Regulation of Hepatitis C Virus Polyprotein Processing by Signal Peptidase Involves Structural Determinants at the p7 Sequence Junctions*

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The hepatitis C virus genome encodes a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases to yield 10 mature protein products (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Although most cleavages in hepatitis C virus polyprotein precursor proceed to completion during or immediately after translation, the cleavages mediated by a host cell signal peptidase are partial at the E2/p7 and p7/NS2 sites, leading to the production of an E2p7NS2 precursor. The sequences located immediately N-terminally of E2/p7 and p7/NS2 cleavage sites can function as signal peptides. When fused to a reporter protein, the signal peptides of p7 and NS2 were inefficiently cleaved. However, when full-length p7 was fused to the reporter protein, partial cleavage was observed, indicating that a sequence located N-terminally of the signal peptide reduces the efficiency of p7/NS2 cleavage. Sequence analyses and mutagenesis studies have also identified structural determinants responsible for the partial cleavage at both the E2/p7 and p7/NS2 sites. Finally, the short distance between the cleavage site of E2/p7 or p7/NS2 and the predicted transmembrane α-helix within the P′ region might impose additional structural constraints to the cleavage sites. The insertion of a linker polypeptide sequence between P-3′ and P-4′ of the cleavage site released these constraints and led to improved cleavage efficiency. Such constraints in the processing of a polyprotein precursor are likely essential for hepatitis C virus to post-translationally regulate the kinetics and/or the level of expression of p7 as well as NS2 and E2 mature proteins.

Whatever the structure and replication strategy of their genomes, all viruses must express their genes as functional messenger RNAs early in infection to direct the translational machinery of the cell to make viral proteins. Although each messenger RNA generally operates as a single translation unit in eukaryotes, RNA viruses have evolved various strategies to derive several separate protein products from a single genome.

One of these strategies is to produce large polyprotein precursors that are processed by proteolytic cleavages to derive their final protein products. This is generally the case for positive strand RNA viruses. A consequence of this strategy is that regulation of the level and/or the kinetics of expression of individual proteins often has to occur post-translationally. Delayed processing and/or partial cleavage leading to the production of precursors are potential strategies to regulate the expression of final products. This has been well illustrated in the case of flavivirus polyprotein where the proteolytic processing at the C-prM junction involves coordinated cleavages at the cytoplasmic and luminal sides of the internal signal sequence (1). The study of the mechanisms leading to delayed and/or partial processing of polyprotein precursors is essential to better understand the replication of these viruses as well as to develop new antiviral strategies.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (2). HCV is a small positive strand enveloped virus that belongs to the Hepacivirus genus in the Flaviviridae family (see Ref. 3 for a recent review). Its genome encodes a single polyprotein precursor of just over 3000 amino acid residues. This polyprotein precursor is co- and post-translationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (see Fig. 1) (4). The structural proteins of HCV, the capsid protein (C) and the envelope glycoproteins E1 and E2, are the components of the viral particle. The p7 polypeptide has been shown to be an ion channel protein (5–8) and has been shown to be essential for infectivity of HCV (9). In addition to its involvement in NS2–3 processing, the role of NS2 in the HCV life cycle has not been determined yet. The other nonstructural proteins, NS3 to NS5B, are involved in other steps of the virus life cycle, e.g. for HCV genome replication (10). Interestingly, all of the HCV polypeptides are associated with the endoplasmic reticulum (ER) or ER-derived membranes (for review see Ref. 4), suggesting that genome replication and assembly occur in association with this compartment.

The structural proteins are released from the polyprotein by host ER signal peptidase(s) (reviewed in Ref. 11). Further processing mediated by a signal peptide peptidase occurs at the C terminus of the capsid protein (12). The cleavage between p7 and NS2 is also mediated by a cellular signal peptidase (13, 14). The nonstructural proteins, i.e. NS2 to NS5B, are released

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¶ The abbreviations used are: HCV, hepatitis C virus; ER, endoplasmic reticulum; HA, hemagglutinin; mAb, monoclonal antibody; endo H, endo-B-N-acetylglucosaminidase H; PFU, plaque-forming unit.
from the polyprotein after cleavage by HCV proteases NS2–3 and NS3–4A (reviewed in Ref. 11). Although most cleavages in the polyprotein precursor proceed to completion during or immediately after translation, partial cleavages at the E2/p7 and p7/NS2 sites lead to the production of an uncleaved E2p7NS2 molecule (13–17). Most of NS2 is progressively cleaved from the E2p7NS2 precursor, whereas at least for most of the HCV strains analyzed, the cleavage between E2 and p7 does not change over time (18). This leads to the production of E2, E2p7, p7, and NS2.

