

# Full-length core sequence dependent complex-type glycosylation of hepatitis C virus E2 glycoprotein

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## Abstract

**AIM:** To study HCV polyprotein processing is important for the understanding of the natural history of HCV and the design of vaccines against HCV. The purpose of this study is to investigate the affection of context sequences on hepatitis C virus (HCV) E2 processing.

**METHODS:** HCV genes of different lengths were expressed and compared in vaccinia virus/T7 system with homologous patient serum S94 and mouse anti-serum M<sub>E2116</sub> raised against *E.coli*-derived E2 peptide, respectively. Deglycosylation analysis and GNA (*Galanthus nivalus*) lectin binding assay were performed to study the post-translational processing of the expressed products.

**RESULTS:** E2 glycoproteins with different molecular weights (~75kDa and ~60kDa) were detected using S94 and M<sub>E2116</sub>, respectively. Deglycosylation analysis showed that this difference was mainly due to different glycosylation. Endo H resistance and its failure to bind to GNA lectin demonstrated that the higher molecular weight form (75kDa) of E2 was complex-type glycosylated, which was readily recognized by homologous patient serum S94. Expression of complex-type glycosylated E2 could not be detected in all of the core-truncated constructs tested, but readily detected in constructs encoding full-length core sequences.

**CONCLUSION:** The upstream conserved full-length core coding sequence was required for the production of E2 glycoproteins carrying complex-type N-glycans which reacted strongly with homologous patient serum and therefore possibly represented more mature forms of E2. As complex-type N-glycans indicated modification by Golgi enzymes, the results suggest that the presence of full-length core might be critical for E1/E2 complex to leave ER. Our data may contribute to a better understanding of the processing of HCV structural proteins as well as HCV morphogenesis.

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## INTRODUCTION

Hepatitis C virus (HCV), the major cause of post-transfusion and community-acquired non-A, non-B hepatitis<sup>[1,2]</sup>, is a member of the Flaviviridae family<sup>[3]</sup>. This virus has a positive-sense, single stranded RNA genome of about 9.6 kb, which encodes a polyprotein precursor of about 3000 amino acids. The polyprotein is further processed into various precursors and mature viral proteins<sup>[4,5]</sup>. The structural proteins are encoded in the order NH<sub>2</sub>-core-E1-E2-P7, which are processed into core (C), E1, E2, and P7 by host membrane-associated signal peptidase(s)<sup>[6-11]</sup>. The downstream nonstructural region is processed by a viral metalloprotease and a viral serine protease located at the N-terminus of NS3<sup>[12-17]</sup>. The core protein is thought to constitute the viral capsid with E1 and E2 being the virus envelope proteins. Numerous studies have shown that E1 and E2 are heavily glycosylated and associate to form a noncovalent heterodimeric complex<sup>[9,10,18,19]</sup>. E1 and E2 are believed to be type I transmembrane proteins with an N-terminal glycosylated ectodomain and a C-terminal hydrophobic anchor.

The lack of an efficient *in vitro* cell culture system for productive HCV propagation<sup>[20-27]</sup> and low levels of HCV particles in the liver tissues or blood of infected patients<sup>[28,29]</sup> have hampered the study of native viral proteins. Fortunately, a variety of prokaryotic and eukaryotic expression systems have proved useful for the production and characterization of HCV encoded proteins<sup>[8, 9, 11, 30-32]</sup>. However, diverse findings have been reported, regarding the molecular weights of E2, which most likely is a reflection of the differences in efficiency of HCV polyprotein processing and post-translational modification achieved in the particular systems<sup>[7-9, 11, 17-19, 30]</sup>. In this study, the E2 expression of recombinant plasmids carrying various length of HCV C-E1-E2 coding sequences was analyzed in the vaccinia virus/bacteriophage T7 RNA polymerase expression system<sup>[33]</sup>. The results suggest that the upstream conserved core coding sequence is required for the production of E2 glycoproteins carrying complex-type N-glycans which react strongly with homologous patient serum and therefore possibly represent more mature forms of E2.

