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Formation of the Flavivirus Envelope: Role of the Viral NS2B-NS3 Protease

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One of the late processing events in the flavivirus replication cycle involves cleavage of the intracellular form of the flavivirus capsid protein (C_{int}) to the mature virion form (C_{vir}) lacking the carboxy-terminal stretch of hydrophobic amino acids which serves as a signal peptide for the downstream prM protein. This cleavage event was hypothesized to be effected by a viral protease and to be associated with virion formation. We have proposed a model of flavivirus virion formation in which processing of the C-prM precursor at the upstream signalase site is upregulated by interaction of the NS2B part of the protease with the prM signal peptide or with an adjacent carboxy-terminal region of the capsid protein in the precursor, and processing of C_{int} by the NS2B-NS3 protease follows the signalase cleavage. Recently, an alternative hypothesis was proposed which suggests a reverse order of these two cleavage events, namely, that cleavage of the C-prM precursor by the NS2B-NS3 protease at the C_{int}→C_{vir} dibasic cleavage site is a prerequisite for the subsequent signalase cleavage of the prM signal peptide. To distinguish between these alternative models, we prepared a series of expression cassettes carrying mutations at the C_{int}→C_{vir} dibasic cleavage site and investigated the effects of these mutations on signalase processing of C-prM and on formation and secretion of prM-E heterodimers. For certain mutated C-prM precursors, namely, for those with Lys→Gly disruption of the dibasic site, efficient formation of prM was observed upon expression from larger cassettes encoding the viral protease, despite the absence of processing at the C_{int}→C_{vir} cleavage site. Surprisingly, formation and secretion of prM-E heterodimers accompanied by late cleavage of prM was also observed for these cassettes, with an efficiency comparable to that of the wild-type expression cassette. These observations contradict the model in which cleavage of the C-prM precursor at the C_{int}→C_{vir} dibasic site is a prerequisite for signalase cleavage.

Flaviviruses are small enveloped viruses with virions composed of three structural proteins designated C, M, and E. Multiple copies of the C protein form the flavivirus nucleocapsid, which contains an infectious single-stranded RNA molecule. Along with lipids of host cell origin, the M and E proteins form the flavivirus envelope. The latter bears major flavivirus antigenic determinants and is believed to be responsible for flavivirus attachment and entry (reviewed in reference 8). Flaviviruses are assembled at intracellular membranes, but the exact intracellular compartments where budding occurs are unknown. The envelopes of cell-associated virions are composed of prM-E heterodimers (where prM denotes precursor of M) which are reorganized after prM cleavage during virus release (44). Evidence has been reported that in the prM-E heterodimer, the prM protein prevents an irreversible conformational change of E during virus secretion through acidified sorting compartments (17, 20, 35). The flavivirus E protein undergoes low-pH-induced conformational rearrangement (18) which appears to be crucial for virus infectivity. Cleavage of the prM protein prior to virus release from cells results in virus-associated M and release of its glycosylated amino-terminal part in the media (29, 35, 46). The function of the M protein in the virions is unknown.

Translation of the flavivirus genome results in formation of a polyprotein precursor which is processed to individual proteins by host cell- and virus-specified proteases. In the precursor, the individual flavivirus proteins are arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, where NS designates nonstructural proteins (reviewed in reference 8). The prM-E-NS1 and NS4A-NS4B junctions are processed by host cell signalases (4, 31, 38, 40, 45), with the former appearing to occur cotranslationally and the latter occurring after internal cleavage of NS4A by a viral protease (22, 32). The catalytic domain of the viral protease was identified within the amino-terminal third of the flavivirus NS3 protein (3, 16). It functions as a NS2B-NS3 complex (7, 10, 14, 33, 43) and is responsible for processing of the NS2A-NS2B-NS3-NS4A and NS4B-NS5 junctions. Cleavages effected by the viral protease occur after the dibasic sequence KR or RR followed by small nonbranched amino acid such as Gly or Ser (36; reviewed in reference 8). Late in flavivirus infection, two other cleavages occur which involve cleavage of prM and further processing of the C protein (reviewed in reference 8). The late cleavage within prM depends on an acidic environment and is believed to be effected by a host cell protease (20, 35) prior to or during release of virions from cells. The second cleavage results in formation of the virion form of protein C (C_{vir}) lacking a hydrophobic sequence present at the carboxy terminus of the intracellular form (C_{int}) (31, 41) and is believed to be closely associated with virion formation (reviewed in reference 8).