In this study, we analyzed the structural constraints leading to the partial/delayed cleavage of E2p7NS2 precursor. Sequence analyses and mutagenesis studies have identified structural determinants responsible for the partial cleavage at both E2/p7 and p7/NS2 sites. The p7 sequence located N-terminally of the signal peptide of NS2 was also shown to reduce the cleavage efficiency at the p7/NS2 site. Finally, insertion of a linker polypeptide sequence between P-3' and P-4' of the cleavage sites also improved cleavage efficiency, suggesting that additional constraints originate from structural elements located at the P' side of the scissile bond. Various structural constraints and their release likely regulate the kinetics and/or the level of expression of p7 as well as NS2 and E2 mature proteins.

EXPERIMENTAL PROCEDURES

Sequence Analyses and Structure Predictions—All of the analyses were made using the Institut de Biologie et Chimie des Proteines HCV data base website facilities (hepatitis.ibcp.fr; 19). The HCV H strain consensus cDNA (20) (GenBankTM accession number AF009606) was used to retrieve the E2, p7, and NS2 sequences of all reported isolates from the EMBL data base using the FASTA homology search program (21). Multiple sequence alignments and amino acid conservation were carried out with the CLUSTAL W program (22). The secondary structure of proteins was predicted using a large set of methods available at the Network Protein Sequence Analysis website (23), including DSC, HNNC, SImpA96, SOPM, GOR4, PHD, and Predator (npsa.phil.ibcp.fr/NPSA and references therein). Various methods (TransMembrane prediction using the hidden Markov model, PHDhtm, TopPred2, and the dense alignment surface method) were combined for the prediction of transmembrane sequences as detailed previously (see Ref. 24 and the web sites and references therein).

Plasmid Constructs—HCV sequences were amplified from clones derived from the H strain and cloned into the pTM1 vector as described previously (25). The pTM1 plasmid contains a polycytoine region immediately downstream of the T7 promoter and the encephalomyocarditis virus internal ribosome entry site cap-independent translation initiation (26). Plasmids pTM1/E2E1, pTM1/SppE1, and pTM1/SpNS2E1 have been described previously (24, 27, 28). Plasmid pTM1/CE1E2p7NS2Myc contains the sequence of a truncated HCV polyprotein including C, E1, E2, p7, and NS2. In addition, the sequence of a linker containing three glycine residues followed by a Myc epitope (EQR1ISEEDL) has been added at the C terminus of NS2 to facilitate the detection of this protein. Plasmid pTM1/E2p7NS2Myc contains the sequence of a truncated HCV polyprotein including E2 with its signal peptide, p7, and NS2 tagged at its C terminus with a Myc epitope. Plasmid pTM1/E2/p7E1 contains the sequence of E2 with its signal peptide, p7 in fusion with E1. Plasmid pTM1/E2p7NS2Myc and pTM1/E2/p7NS2Myc contain the sequence of a truncated HCV polyprotein E2/p7NS2Myc with a Thr residue inserted at position two of the p7 polypeptide, a Pro residue inserted at position three in NS2, or both mutations, respectively (see Table I). Plasmids pTM1/E2HAp7NS2Myc and pTM1/E2p7HANS2Myc derive from plasmid pTM1/E2p7NS2Myc with a HA epitope (YFYDVPFDYA) surrounded by three glycosyl residues, inserted after the third amino acid of p7 or NS2, respectively (see Table I).