## MATERIALS AND METHODS

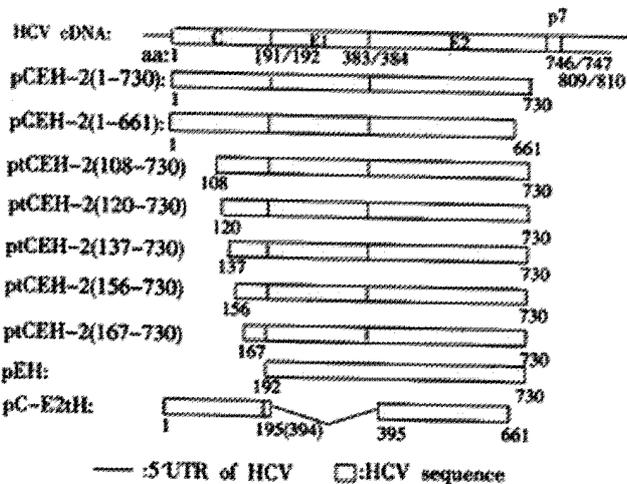
### Cells and viruses

Human HeLa (ATCC #CCL-2) and monkey BS-C-1 (ATCC #CCL-26) cells were maintained in Dulbecco's modified essential medium (DMEM/HG) supplemented with 5% heat inactivated fetal calf serum (FCS) at 37°C in a 5% CO<sub>2</sub> atmosphere. Recombinant vaccinia virus vTT7 that expresses the bacteriophage T7 RNA polymerase gene under the control of vaccinia virus early/late promoter P7.5 was generated and propagated as previously described<sup>[34]</sup>. PFU (plaque forming unit) titration was performed on BS-C-1 cell monolayers.

### Plasmid constructions

The vaccinia virus/T7 promoter expression vector pTM1 was kindly provided by Bernard Moss (NIH, Bethesda, USA) and all of the expression plasmids carrying HCV cDNA encoding structural proteins described below were derived from pTM1. Figure 1 depicts the HCV gene fragment in the expression plasmids. Plasmids pCEH-2 (1-730)

and pEH containing HCV C, E1 and E2 gene of subtype 1b<sup>[35]</sup> (GENBANK accession #D10934) were described previously<sup>[34]</sup>. Briefly, cDNA sequences encoding HCV polyprotein amino acids 192 to 730 were inserted into pTM1 to obtain pEH. HCV sequences encoding complete C were fused with the E1/E2 sequences of pEH to result in plasmid pCEH-2(1-730). The latter plasmid served as basis for PCR cloning to generate plasmids pCE1(1-341), ptCEH-2(108-730), ptCEH-2(120-730), ptCEH-2(137-730), ptCEH-2(156-730), ptCEH-2(167-730), pCEH-2(1-661) and pTM1/EH(192-661) for the expression of 5' or/and 3' truncated HCV sequences encoding HCV polyprotein amino acids as indicated by numbers in parenthesis. Plasmid pC-E2H(1-195/394-661) was generated by deleting E1 coding sequences from pCEH-2(1-661) and linking polyprotein amino acids aa194 and aa394 together. Authenticity of HCV cDNA sequences in all constructed plasmids was confirmed by automatic sequencing.



**Figure 1** Schematic maps of HCV coding sequences inserted into the plasmid pTM1 and expressed under transcriptional control of the bacteriophage T7 pol promoter. Numbers refer to amino acids of the HCV polyprotein.

### Transient expression of recombinant genes using vaccinia virus/T7 RNA polymerase (VV-T7pol)

HeLa cells grown to 80% confluency were infected with recombinant vaccinia virus vTT7 at a multiplicity of infection (MOI) of 10 PFU per cell to allow for production of recombinant T7 RNA polymerase. At 2h post-infection, the inoculum was removed and DNA of pTM1 based expression plasmids was transfected using DOTAP liposomal transfection reagent as described by the manufacturer (Roche Molecular Biochemicals, Mannheim). After 24 hours of incubation, infected/transfected cells were washed twice with PBS, harvested by scraping, pelleted upon brief centrifugation, and resuspended in a small volume of PBS. Cell lysates were prepared by adding SDS-PAGE sample loading buffer and stored at -80°C until further analysis.

