The addition of N-linked oligosaccharides to Asn-X-(Ser/Thr) sites is catalyzed by the oligosaccharyltransferase, an enzyme closely associated with the translocon and generally thought to have access only to nascent chains as they emerge from the ribosome. However, the presence of the sequon does not automatically ensure core glycosylation because many proteins contain sequons that remain either nonglycosylated or glycosylated to a variable extent. In this study, hepatitis C virus (HCV) envelope protein E1 was used as a model to study the efficiency of N-glycosylation. HCV envelope proteins, E1 and E2, were released from a polyprotein precursor after cleavage by host signal peptidase(s). When expressed alone, E1 was not efficiently glycosylated. However, E1 glycosylation was improved when expressed as a polyprotein including full-length or truncated forms of E2. These data indicate that glycosylation of E1 is dependent on the presence of polypeptide sequences located downstream of E1 on HCV polyprotein.

Proteins that are transported and sorted by the secretory pathway begin their journey at the endoplasmic reticulum (ER) membrane. It is here that nascent secretory and membrane proteins are translocated across or integrated into the membrane. However, before moving to the final destination, these proteins have to be appropriately modified, folded, and assembled. Among the modifications affecting proteins targeted to the secretory pathway of eukaryotic cells, N-linked glycosylation is often observed. This modification plays an important role in regulating the activity, stability, and antigenicity of mature proteins. In addition, N-linked glycosylation allows newly synthesized glycoproteins to interact with a lectin-based chaperone system in the ER, which plays a major role in protein folding and quality control.

The addition of N-linked oligosaccharides is catalyzed by the oligosaccharyltransferase, an enzyme closely associated with the translocon and generally thought to have access only to nascent chains as they emerge from the ribosome. It has been shown in a cell-free translation system that oligosaccharyltransferase transfer only occurs when 12–14 amino acids C-terminal to a sequon have been translocated into the ER lumen. This suggests that the active site of the oligosaccharyltransferase and dolichol oligosaccharide donor, which are tethered to the luminal surface, are projected 30–40 Å into the ER lumen. Core glycosylation determines the number of individual oligosaccharides attached to a given polypeptide and involves the transfer of a presynthesized Glc3Man9GlcNAc2 unit from a membrane-associated donor, oligosaccharide-pyrophosphodolichol, to asparagine residues in the tripeptide acceptor sequon Asn-X-(Ser/Thr), where X is any amino acid except Pro. Although the sequon is essential for core glycosylation, it is observed that all potential glycosylation sites are not utilized, and some sequons are inefficiently glycosylated. Recent studies have demonstrated that the amino acid present at the X position of the sequon may modulate the efficiency of core glycosylation. In addition, inhibition of disulfide bond formation may increase the level of modification of a naturally occurring glycosylation sequon, suggesting that there may be a link between folding and utilization of glycosylation sequons in vivo.

In a study using carboxypeptidase Y in Saccharomyces cerevisiae as a model system, the introduction of new sites for N-glycosylation at positions buried in a folded protein structure did not necessarily lead to glycosylation of these sites, indicating that folding and glycosylation can compete in vivo and that glycosylation does not necessarily precede folding.

The maturation of viral proteins in infected cells involves mostly the host cell metabolic pathway, including localization mechanisms, folding proteins, and enzymes that modify the primary translation product. For this reason, viral envelope proteins have often been used as tools for cell biology studies. In this work, we used hepatitis C virus (HCV) envelope protein E1 as a model to study N-glycosylation in a cell culture system. The HCV genome encodes two envelope proteins, E1 and E2, which form a noncovalent heterodimer. These two envelope proteins are released from HCV polyprotein precursor after cleavage by host signal peptidase(s). They are transmembrane proteins with a large N-terminal ectodomain and a C-terminal hydrophobic anchor, and they are heavily modified by N-linked glycosylation. E1 has been shown to be glycosylated at positions 196, 209, 234, and 305 (positions on the polyprotein). Here, we show that E1 was not efficiently glycosylated when expressed alone. However, glycosylation of E1 was improved when expressed as a polyprotein with the full-length or a truncated form of E2, indicating that glycosylation of E1 is dependent on the presence of polypeptide sequences located downstream of E1 on HCV polyprotein.
Efficiency of Glycosylation of a Viral Envelope Protein