Cell Culture—The HepG2, CV-1, and 143B (thymidine kinase-deficient) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell monolayers were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

Generation and Growth of Viruses—Vaccinia virus recombinants were generated by homologous recombination essentially as described (29) and plaque-purified twice in 143B cells under bromodeoxyuridin selection (50 μg/ml). Stocks of vTF7–3 (a vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase) (30), the wild-type vaccinia virus strain, Copenhagen, its thermosensitive derivative ts7 (31), vaccinia virus recombinant expressing the full-length HCV polyprotein (14), and vaccinia virus recombinants expressing HCV proteins studied in this work were grown and titrated on CV1 monolayers. The genes of HCV proteins expressed in this work are under the control of a T7 promoter, and expression of the proteins of interest is achieved either by co-infection of vTF7–3 with the appropriate vaccinia virus recombinant or by infection with vTF7–3 followed by transfection with the appropriate pTM1 plasmid.

Antibodies—Monoclonal antibodies (mAbs) 3/11 (anti-E2) kindly provided by J. McKeating (32), A4 (anti-E1) (16), H47 (anti-E2) (33), and anti-Myc (ATCC CRL-1729) were produced in vitro by using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. The polyclonal anti-NS2 (15) was kindly provided by C. M. Rice.

Metabolic Labeling, Immunoprecipitation, and Endoglycosidase Digestions—For protein expression, HepG2 cells were either co-infected with vTF7–3 and the appropriate vaccinia virus recombinant or infected with vTF7–3 followed by transfection with the appropriate pTM1 plasmid by using FuGENE (Roche Applied Science). Cells expressing recombinant proteins were metabolically labeled with [35S]-protein-labeling mix (530 MBq/ml; Amersham Biosciences) as described previously (16). The cells were lysed in RIPA buffer (0.05 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Igepal) and immunoprecipitations were carried out as described previously (17). The amounts of proteins immunoprecipitated were determined by phosphorimaging and normalized relative to their methionine and cysteine content. For endoglycosidase digestion, immunoprecipitated proteins were eluted from protein A-Sepharose in 30 μl of dissociation buffer (0.5% SDS and 1% 2-mercaptoethanol) by boiling for 10 min. The protein samples were then divided into equal portions for digestion with either endo-β-N-acetylglucosaminidase H (endo H) or left untreated (control). The digestions were carried out for 1 h at 37 °C in the buffer provided by the manufacturer. Digested samples were mixed with an equal volume of 2× Laemmli sample buffer and analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Sequence Analyses in the Regions of E2/p7 and p7/NS2 Cleavage Sites—Based on the hydrophobicity of the regions located just N-terminally of the cleavage sites, the dependence of cleavage of the polyprotein on the presence of membranes, and mutagenesis of cleavage sites into suboptimal substrates for signal peptidase, it has been shown that signal peptides are involved in E2/p7 and p7/NS2 cleavages (reviewed in Ref. 11) (Fig. 1). Indeed, the C terminus of E2 and p7 contain a sequence for reinitiation of translocation, and when fused to a reporter protein, these sequences function as signal peptides (24, 28). For this reason, the translocation reinitiation sequence, which is present at the C terminus of E2, has been named the signal peptide of p7 (Spp7) (Fig. 1). Similarly, the C-terminal hydrophobic stretch of p7 has been named the signal peptide of NS2 (SpNS2). Cleavage at the E2/p7 and the p7/NS2 sites is partial or delayed, producing an E2p7NS2 precursor (13–17). Here, we were interested in identifying the structural features responsible for the partial/delayed cleavage of E2p7NS2 precursor.

All of the secondary structure prediction methods tested indicate that the region of E2/p7 cleavage site might adopt an α-helical fold (Fig. 2). In addition, a coiled-coil structure including the E2/p7 site is slightly predicted by the method of Lupas et al. (34) (data not shown), indicating again that this region exhibits a strong propensity to adopt a well folded structure.
Regulated Processing in HCV Polyprotein

The p7/NS2 cleavage site region is also predicted to fold into α-helix (Fig. 2), suggesting that it might also be involved in a well structured element. In summary, both E2p7 and p7/NS2 cleavage site regions show a clear propensity to be well structured element. In summary, both E2/p7 and p7/NS2 cleavages by mutagenesis in the context of the full-length HCV polyprotein, our data indicate that it is not dramatically influenced by sequences located N- and C-terminally on the polyprotein.