### Western blot analysis

Cell lysates were separated by reductive SDS-PAGE and then transferred onto nitrocellulose membranes (Schleicher & Schuell). Blocking was done using 5% fat-free milk powder. For immunodetection of HCV proteins, blots were incubated with primary antibodies, washed, and incubated with 1000 fold diluted HRP-protein A (Sigma). The membranes were then washed again and reactive proteins were detected using the ECL system (Amersham Pharmacia Biotech) according to the manufacturers' instructions. The primary antibodies used in this study include: anti-HCV human serum S94 at a dilution of 1:500 (kindly provided by Wang, Y., Beijing

University, China), anti-HCV human serum S268 at a dilution of 1:500 (kindly provided by Lu Z., Shanghai Ruijin Hospital, China), anti-E2 mouse polyclonal antibody M<sub>E2116</sub><sup>[36]</sup> at a dilution of 1:300 (raised against E. Coli-derived HCV E2 polypeptide aa450 to 565), and anti-E1 rabbit polyclonal antibody R<sub>E1135-C</sub> at a dilution of 1:250 (raised against an E. coli-derived C-terminally truncated HCV E1 fragment).

### Characterization of N-glycans on expressed E2 glycoproteins

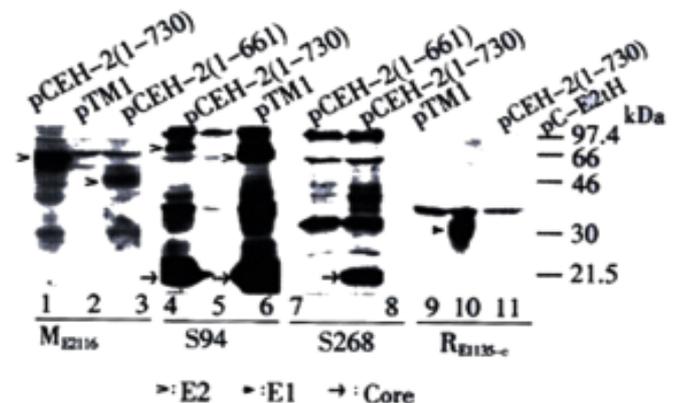
For deglycosylation analysis, cell pellets were directly lysed in denaturing buffer provided by the manufacturer and digested with PNGase F (NEB) or Endo H (NEB) for 2 hours at 37°C.

The type of N-glycans on expressed E2 glycoproteins was also analyzed by testing its ability to bind to GNA (Galanthus nivalus) lectin. HeLa cells infected with vTT7 and transfected with pCEH-2(1-730) were collected by scraping, washed in cold PBS, and then lysed with lysis buffer (50mM Tris-HCl [pH8.0], 150mM NaCl, 0.5% Nonidet P-40, 1mM PMSF). After centrifugation at 10000g, the supernatant was allowed to bind to GNA-agarose (Sigma). The flow-through fraction was collected and the gel matrices were washed with lysis buffer. Bound proteins were eluted with 1M  $\alpha$ -D-mannopyranoside (Sigma) in lysis buffer. Samples were then analyzed by Western-blotting.