EXPERIMENTAL PROCEDURES

**Cell Culture**—The HepG2 cell line was obtained from the American Type Culture Collection, Manassas, VA. Cell monolayers were grown in Dulbecco’s modified essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

**Generation and Growth of Viruses**—Plasmids expressing proteins of interest were constructed as described previously (22). Vaccinia virus recombinants were generated by homologous recombination essentially as described previously (23). The following vaccinia virus recombinants have been described previously: vTF7-3 (expressing the T7 DNA-dependent RNA polymerase) (24), vaccinia viruses expressing E1 or glycosylation mutants of E1 (N1, N2, N3, N4, N2-3, N1-2-3 and N1-2-3-4) (22), E1E2-524 (expressing the signal sequence of E1, E1 and a truncated form of E2 ending at residue 524) (25), E1E2-661 and E1E2-715 (26), E1E2-524, E1E2-661 and E1E2-715 (27).

**Metabolic Labeling, Immunoprecipitation, and Endoglycosidase Digestions**—Cells expressing HCV proteins were metabolically labeled with 35S-protein labeling mix (100 μCi/ml, NEN Life Science Products) as described previously (28). Cells were lysed with 0.5% iegpal CA-630. Phosphor imaging quantifications were also performed.

**Western Blotting**—Proteins bound to nitrocellulose membranes (Hybond-C extra, Amersham Pharmacia Biotech) were revealed by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech) as recommended by the manufacturer. Briefly, after separation by SDS-PAGE under reducing conditions, proteins were transferred to nitrocellulose membranes by using a trans-blot apparatus (Bio-Rad) and detected with specific mab (A4, see Ref. 28, dilution 1:1000) followed by rabbit anti-mouse immunoglobulin conjugated to peroxidase (dilution 1:1000, DAKO).

**RESULTS**

**E1 Expressed Alone Is Not Efficiently Glycosylated**—Some RNA viruses synthesize their polypeptides as a polypeptide precursor that is cleaved co- or post-translationally by viral and/or host proteases. The fact that the envelope proteins of this type of virus are translated from a single coding region implies that internal signal peptides must be cleaved. HCV envelope protein E1 has its signal sequence located downstream of the mature form of the capsid protein (C) (21) (Fig. 1, top). In addition, a hydrophobic sequence present in the second half of the transmembrane domain of E1 is the signal sequence for the other envelope protein E2 located immediately downstream of E1 on the polypeptide. Previous work has shown that the expression of E1 alone with its signal sequence leads to its translocation in the ER lumen and anchorage of its C-terminal hydrophobic sequence in the ER membrane (30). However, E1 is often resolved as a multiband protein when analyzed by SDS-PAGE, suggesting that different glycoforms of this protein might exist (31–33). To analyze whether some glycosylation sites differ in their degree of glycosylation, E1 and glycosylation mutants were expressed in HepG2 cells by vaccinia virus recombinants, and their electrophoretic mobilities were compared after separation by SDS-PAGE followed by Western blotting. As shown in Fig. 2a, the bands detected for wild-type E1 comigrated with the highest molecular-weight bands of mutants lacking one (E1-N2), two (E1-N2-3) and three (E1-N1-2-3) glycans (a), or to wild-type E1 digested by PeptideN-glycosidase F (PNGase F) or endo-β-N-acetylgalactosaminidase H (Endo H) (b).

**Efficiency of Glycosylation of a Viral Envelope Protein**

![Image](89x586 to 257x729)

**FIG. 1.** Processing of the N-terminal region of HCV polyprotein generating the envelope proteins E1 and E2, and schematic representation of the proteins expressed in these studies. Signal sequences and transmembrane domains are indicated by solid boxes. The arrows indicate the sites that are cleaved by a signal peptidase. The amino acid positions of the first residues of E1, E2, and p7 are indicated above the arrows. The N-linked glycosylation sites of E1 are indicated by asterisks. These sites are located at amino acid positions Asn-196 (N1), Asn-209 (N2), Asn-234 (N3), and Asn-305 (N4), respectively. Numbers reported at the end of truncated E1E2 polyproteins indicate the positions of the last residue of E2 present in the truncated polyprotein.