Although the processing of the E2p7NS2 precursor seems to be slightly more efficient in the context of the full-length HCV polyprotein, our data indicate that it is not dramatically influenced by sequences located N- and C-terminally on the polyprotein. Together, these data show that the processing at the E2/p7 and p7/NS2 sites can be analyzed in the context of an E2p7NS2 polyprotein.

Processing Occurs Independently at E2/p7 and p7/NS2 Cleavage Sites—Because the E2/p7 and p7/NS2 cleavage sites are relatively close to each other (63 amino acids), we wondered whether cleavage at one of these sites might be dependent on the cleavage of the other site. To determine whether these cleavages are independent or not, we chose to block one of the cleavage sites and analyzed the efficiency of processing of the unmodified site. To abolish the signal sequence cleavage between E2 and p7 or between p7 and NS2, the residue at position P1 of the cleavage site was mutated from Ala to Arg in the context of E2p7NS2Myc (Table I, E2/p7 and p7/NS2). This mutation is known to abolish signal peptide cleavage (38).

Processing of the mutated proteins was analyzed by immunoprecipitation of the precursor protein and its cleaved products with the anti-Myc antibody. As shown in Fig. 4, none of the mutations blocked the other cleavage site. Indeed, a NS2Myc protein was detected when the E2/p7 cleavage site was abolished (Fig. 4, E2/p7NS2Myc). Similarly, a p7NS2Myc polypeptide was observed when the p7/NS2 cleavage site was abolished (Fig. 4, E2p7:NS2Myc). Together, these observations indicate that processing can occur independently at E2/p7 and p7/NS2 cleavage sites.

Identification of a Structural Constraint Located N-terminally of the Signal Peptide of NS2—As discussed above, when fused to a reporter protein, the hydrophobic stretches located N-terminally of E2/p7 and p7/NS2 cleavage sites (Fig. 2) have been shown to function as signal sequences (24, 28). To determine whether structural features in these sequences potentially lead to partial cleavages at the E2/p7 and p7/NS2 sites, we analyzed the efficiency of cleavage of a reporter protein fused to them. HCV glycoprotein E1 was used as a reporter protein because it is a glycosylated protein, and we can easily discriminate between glycosylated and nonglycosylated forms.

* J. Dubuisson, unpublished data.
E1 has indeed been shown to be a good reporter protein in some of our previous works (24, 28).

Processing of chimeras was analyzed by immunoprecipitation as above using an anti-E1 antibody. Similarly to previous observations (24, 28), the signal peptides of p7 (Spp7) and NS2 (SpNS2) led to the translocation of the reporter E1 glycoprotein as shown by its glycosylation (Fig. 5). Indeed, E1 expressed with Spp7 or SpNS2 migrated as three bands of 28–32 kDa when analyzed by SDS-PAGE (Fig. 5, Spp7E1 and SpNS2E1, Endo H/H11002).

Table I

| Sequences of the constructs at the junctions of the cleavage sites |
|---------------------------------------------------------------|
| **E2** | **p7** |
| ...MILLISQAELGENLVIINA... | ... |
| E2p7 | p7 |
| ...MILLISQAELGENLVIINA... | ...MILLISQAELGENLVIINA... |
| E2p7 | p7 |
| ...MILLISQAELGENLVIINA... | ...MILLISQAELGAGGYDVPDYAGGNLVILNA... |
| E2p7 | p7 |
| ...MILLISQAELGENLVIINA... |

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infection, the cells were labeled for 1 h with \(^{35}\)S-protein labeling mix. At 5 h post-transfection, the cells were co-infected with vTF7–3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 PFU/cell.

These experiments are shown at the top of the figure. HepG2 cells were co-infected with vTF7–3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 PFU/cell. At 5 h post-infection, the cells were labeled for 1 h with \(^{35}\)S-protein labeling mix. The cell lysates were immunoprecipitated with the anti-Myc mAb. The samples were analyzed as in Fig. 3. Immunoprecipitated proteins are indicated on the right. The sizes (in kDa) of protein molecular mass markers are indicated on the left.