## RESULTS

### Detection of E2 glycoproteins of different Molecular Weights using antibodies of distinct origins

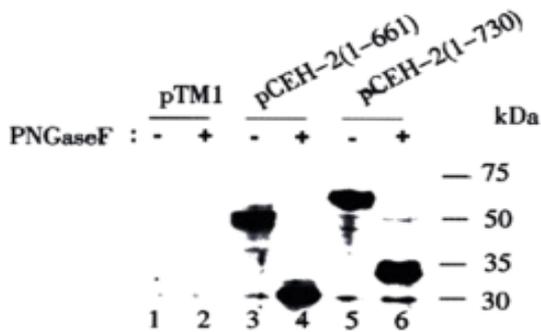
The hybrid vaccinia virus/T7 bacteriophage RNA polymerase expression system was used to study the expression of the HCV structural proteins. The transient expression products of plasmids pCEH-2(1-730) and pCEH-2(1-661), which contain HCV cDNA encoding the structural region terminating at amino acid 730 and 661 of the polyprotein respectively, were analyzed by Western blot using polyclonal mouse serum M<sub>E2116</sub> raised against E.coli-derived E2 protein<sup>[36]</sup>. E2 products with apparent molecular weights (MWs) of ~60kDa and ~50kDa were detected for pCEH-2(1-730) and pCEH-2(1-661) respectively (Figure 2, lane 1, 3). The apparent molecular weights were higher than calculated values, which, along with the heterogeneous appearance of the detected bands, suggested that these were glycosylated expression products. The lower molecular weight of the E2 species obtained from expression of pCEH-2(1-661) in comparison to pCEH-2(1-730) was consistent with the introduced truncation at the 3' end of the E2 coding sequences leading to the loss of 70 amino acids in the recombinant polypeptide backbone.



**Figure 2** Detection of E2 glycoprotein species of different molecular masses. Transient expression products were analyzed by Western blot with antibodies of distinct origin: mouse polyclonal antibody M<sub>E2116</sub> (lane 1, 2, 3); HCV patient serum S94 (lane 4, 5, 6); HCV patient serum S268 (lane 7, 8); rabbit polyclonal antibody R<sub>E1135-C</sub> (lane 9, 10, 11). Empty vector pTM1 was used as the negative control. HCV-specific protein bands are indicated by arrowheads. The plasmids used for transfection are indicated at the top of the lanes.

The expression products were also analyzed with HCV patient serum S94. Multiple prominent bands were detected for pCEH-2(1-730) and pCEH-2(1-661), but not for vector plasmid pTM1, representing expressed HCV structure proteins and possibly some precursors. The bands of  $\sim 75$  kDa and  $\sim 66$  kDa (Figure 2, lane 4, 6), which again consistent with the different length of E2 coding sequence in both plasmids, should represented the E2 proteins, although their MWs were higher than that detected by  $M_{E2116}$ . It is worth noting that the analyzed HCV cDNA originated from the same patient from whom S94 was collected. The core antigen and some precursors of expression products could also be detected by another HCV patient serum S268, but we could not detect the E2-specific 75kDa band (Figure 2, lane 8), which could be attributed to the high variability of the E2 glycoprotein<sup>[37, 38]</sup>.

The antibody dependent detection of different E2 glycoprotein species was surprising. To rule out that the E2 bands of higher MW is the uncleaved E1-E2 precursors, the expression products from pCEH-2(1-730) were analyzed with anti-E1 rabbit sera. A heavy band of about 30kDa was detected, possibly representing multiple forms of E1 proteins (Figure 2, lane 10), while no E1-specific band with higher MW was detected. Another possible explanation could be that E2 polypeptides of varying sizes were synthesized due to incomplete or irregular processing of the polyprotein. This hypothesis was abandoned when we subjected recombinant proteins to deglycosylation with PNGase F prior to Western blot analysis with mouse polyclonal antibody  $M_{E2116}$ . After PNGase F treatment, only one E2-specific band with apparent molecular weight of 30 kDa was detected for pCEH-2(1-661) expression products, while an E2-specific doublet band of 33/34 kDa was detected for pCEH-2(1-730) expression products (Figure 3), which is consistent with the MWs of calculated E2 polypeptide backbones. It suggests that difference of the MWs of E2 species detected by  $M_{E2116}$  and S94 from the same transient expression products (pCEH-2(1-730):  $\sim 60$ kDa and  $\sim 75$ kDa, pCEH-2(1-661):  $\sim 50$ kDa and  $\sim 66$ kDa) is mainly due to different N-glycosylation. The detection of a E2 peptide doublet with pCEH-2(1-730) but not with pCEH-2(1-661) is in agreement with the fact that E2 contains a PKR-eIF2a phosphorylation site (PKR: RNA-activated protein kinase) at aa659-670<sup>[39]</sup>, which is largely deleted in the pCEH-2(1-661) construct.