![Image](368x535 to 494x729)

**FIG. 2.** Glycosylation of E1 expressed in the absence of E2. HepG2 cells were coinfected with vTF7-3 and the appropriate vaccinia virus recombinant at a multiplicity of 5 pfu/cell. Infected cells were harvested at 7-h postinfection, the proteins were separated by SDS-PAGE (13% acrylamide), and E1 was revealed by Western blotting with the anti-E1 mab A4. The migration profile of E1 expressed in the absence of E2 (E1Nt) was compared with glycosylation mutants of E1 lacking one (E1-N2), two (E1-N2-3), three (E1-N1-2-3) or 4 (E1-N1-2-3-4) glycans (a), or to wild-type E1 digested by Peptide-N-glycosidase F (PNGase F) or endo-β-N-acetylgalactosaminidase H (Endo H) (b).
Coexpression of E1 and E2 in Cis Improves the Efficiency of Glycosylation of E1—We have shown that E1 needs to be coexpressed with E2 to efficiently form its intramolecular disulfide bonds, indicating that the folding of E1 is assisted by E2 (27, 34). Although E2 is located immediately downstream of E1 on the HCV polyprotein, we wanted to determine whether the coexpression of the two envelope proteins would have some effect on the efficiency of E1 glycosylation. To answer this question, the electrophoretic mobility of E1 coexpressed with E2 was analyzed by SDS-PAGE followed by Western blotting and compared with E1 expressed by itself. Expression of E2 in cells infected with vaccinia virus recombinants expressing E2E1, E1E2, or E1+E2 was confirmed by Western blotting with an anti-E2 mab (data not shown). To be sure that all the cells would express both E1 and E2 when produced by different vaccinia virus recombinants, a multiplicity of infection of 5 pfu/cell was used for each virus. As shown in Fig. 4a, the ratio of fully glycosylated E1 was higher when the two envelope proteins were coexpressed (E1E2; approximately 75% of total E1), whereas only approximately 50% of E1 expressed alone (E1) was glycosylated at all four sites. Similar results were obtained by using Sindbis virus recombinants (data not shown), indicating that this observation is independent of the expression vector. It is worth noting that when analyzed in pulse-chase experiments, the intensity of E1 coexpressed with E2 was lower during the pulse, and a smear was observed above the E1 band (Fig. 4b, compare E1 and E1E2). This suggests that synthesis of the polyprotein is not terminated at this time point and that cleavage between E1 and E2 is not yet complete. The difference in the efficiency of glycosylation of E1 expressed in the presence or absence of E2 indicates that the presence of E2 improves the efficiency of glycosylation of E1. When HepG2 cells were infected by vaccinia virus recombinants expressing E1 and E2 separately (E1+E2), no change in the glycosylation profile of E1 was observed (Fig. 4a, compare E1 and E1+E2), indicating that E1 and E2 need to be expressed from the same transcript to increase the efficiency of glycosylation of E1. However, when the positions of E1 and E2 were inverted on the polyprotein (E2E1), instead of observing a relative increase in the intensity of the fully glycosylated E1, a slight decrease was repeatedly detected (Fig. 4a, compare E2E1 and E1), indicating that the position of E1 and E2 on the polyprotein is important for efficient glycosylation of E1. Altogether, these data indicate that coexpression of E1 and E2 in cis, with E2 downstream of E1, improves the efficiency of glycosylation of E1.

The N Terminus of E2 on HCV Polyprotein Is Sufficient to Improve the Efficiency of Glycosylation of E1—To determine whether the whole sequence of E2 is necessary to improve the efficiency of glycosylation of E1, deletions were introduced in E2. As shown in Fig. 5a, C-terminal deletions of E2 ending at position 715 (E1E2–715), 661 (E1E2–661), and 524 (E1E2–524) did not reduce the efficiency of glycosylation of E1, indicating that the presence of the N terminus of E2 is responsible for E2-dependent glycosylation of E1. We also wondered whether the sequence necessary to improve the glycosylation of E1 needs to be a specific one. We therefore replaced the N terminus of E2 by a short amino acid sequence corresponding to a Myc epitope (EQKLISEEDL) plus three Gly residues at the junction with E1 (E1-Myc). The last residue at the C terminus of E1 (Ala) was replaced by an Arg to avoid partial signal sequence cleavage which would interfere with interpretation. Although the effect was less dramatic, the presence of another sequence downstream of E1 is sufficient to improve its glyco-