**Fig. 4. Processing occurs independently at E2/p7 and p7/NS2 cleavage sites.** HepG2 cells were infected with vTF7–3 at a multiplicity of infection of 5 PFU/cell for 1 h and then transfected with the appropriate plasmid. At 3.5 h post-transfection, the cells were labeled for 1 h with \(^{35}\)S-protein labeling mix. The cell lysates were immunoprecipitated with the anti-Myc mAb. The samples were analyzed as in Fig. 3. Immunoprecipitated proteins are indicated on the right. The sizes (in kDa) of protein molecular mass markers are indicated on the left.

**Fig. 5. Cleavage of the signal peptides fused to a reporter protein.** A schematic representation of the chimeric proteins used in these experiments is shown at the top (A and B) of the figures. HepG2 cells were co-infected with vTF7–3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 PFU/cell. At 5 h post-infection, the cells were labeled for 1 h with \(^{35}\)S-protein labeling mix. Bottom panels, the cell lysates were immunoprecipitated with mAb A4 (anti-E1) and treated or not with endo H. The samples were analyzed by SDS-PAGE (12% polyacrylamide) followed by autoradiography. The proteins are indicated on the right. The deglycosylated proteins are indicated by an asterisk.

To confirm that the sequence located N-terminally of the signal peptide of NS2 in cleavage inefficiency. A schematic representation of the chimeric proteins used in these experiments is shown at the top of the figure. HepG2 cells were co-infected with vTF7–3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 PFU/cell. At 5 h post-infection, the cells were labeled for 1 h with \(^{35}\)S-protein labeling mix. The cell lysates were immunoprecipitated with the anti-Myc mAb. The samples were analyzed as in Fig. 3. The proteins are indicated on the right. The sizes (in kDa) of protein molecular mass markers are indicated on the left.

To confirm that the sequence located N-terminally of SpNS2 induces a structural constraint limiting p7/E1 cleavage by the signal peptidase.

**Fig. 6. Role of the sequence located N-terminally of the signal peptides of NS2 in cleavage inefficiency.** A schematic representation of the chimeric proteins used in these experiments is shown at the top of the figure. HepG2 cells were co-infected with vTF7–3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 PFU/cell. At 5 h post-infection, the cells were labeled for 1 h with \(^{35}\)S-protein labeling mix. The cell lysates were immunoprecipitated with the anti-Myc mAb. The samples were analyzed as in Fig. 3. The proteins are indicated on the right. The sizes (in kDa) of protein molecular mass markers are indicated on the left.

sequence of p7 within residues 747–780, which are located N-terminally of SpNS2, induces a structural constraint limiting p7/E1 cleavage by the signal peptidase.

To confirm that the sequence located N-terminally of SpNS2 induces a structural constraint on p7/NS2 cleavage site, we made several deletions of sequences located N-terminally of the p7/NS2 cleavage site in the context of E2p7NS2Myc. As shown in Fig. 6, the efficiencies of cleavage of p7NS2Myc and E2p7NS2Myc were very similar. However, when p7 was deleted of its first transmembrane domain (i.e., SpNS2NS2Myc), the cleavage was improved, confirming the above observation made when E1 was used as a reporter protein. Although the cleavage was clearly improved in the absence of the first transmembrane domain of p7, a residual uncleaved product was still detected, indicating that an additional constraint is also present at the P’ side of the p7/NS2 cleavage site.