**Figure 3** Deglycosylation analysis of the expressed HCV E2 proteins with PNGase F. Transient expression products were subjected to Western blot analysis with  $M_{E2116}$  as the primary antibody. pTM1 transfected cells were used as control. The plasmids used for transfection are indicated at the top of the lanes. Samples incubated with deglycosylation buffer were run in parallel. +: samples digested with PNGase F, -: samples incubated with PNGase F reaction buffer.

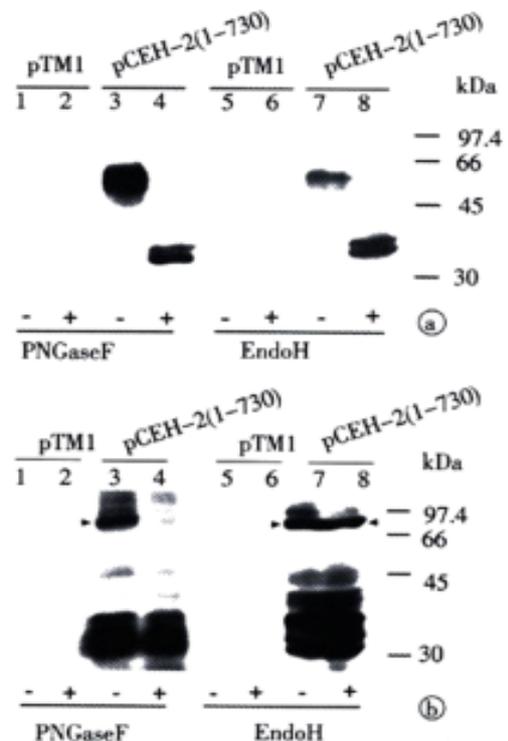
Altogether, the above results suggest that the 75kDa and 66kDa bands detected by S94 are HCV E2 glycoproteins of heavier glycosylation and thus higher molecular weight.

### ***$M_{E2116}$ - and S94-reactive E2 glycoproteins carried different types of N-glycans***

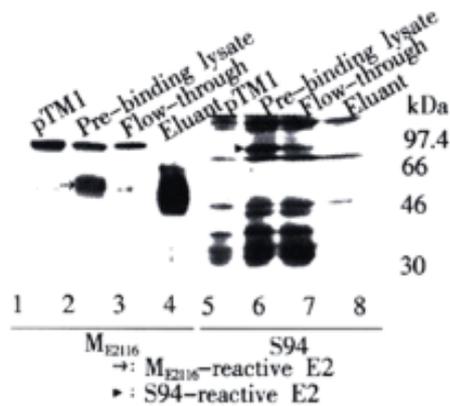
Since  $M_{E2116}$ - and S94-reactive E2 species had polypeptide backbones

of the same size, the difference in apparent molecular weight and antibody reactivity could only be attributed to differences in the degree and/or type of glycosylation. The glycan type on different E2 species expressed from pCEH-2(1-730) was then analyzed by testing their sensitivity to PNGase F and Endo H. PNGase F hydrolyzes all types of N-glycan chains from glycopeptides and glycoproteins unless they carry  $\alpha$ -1-3 linked core fucose residues present in insect and plant glycoproteins<sup>[40]</sup>, while Endo H cleaves only high mannose structures and hybrid structures on N-linked oligosaccharides of glycoproteins<sup>[41]</sup>. Figure 4A shows that the  $M_{E2116}$ -reactive E2 species was sensitive to both PNGase F and Endo H digestion. The S94-reactive E2 species disappeared after PNGase F digestion (Figure 4B). It was difficult to detect the deglycosylated E2 with S94 after PNGase F treatment, because there were multiple HCV polyprotein precursor proteins of about 30000 reacted strongly with S94 and unglycosylated E2 seemed to react weakly with S94 (unpublished data). However, the highly glycosylated, S94-reactive E2 band remained after Endo H digestion (Figure 4B). The glycan type of different E2 species expressed from pCEH-2(1-730) were also analyzed by testing their ability to bind to GNA lectin. GNA is specific for the non-reducing end of  $\alpha$ -D-mannosyl residue of glycoconjugate and therefore can be used to probe the presence of high mannose type or hybrid type glycans on glycoproteins<sup>[42, 43]</sup>. The  $M_{E2116}$ -reactive E2 species could quantitatively bind to and be eluted from GNA-agarose, whereas no obvious binding could be demonstrated for S94-reactive E2 species (Figure 5).