**FIG. 3.** Glycosylation of E1 mutated at glycosylation site N1, N2, N3, or N4. HepG2 cells were coinfected with vTF7-3 and the appropriate vaccinia virus recombinant(s) at a multiplicity of 5 pfu/cell. Infected cells were harvested at 7-h postinfection, the proteins were separated by SDS-PAGE (13% acrylamide), and E1 was revealed by Western blotting with the anti-E1 mab A4. Bands corresponding to the presence of one (1g), two (2g), three (3g), or four (4g) glycan(s) are indicated. Gel autoradiographs were exposed in the linear range and analyzed by densitometric scanning. The 4-glycan (E1) or 3-glycan (N1, N2, N3, or N4) bands were quantified and expressed as percent of total or mutant E1 molecules that are fully glycosylated (b).

**FIG. 4.** Comparison of the glycosylation of E1 expressed alone or coexpressed with E2. a, HepG2 cells were coinfectected with vTF7-3 and the appropriate vaccinia virus recombinant(s) at a multiplicity of 5 pfu/cell. Infected cells were harvested at 7-h postinfection, the proteins were separated by SDS-PAGE (13% acrylamide), and E1 was revealed by Western blotting with the anti-E1 mab A4. Bands corresponding to the presence of one (1g), two (2g), three (3g), or four (4g) glycans are indicated. b, pulse-chase analysis of E1 expressed alone or as an E1E2 polyprotein. HepG2 cells were coinfected with vTF7-3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 pfu/ml. At 4.5-h postinfection, cells were pulse-labeled for 5 min and chased for the indicated times. Cell lysates were immunoprecipitated with mab A4, and samples were separated by SDS-PAGE (13% acrylamide). Bands corresponding to the presence of two (2g), three (3g) or four (4g) glycans are indicated.
indicated. It has been shown that E1 needs to be coexpressed with E2 to fold properly (27), and here we show that expression of E1 and E2 from the same polyprotein improves the efficiency of glycosylation of E1. We therefore wanted to know whether these two functions of E2 are linked. Because the presence of a truncated form of E2 on HCV polyprotein is sufficient to improve the glycosylation of E1, we analyzed whether a truncated form of E2 also improves the folding of E1. For this purpose, we monitored disulfide bond formation by SDS-PAGE under nonreducing conditions as described previously (29). This method takes advantage of an increase in mobility as a protein acquires a compact conformation stabilized by the formation of intramolecular disulfide bonds. An oxidized form of E1, which appeared slowly, was clearly detected in the context of E1E2 (Fig. 6) as previously observed (29). However, the intensity of the oxidized form of E1 was lower when the polyprotein was truncated at the C terminus of E2 (Fig. 6, E1E2–661) or in the absence of E2 (Fig. 6, E1). Quantitative analyses showed approximately a 50% reduction in the formation of oxidized E1 when E2 was truncated. It has to be noted that part of E1, when separated under nonreducing conditions, remains poorly understood why some sequons are glycosylated to a variable extent. Here, we report that a sequence located far downstream of the glycosylation sites of HCV envelope protein E1 modulates the efficiency of glycosylation of these sites.

HCV envelope protein E1 is not efficiently glycosylated when expressed in the absence of E2. Indeed, approximately 50% of E1 was partially glycosylated when expressed in HepG2 cells by a vaccinia virus recombinant. Residues like Trp, Leu, Asp, and Glu at position X have been shown to be associated with less efficient core glycosylation in a cell-free system (16). Indeed, large hydrophobic amino acids may inhibit core glycosylation by producing an unfavorable local conformation, and the charge of the X residue may influence the ability of oligosaccharyltransferase to bind simultaneously to the sequon and the negatively charged dolichol-PP-oligosaccharide precursor (13, 35). A Ser (N1 and N2), an Ala (N3), and a Cys (N4) occupy the X positions in the sequence of the E1 protein used in our study (22), and these residues are usually associated with highly efficient core glycosylation. However, the Cys residue is uncommon at the X position in core-glycosylated sequons (36). This probably reflects the potential of the Cys residues to participate in disulfide bonding (37, 38). Therefore, one hypothesis to explain the lower efficiency of E1 glycosylation at site 4 is that in the absence of E2, the Cys residue might be partially reactive to form disulfide bonds. More recently, it has been shown that the residue in position Y can also influence the efficiency of glycosylation in the sequon Asn-X-Ser-Y (39), and the presence of an Ile at position Y as seen at site 4, is not the most favorable residue for maximum efficiency of glycosylation. This might also explain why in vitro core glycosylation is partial at site 4 (22). Core glycosylation can also be influenced by the position of the sequon in a protein (6, 14, 40), and the location of site 1 of E1, five residues from the N terminus, is not favorable for glycosylation. However, when the efficiency of glycosylation of this site was studied in a cell-free system, it was fully glycosylated (22), suggesting that other factors probably involving folding (18, 19, 37) might play a role in modulating the efficiency of its glycosylation.

