structures of trypsin-like serine proteinases and conserved sequence patterns of various HCV isolates, Pizzi and coworkers have recently proposed a model for the NS3 proteinase SBP (41). They suggest that hydrophilic amino acids with small side chains (ideally Cys or Ser) would be preferred at the P1 position of the substrate. We found that, consistent with their model, an amino acid with a large hydrophobic or aromatic side chain at the P1 position (Ile or Phe, respectively) drastically reduced cleavage at the mutated site. Furthermore, substitutions of the P1 residues by a polar amino acid (Asn) or a small hydrophobic residue (Ala) reduced or blocked processing at the *trans* cleavage sites (Table 1). Although these findings are in general agreement with the model, it should be noted that the conservative P1 Cys→Ser substitution at the NS4A/4B site greatly reduced processing at this site. The reason for this finding is not clear, but it will be interesting to see whether mutations in the SBP analogous to the ones described for dengue virus 2 (42) will influence substrate determinants around the scissile bond.

A remarkable flexibility for amino acid substitutions was found at the NS3/4A site, where cleavage occurs only intramolecularly. Interestingly, a Thr residue is found at the P1 position of this site, compared with a P1 Cys present at all *trans* cleavage sites. Although it is suggestive, the inability to cleave the NS3/4A site in *trans* cannot be explained by the nature of the P1 residue alone, because an NS3′-5B polyprotein substrate in which the P1 Thr was substituted by a Cys still was not cleaved in *trans* at this site (1). Furthermore, introducing a Thr at the NS4A/4B site or the NS5A/5B site still allows *trans* cleavage, albeit with reduced efficiency (Table 1). Thus, *cis* cleavage at the NS3/4A site is probably governed by the local folding of the single polypeptide chain encompassing both the proteinase and the substrate. As long as the overall folding of this protein is not significantly disturbed, a peptide bond in close proximity to the catalytic center of the enzyme would be cleaved and only slightly influenced by the nature of the amino acids forming the scissile bond. However, given this model, it still is difficult to envisage how site specificity is achieved if NS3 can fit almost any amino acid residue into its S1 binding pocket. One explanation might be that an alternative processing site(s) exists in close proximity. Such a site(s) normally is not cleaved, or is only very inefficiently cleaved, and therefore it is detected only when cleavage at the natural site is blocked by P1 residues which do not fit in the SBP. Interestingly, a Ser or Thr is found at the P2′ position and is followed by a Trp. Furthermore, if processing would occur between P2′ and P3′, an acidic amino acid would be present at the new P6 position (Fig. 1B). A similar flexibility has been described for subtilisin (32). This proteinase normally cleaves peptide bonds following an aromatic side chain at P1, but it will accept nonaromatic apolar side chains and even charged side chains. In most cases in which P1 is an "abnormal" residue, the enzyme can cleave carboxy terminal to P3 or P4 substrate residues if they are hydrophobic, and this cleavage can be ascribed to an elaborate S4 binding crevice that can accommodate such side chains (reference 32 and references cited therein). However, for HCV the existence of such alternative cleavage sites between NS3 and NS4A remains to be determined.

More stringent requirements for the primary sequence around the scissile bond were found at the *trans* cleavage sites, suggesting that processing at these sites is governed not only by the structure of the polyprotein substrate but also by specific recognition between the proteinase and its substrate at or around the cleavage site. Different requirements for *cis* and *trans* cleavage are not unique for HCV polyprotein processing because similar observations have been reported for cleavage at the VP1/2A site by poliovirus $2A^{pro}$ (21) and for cleavage at the NS2A/2B site (cleaved primarily in *cis*) (39) and the NS4B/5 site (cleaved primarily in *trans*) (35) by the yellow fever virus NS2B/3 proteinase.

Several additional observations also support the role of polyprotein structure in cleavage site recognition. On the basis of the NS3 proteinase cleavage site consensus sequences, five potential cleavage sites within the HCV polyprotein of our isolate can be identified. Given that the P6 acidic residue is not essential or that an acidic residue may also be present at the P site at various positions, this number is even higher. Yet, apart from a possibly heterogenous processing at the amino terminus of NS5A (23), no evidence for alternative cleavages exists so far. Furthermore, we have recently described NS3 deletion mutants within the helicase domain unable to cleave at the NS3/4A site (2). As these deletion mutants still contain an enzymatically active proteinase able to cleave a polyprotein substrate in *trans* (31), the absence of *cis* cleavage with these proteinases probably is due to incorrect folding, rendering the NS3/4A cleavage site inaccessible for the catalytic center of the enzyme.

Processing of viral polyproteins can occur sequentially, and in some cases cleavage at a certain site is a prerequisite for processing at another site. For example, cleavage at the 4A/2K site of yellow fever virus polyprotein is required for subsequent signalase cleavage at the 2K/4B site (34). Using an NS3′-5B polyprotein expressed by recombinant vaccinia viruses in mammalian cells, we also examined the influence of the introduced

mutation on cleavage at the unaltered sites. In no case did mutations which blocked or reduced cleavage at a certain site significantly influence processing at any of the other sites. Thus, in a circumstance analogous to the situation observed for the yellow fever virus (35), our results suggest that even though processing of the NS proteins appears to occur in a preferred order (3), alternative pathways are possible. Processing of the HCV NS proteins most likely is essential to generate mature, enzymatically active viral proteins from an inactive precursor polyprotein for which the NS3-encoded serine-type proteinase plays a pivotal role. The study described here provides further insight into the substrate specificity of this important enzyme and therefore may provide a basis for the rational design of antiviral drugs.

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