The resistance of S94-reactive E2 glycoprotein species to Endo H digestion together with the fact that it could not bind to GNA indicates that the S94-reactive E2 protein carries complex-type glycans.



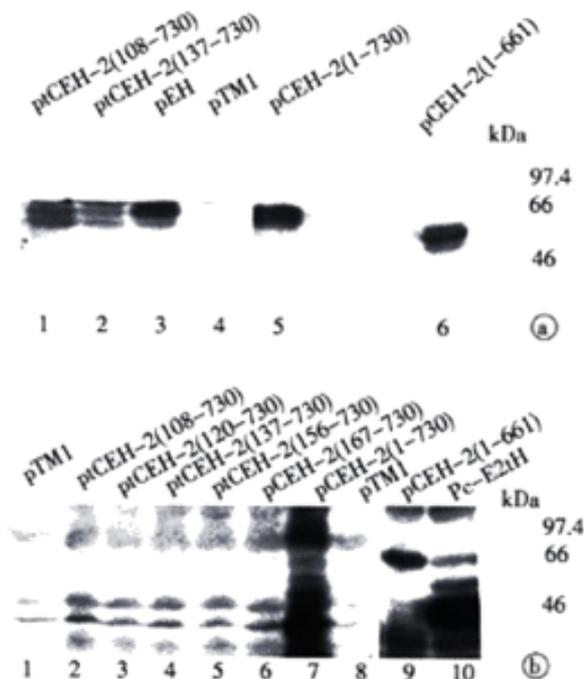
**Figure 4** Different sensitivities of two glycosylated E2 species to PNGase F and Endo H. Transient expression products of pCEH-2(1-730) were digested with PNGase F or Endo H, respectively. The digested samples were analyzed by Western blot with  $M_{E2116}$  (A) and S94 (B). Empty vector pTM1 was used as negative control. Samples incubated with deglycosylation buffer were run in parallel. +: samples digested with Endoglycosidase, -: samples incubated with Endoglycosidase reaction buffer. The plasmids used for transfection are indicated at the top of the lanes. Endoglycosidases used in this study are indicated at the bottom of the lanes. S94-reactive E2 proteins are indicated by arrowheads.



**Figure 5** Different ability of differently glycosylated E2 species to bind to GNA-agarose. HeLa cells infected with vTT7 and transfected with pCEH-2(1-730) were collected, washed and lysed with lysis buffer. The cleared supernatant was then allowed to bind to GNA-agarose. The gel beads were washed and eluted with 1M  $\alpha$ -D-mannopyranoside in lysis buffer. Pre-binding lysate, flow-through and eluant fractions were analyzed by Western blot analysis. HeLa cells infected with vTT7 and transfected with pTM1 were served as negative control. The sera used as primary antibodies are indicated at the bottom of the lanes. E2 proteins are indicated by arrowheads.

### HCV core sequence dependent formation of complex-glycosylated E2

We also assessed if co-expression of core or E1 coding sequences had any effect on the production of the E2 proteins, which was readily recognized by homologous patient serum S94. A set of expression plasmids containing HCV cDNAs with various deletions in the core sequence (ORF starting at aa108, aa120, aa137, aa151, or aa167, respectively) were constructed. The lysates from vTT7-infected and plasmid DNA transfected HeLa cells were analyzed by Western blot with either the mouse anti-serum M<sub>E2116</sub> or the patient serum S94 (Figure 6).



**Figure 6** Requirement of the core sequence for the expression of complex-type glycosylated E2. Expression products of differently truncated HCV structural genes were analyzed by Western blot analysis. Blots were probed with M<sub>E2116</sub> (A) or with S94 (B). Empty vector pTM1 was used as negative control. The plasmids used for transfection are indicated at the top of the lanes.