Earlier, we suggested a model which describes late events occurring during formation of flavivirus virions (46). In this model, processing of the C-prM precursor by signalases with formation of C_{int} and prM occurs efficiently only in the presence of the viral NS2B-NS3 protease, with NS2B responsible for mediation of signalase cleavage of the precursor. This model retains the originally suggested (8) sequential order of the two cleavage events which are involved in processing of the C-prM precursor, i.e., that signalase cleavage of C-prM occurs

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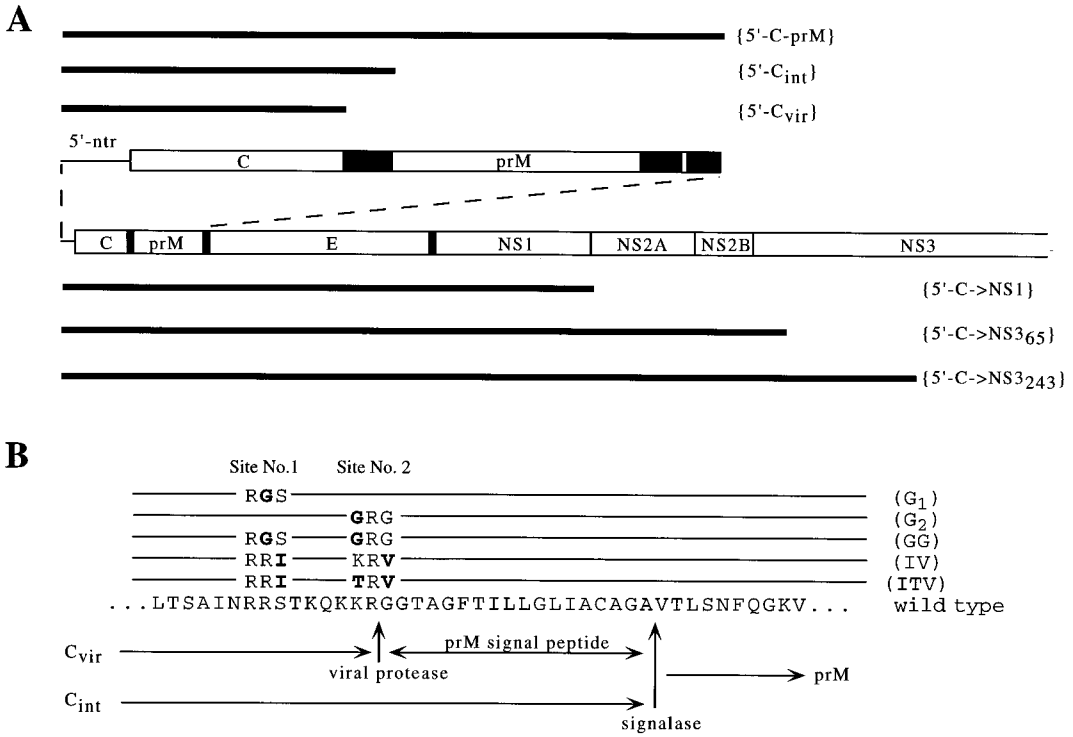

FIG. 1. Schematic representation of proteins encoded by expression cassettes used in this study. (A) The 5′-terminal half of the WN genome, with the amino-terminal half of the encoded polyprotein drawn as an open bar, is shown in the middle. Individual flavivirus proteins are marked, with the vertical lines denoting their boundaries. Hydrophobic regions serving as signal and stop-transfer sequences are represented by the thick vertical lines. Above, a second open bar represents the part of the WN genome encoding the 5′ nontranslated region (5′-ntr) and the C-prM precursor. The C_{int} membrane anchor/prM signal peptide, and the prM membrane anchor consisting of a prM stop-transfer sequence and the E signal peptide divided by a single Arg residue, are designated by filled boxes. Above, the thick horizontal lines show gene cassettes encoded by in vitro transcription plasmids, designated as in Materials and Methods, which were used for in vitro protease assays. In the lower part of the figure, another set of thick horizontal lines represents the expression cassettes used in vaccinia virus-T7 polymerase system. (B) Single-letter amino acid representation of the wild-type WN polyprotein sequence at the C-prM junction. The carboxy termini of C_{vir} and C_{int} and the amino terminus of prM are denoted by the horizontal arrows; the viral protease and signalase cleavage sites are marked by the vertical arrows below the sequence. Two dibasic sites, RRS and KRG, which were subjected to mutagenesis are marked as No. 1 and No. 2. The mutations which were introduced are shown above the sequence, with lines showing unchanged sequences. Abbreviations for the mutated cassettes used in the text are shown on the right.