Although the signal peptides of p7 and NS2 are efficiently cleaved when fused to a reporter protein, we cannot exclude a relationship between the signal peptide and the N terminus of p7 or NS2 that would modulate cleavage efficiency. If such a relationship exists, the efficiency of cleavage should be modified in the context of a foreign signal peptide instead of the natural signal peptide of p7 or NS2. We therefore fused a signal peptide known to be cleaved co-translationally, the signal peptide of CD4, at the N termini of p7 and NS2 (Table I). These proteins were expressed in HepG2 cells, and their electrophoretic mobility was analyzed by SDS-PAGE. Two bands were detected when p7 was expressed with the signal peptide of CD4, indicating that signal peptide cleavage was partial (Fig. 7). Indeed, the more slowly migrating band likely corresponds to p7 with an uncleaved signal peptide, whereas the quickly migrating band corresponds to p7 without any signal peptide. When NS2 was expressed with the signal peptide of CD4, the efficiency of cleavage was very low, because only a minor proportion of the protein was in its mature form (Fig. 7). Similar results were obtained when NS2 was expressed with the signal
peptide of HCV glycoprotein E2 (data not shown). Together, these data suggest that constraints located at the P′ sides of the cleavage sites reduce the efficiency of cleavage and that a relationship between the native signal peptide and the N terminus of NS2 might improve cleavage efficiency. In addition, this suggests that there is cooperativity between the sequences located on both sides of the cleavage sites to modulate cleavage efficiency.

Identification of a Structural Constraint in the E2p7 Cleavage Site—Sequence analyses of E2p7 and p7/NS2 cleavage sites and their contiguous regions have suggested that structural determinants are present in these cleavage sites and might play a role in the inefficiency of E2p7NS2 precursor processing (Fig. 2). As shown in Fig. 2, the E2p7 cleavage site region is predicted as showing a propensity to be folded into α-helix. To destabilize this putative structure, various mutation/insertion of amino acids have been tested theoretically using sequence prediction tools. Residues of E2p7 cleavage site have been kept intact, and drastic changes were excluded to allow us further coherent interpretations. It appeared that the insertion of a Thr residue in the second position of p7 was a good compromise because it introduces a shift in the sequence and in the predicted α-helix, and it suppresses the coiled-coil prediction in this region. The insertion of this Thr residue might thus disturb potential α-helices interactions involving the E2p7 cleavage site region. Experimentally, this Thr residue was inserted in the E2p7NS2Myc polyprotein (Table I, E2 Tp7).

The processing of wild-type and mutated E2p7NS2Myc polyproteins was analyzed as above after metabolic labeling of cells expressing these proteins followed by immunoprecipitation with the anti-Myc antibody. As for the wild-type polyprotein, the anti-Myc antibody immunoprecipitated the E2p7NS2Myc, p7NS2Myc, and NS2Myc proteins when the Thr residue was introduced between the P1 and P2′ residues of E2p7 cleavage site (Fig. 8, E2 Tp7NS2Myc). However, the efficiency of cleavage at the E2p7 cleavage site was improved. Indeed, a decrease in the amount of the E2p7NS2Myc precursor from 48 to 30% and an increase in the amount of p7NS2Myc cleavage product from 19 to 46% were observed when the Thr residue was introduced. These data indicate that the E2p7 cleavage has been improved by introducing a Thr residue near the scissile bond, indicating that the N terminus of p7 plays a role in the inefficient processing by the signal peptidase. Together, these data indicate that a structural determinant located at the P′ side of E2p7 cleavage site contributes to the regulation of cleavage in this region of HCV polyprotein.

As for the E2p7 site, the p7/NS2 cleavage site region was also predicted to be potentially well structured, possibly forming a short α-helix. This putative helix involves the two negatively charged residues 2 and 4 of NS2 that are strictly conserved, pointing to an essential structural and/or functional role. To break this putative α-helix and to destabilize the electric charge pattern, a Pro residue was inserted in the third position of NS2 in the E2p7NS2Myc polyprotein (Table I, p7NS2Myc). Although no global improvement in the cleavage efficiency of E2p7NS2Myc was observed when compared with E2p7NS2Myc (44 and 52%, respectively; Fig. 8), the p7NS2Myc/NS2Myc ratio was clearly lower for this mutant (compared 8 of 36 for E2p7NS2Myc lane with 19 of 33 for E2p7NS2Myc lane). This indicates that the Pro insertion had no obvious effect on E2p7 cleavage, whereas it facilitated p7/NS2 cleavage, pointing to a role of the structural element located at the P′ side of p7/NS2 cleavage site.