When using mouse antibody M<sub>E2116</sub>, recombinant E2 of  $\sim$ 60kDa and  $\sim$ 50kDa could be detected upon expression of all constructs tested (Figure 6A). Patient serum S94 allowed detection of E2 for pCEH-2(1-730) and pCEH-2(1-661) with full-length core coding sequences (Figure 6B, lanes 7, 9), similar to that described in Figure 2. In contrast, no E2 products could be visualized after expression of constructs containing no or only partial core sequences (Figure 6B, lanes 1-6). Interestingly, deletion of E1 coding sequences had no significant effect on the synthesis of S94 detectable E2 protein (Figure 6B, lane 10). These results suggest that the presence of complete HCV core sequence is crucial for the expression and/or post-translational processing of the complex-type glycosylated form of E2.

### DISCUSSION

In this study, various constructs of HCV cDNAs placed under transcriptional control of the bacteriophage T7 promoter were transiently expressed using vaccinia virus/T7 system. Upon characterization of the HCV gene products with different antibodies, two species of E2 with different MWs were identified in the expression products of the same plasmid. The high molecular weight forms of E2 were readily recognized by a patient serum, but displayed weak reactivity with antibodies raised against E. coli derived E2. These high molecular weight forms of E2 were not likely produced from inefficient proteolytical processing at the E1/E2 boundary as these proteins were not stained with E1-specific antibodies. Efficient processing at E1/E2 was confirmed by deglycosylation analysis. The difference of the MWs of E2 species detected by S94 and by M<sub>E2116</sub> was therefore mainly due to different N-glycosylation. The S94-reactive E2 glycoproteins, which were resistant to Endo H digestion and could not bind to GNA, carry complex-type glycans.

The specific recognition of the complex-type glycosylated E2 but not the high-mannose-type glycosylated E2 by homologous patient serum S94 suggested that the former could be a better representation of native E2 proteins on HCV virions. Similar results were also reported by Inudoh *et al*<sup>[44]</sup>. By comparing the reactivity of complex-type glycosylated E2 and the high-mannose-type glycosylated E2 with different patient sera, they demonstrated that the former is superior in diagnosing HCV infection. Their results and our results reported here are in concordance with the finding that E2 protein on patient derived virions contained complex-type sugars indicating Golgi-specific modification<sup>[45]</sup>.

Expression of full-length or C-terminally truncated envelop proteins in eukaryotic cells has demonstrated that E1 and E2 are retained within the ER membrane system due to the presence of ER-retention signals in the C-termini of both envelope proteins<sup>[46-50]</sup>. However, recent study indicates that HCV E2 proteins could also present in the Golgi apparatus of the stably transfected cell line expressing HCV C-E1-E2-NS2 fragment. A possible explanation could be that Martire *et al*<sup>[51]</sup> used an HCV gene fragment including full-length core sequences in their study while structural protein sequences without full-length core sequences were used to study the localization of envelop proteins. The results reported here demonstrated that the complex-type glycosylated, possibly more mature form of E2 is only detectable upon co-expression of the complete HCV core coding sequence. Deletion of the first 107 N-terminal core amino acid residues was obviously sufficient to abrogate production of complex-glycosylated E2. This result suggest that the core protein might allow for targeting the envelope glycoproteins to Golgi-specific modification, which could be a key step in the morphogenesis of HCV virions. HCV-like particles were observed when HCV cDNA encoding whole core, E1 and E2 was expressed in baculovirus-insect expression system<sup>[52]</sup>. After binding of core to the E1-E2 complex statically located on the ER membrane, virus-like particles might be formed and the conformation of E1-E2 complex changed, which could result in the abrogation of the ER-retention

signal for the E1-E2 complex. Then the virus-like particles might migrate along the secretion pathway, where E2 (and E1) proteins undergo more complex glycosylation by the Golgi enzymes.

In summary, upon expression of recombinant HCV core, E1, and E2 sequences, the E2 proteins of different glycosylations could be identified. The complex-glycosylated E2 protein might represent a more mature form of E2 and its formation required the conserved core coding sequences. Our data may contribute to a better understanding of the processing of HCV structural proteins as well as HCV morphogenesis.

## ACKNOWLEDGMENT

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