prior to viral protease-mediated cleavage of C_{int}. Recently an alternative hypothesis, also suggesting a crucial role of the NS2B-NS3 protease for signalase cleavage of the C-prM precursor, was proposed (1, 24). However, the alternative model specifies the reverse order of the cleavage events, with cleavage of the C-prM precursor at the C_{int}→C_{vir} dibasic site by the viral protease as a prerequisite for efficient processing at the signalase cleavage site. In both of these reports, the model was based on observations that inactivation of the protease decreased formation of the prM protein to an undetectable level. However, inactivation of the protease would result in formation of NS2A-NS2B-NS3$_x$ precursors (1, 7, 10, 12, 14, 24, 33, 43), and suppression of C-prM signalase cleavage was observed in the presence of such precursors (46). Thus, observations based on inactivation of the protease have not provided conclusive evidence for a specific processing scheme of the C-prM precursor.

To distinguish between the two alternative models, we have introduced mutations which abolish cleavage at the C_{int}→C_{vir} cleavage site and investigated their effects on the processing and secretion of prM-E heterodimers. In this study, we demonstrate that cleavage at this site does not appear to be necessary either for efficient processing of the C-prM precursor and prM formation or for secretion of prM-E heterodimers.

MATERIALS AND METHODS

Cells, viruses, and antisera. A vaccinia virus recombinant, vW{5′-C→NS3$_{243}$}, carrying a cDNA genome fragment of West Nile virus (WN) has been described previously (46). Along with the WN structural proteins and proteins NS1 and NS2A, this recombinant expresses the active WN protease consisting of proteins NS2B and 243 N-terminal amino acids of protein NS3. HeLaT4 cells (25) were used throughout this study as described previously (46). An anti-WN hyperimmune ascites fluid was obtained from the American Type Culture Collection.

Construction of gene cassettes. Standard procedures for DNA manipulations and cloning were followed (26). For PCR amplification, Vent thermophilic DNA polymerase (New England Biolabs, Beverly, Mass.) was chosen because of higher fidelity (28) and was used according to the manufacturer's recommendations. All recombinant plasmids were characterized by restriction analysis and sequencing. If not mentioned otherwise, enzymes were purchased from New England Biolabs, except for *Asp* 718, which was obtained from Boehringer Mannheim (Indianapolis, Ind.). Gene cassettes used in this study are depicted in Fig. 1.

Construction of the W{5′-C-prM}, W{5′-C→NS1}, W{5′-C→NS3$_{65}$}, and W{5′-C→NS3$_{243}$} cassettes has been described earlier (46). The {C-prM} cassette is controlled by the SP6 promoter; the next two are assembled under control of the T7 promoter. The last cassette is cloned in pVT7Dneo (47) and is controlled by a tandem of the P7.5 and T7 promoters. Construction of W{5′-C_{vir}} and W{5′-C_{int}} cassettes, which code for the authentic virion and intracellular forms of the WN capsid protein, has also been described previously (48); in both, transcription is controlled by the T7 promoter.

Variants of the {C-prM} cassette with point mutations in the C_{int}→C_{vir} cleavage site and adjacent region (see Fig. 1 for details) were constructed by PCR-mediated point mutagenesis. In general, mutated cassettes were synthesized by two-step PCR amplification (21) of WN cDNA segments (46). The 5′-C-coding half was amplified with primers corresponding to the SP6 promoter (as direct) and to the nucleotide (nt) 385–411 region (as reverse). The reverse primer