A double mutant E2Tp7NS2Myc was created to analyze the efficiency of E2p7NS2 processing in the context of mutations improving cleavage efficiency at both E2p7 and p7/NS2 sites. Interestingly, the efficiencies of processing of E2Tp7NS2Myc and E2Tp7NS2Myc were very similar, suggesting that increasing the efficiency of cleavage at E2p7 site has a dominant effect over any increased efficiency at the p7/NS2 site.

**Introduction of a Linker Sequence at the E2p7 and p7/NS2 Sites Improves the Efficiency of E2p7NS2 Processing**—In the
These data show that inserting a 15-residue linker polypeptide in the P' region within three residues of the scissile bond improves the efficiency of processing of the E2p7NS2Myc precursor, indicating that sequences located at the P' side of the E2p7 and p7/NS2 cleavage sites impose additional constraints on signal peptidase processing.

**DISCUSSION**

Production of an E2p7NS2 precursor as well as a stable E2p7 protein raises some questions about the role of these components in the biology of HCV life cycle. In this study, we analyzed the structural constraints at E2/p7 and p7/NS2 cleavage sites. Sequence analyses and mutagenesis studies have highlighted the presence of structural determinants responsible for the inefficiency of cleavage at the E2/p7 and p7/NS2 sites. In addition, insertion of a linker polypeptide sequence between P-3' and P-4' of the cleavage sites also improved cleavage efficiency, whereas the N-terminal sequences of p7 and NS2 decreased the cleavage efficiency when expressed in the presence of a foreign signal peptide, suggesting that additional constraints exist in the P' region of the cleavage sites. Finally, the sequence of p7 located N-terminally of the signal peptide of NS2 was also shown to reduce the cleavage efficiency at the p7/NS2 site. Together, these results indicate cooperativity between N- and C-terminal sequences on both sides of the cleavage sites to modulate cleavage efficiency. These N- and C-terminal constraints in the processing of a polyprotein precursor are likely essential for HCV to post-translationally regulate the kinetics and/or quantitative expression of some of its final protein products. In particular, the involvement of both sides of p7 sequence in the two partial/delayed signal peptidase cleavage sites identified in HCV polyprotein points to a central role for p7 in the regulation of its release.

The C termini of E2 and p7 contain a sequence for reinitiation of translocation, and when fused to a reporter protein, these sequences function as signal peptides (24, 28). As shown in this work, in the absence of any additional sequence at their N terminus, these signal peptides were totally cleaved from the reporter protein, indicating that they function efficiently. Therefore, the partial cleavages observed in the context of HCV polyprotein suggests that sequences located at the P' region of E2p7 and p7/NS2 cleavage sites likely induce conformational constraints at the level of these sites. A signal peptide exhibits a specific structure that contains a short positively charged region at the N terminus, a central hydrophobic region (h-region), and a more polar C-terminal region containing the site that is cleaved by the signal peptidase (41). The hydrophobic region is helical, whereas the C-terminal region must adopt an extended conformation to fit into the catalytic site of the signal peptidase and be eventually cleaved (42, 43). Because an extended conformation is energetically unstable by itself in the hydrophobic environment of the membrane in the absence of stabilizing intermolecular hydrogen-bonds, it is likely that the C-terminal region of the signal peptides (Spp7 and SpNS2) can display a more stable conformation (e.g. α-helix as predicted) leading to partial cleavage of E2p7 and p7/NS2.

How could the cleavage sites be structurally stabilized to reduce their accessibility? One possibility is that the conformation of the cleavage site is dependent on the sequence located at the P' region. Indeed, our sequence analyses as well as our mutagenesis studies suggest that the E2/p7 cleavage site together with the N-terminal sequence of p7 might form an α-helix. Such a helix is particularly favored in a hydrophobic environment because of its stabilizing network of hydrogen bonds. The disruption of such a helix involving the signal peptide cleavage site to an extended conformation is energetically demanding and might easily explain the inefficient/de-
Regulated Processing in HCV Polyprotein

In conclusion, we show in this work that signal peptidase cleavage can be controlled by elements present outside of the signal peptide. Indeed, structural determinants located N- and C-terminally can modify the accessibility of the cleavage site likely by changing its conformation. This allows HCV to exert post-translational control on the expression of the mature form of some of the proteins it encodes and to regulate their activity.

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