Our data indicate that glycosylation of E1 is improved when expressed as a polypeptide including full-length or truncated forms of E2, indicating that glycosylation of E1 is dependent on the presence of a polypeptide located downstream of E1 on HCV polyprotein. How can such a sequence, which is rapidly cleaved from the polyprotein by signal peptidase, improve the efficiency of glycosylation of E1? Recently, it has been shown that some mutations of the MHC class II-associated invariant chain (II) lead to a reduced interaction with the ribosome-associated membrane protein 4 and inefficient N-glycosylation, suggesting that glycosylation can be individually regulated by an interaction with ribosome-associated membrane protein 4 (41). However, this mechanism might be specific for II. In this model, ribosome-associated membrane protein 4 is proposed to mediate a translocation pause by interacting with a sequence of II immediately downstream of the two glycosylation sites. In the case of E1, the sequence responsible for improvement of glycosylation is located 188 and 79 residues downstream from

**TABLE 6. Analysis of intramolecular disulfide bond formation in E1 expressed as a full-length (E1E2) or a truncated (E1E2–661) polyprotein or expressed alone (E1).** HepG2 cells were coinfected with vTF7-3 and the appropriate vaccinia virus recombinant at a multiplicity of infection of 5 pfu/ml. At 4.5-h postinfection, cells were pulse-labeled for 5 min and chased for the indicated times. Cell lysates were immunoprecipitated with mab A4, and immunoprecipitates were analyzed under nonreducing condition by SDS-PAGE (10% acrylamide). Red, reduced; α, oxidized.
sites N1 and N4, respectively. In addition, there seems to be no specificity in the sequence involved in assisting glycosylation. A study using rabies virus glycoprotein as a model has shown that core glycosylation can be influenced by the presence or absence of regions more than 68 amino acids C-terminal to a specific glycosylation site (42). This is in agreement with recent data showing that, in the absence of a stop transfer sequence between the glycosylation acceptor sequon and the C terminus, the efficiency of glycosylation increases as the stop codon is moved further away from the sequon and plateaus at a distance of ~60 residues or more (43). These data suggest that glycosylation is inefficient when chain termination happens before the acceptor site reaches the oligosaccharyltransferase active site. Indeed, in a nascent polypeptide, the distance between the ribosomal peptidyl transferase site, and the oligosaccharyltransferase active site has been estimated to be ~65 residues (44). In the case of E1 expressed alone, the N4 site is located 79 residues from the stop codon, which had been introduced at the C terminus of E1. However, the stop transfer sequence of E1 was not deleted and the N1 site, which is also less efficiently recognized, is 188 residues from the C terminus of E1. An alternative explanation of E2-dependent glycosylation of E1 is that the folding of this protein might be different depending on whether it is expressed alone or coexpressed with E2, and the folding of E1 expressed alone might compete with its glycosylation. In vitro studies have shown that the acceptor peptide probably has to adopt a specific conformation, the Asn-X-Ser/Thr domain, which is more favorable for N-glycosylation. This conformational change might have some impact on the folding of the ectodomain of E1 and potentially on its glycosylation.

Because the glycosylation of a protein can influence its expression and function, it is important to understand why some proteins are only partially glycosylated. It has been shown that the type of amino acid present at the X position of the sequon can modulate the efficiency of core glycosylation in vitro (16), and in some instances, folding has been shown to compete with glycosylation (17–19). Control of glycosylation as described for E1 has never been reported before and it is likely because of the constraints imposed by the way HCV expresses its proteins. Whether it is unique to HCV envelope proteins or it applies to other glycoproteins synthesized as polyprotein precursors remains to be shown.

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