introduced C→A and T→G point mutations at positions 392 and 407, causing replacement of an AGC triplet (Ser) for the ATC triplet (Ile) and an AAA triplet (Lys) for the ACA triplet (Thr). The prM half was separately amplified by using primers corresponding to nt 394–422 (as direct) and nt 952–971 (as reverse) regions. The direct primer in the last case introduced two point mutations at positions 407 (A→C) and 413 (G→T), causing replacement of an AAA triplet (Lys) for the ACA triplet (Thr) and a GGA triplet (Gly) for the GTA triplet (Val). After a second round of PCR with the amplified halves by using the first direct and second reverse primers and digestion with *Eco*RI (12 nt upstream from the beginning of the WN 5′ region) and *Mlu*I (position 920; at the end of the prM gene), the resulting fragment was exchanged with the *Eco*RI-*Mlu*I fragment of the wild-type {5′-C-prM} cassette. Individual IV and ITV variants of the wild-type cassette (Fig. 2) were identified by sequencing. Construction of the {5′-C(G_1)-prM}, {5′-C(G_2)-prM}, and {5′-C(GG)-prM} variants of the wild-type cassette has been described previously (48).

The *Eco*RI-*Mlu*I fragments from the individual characterized mutant variants of the {5′-C-prM} cassette were subsequently exchanged with the same fragments in the {5′-C→NS1}, {5′-C→$NS3_{65}$}, and {5′-C→$NS3_{243}$} cassettes and designated the IV, ITV, G_1, G_2, and GG variants (Fig. 1); structures of the resulting plasmids were verified by sequencing.

Expression of the wild-type and mutated cassettes in vivo and in vitro. In general, expression using the vaccinia virus-T7 polymerase system (15), cell lysis, and immunoprecipitation were done as described earlier (46) except that DNA transfection was performed by the procedure described by Rose et al. (37) either for 5 h or overnight, followed by labeling and chases as described previously (46). For deglycosylation studies with endoglycosidase (endo) H and peptide *N*-glycosidase (PNGase) F (both from New England Biolabs), recommendations of the manufacturer were followed. RNA synthesis, cell-free translation in a rabbit reticulocyte lysate (Boehringer Mannheim), and viral protease assays were performed as previously reported (48). Routinely, total radioactivity of all immunoprecipitated samples was determined by scintillation counting; roughly equal amounts of radioactivity were used for polyacrylamide gel electrophoresis (PAGE) analysis as described elsewhere (46, 48). Where specified, dried gels were scanned with a model 445SI PhosphorImager (Molecular Dynamics Inc., Sunnyvale, Calif.) and quantitated by using the IPLab Gel package (Signal Analytics Corp., Vienna, Va.).

RESULTS

Description of in vivo and in vitro expression cassettes. Earlier, two major observations were made concerning expression of the WN{5′-C→$NS3_{243}$} cassette (46), which encodes components of the viral protease. First, in contrast to shorter cassettes which did not encode the viral protease components, efficient processing of the C-prM precursor by signalases with formation of prM was observed. Second, expression resulted in formation and secretion of prM-E heterodimers and late cleavage of prM, thus apparently reproducing late processing events in the flavivirus replication cycle. While formation of prM upon expression of a W{5′-C→NS1} cassette was less efficient but still readily detectable, almost no prM was observed with a W{5′-C→$NS3_{65}$} cassette, which yielded a noncleaved NS2A-NS2B-$NS3_{65}$ precursor. No secretion of prM and E was detected in either case. Thus, both efficient prM formation and prM-E secretion appeared to be dependent on the presence of the active protease. To further investigate the role of the viral protease, five {5′-C→$NS3_{243}$} cassettes with different mutations at the carboxy terminus of C_{vir} have been constructed (Fig. 1) and cloned under the control of P7.5-T7 tandem promoters (47), thus enabling transient expression from plasmids in the vaccinia virus-T7 polymerase system (15).

From sequence data, it was suggested (6) that C_{int}→C_{vir} processing for WN occurs at the KR/G site, and recently this has been demonstrated experimentally in vitro (48). However, several amino acids upstream from the KR/G cleavage site, a similar sequence, RRS, occurs in the WN capsid protein (6) which theoretically satisfies the dibasic consensus requirements (reviewed in reference 8). Although the KR/G configuration is found at most WN cleavage sites mediated by the viral protease (43), a KR/S cleavage site is present between the WN NS3 and NS4A proteins (5). In addition, it was shown that R→K mutations in the dibasic consensus are tolerated to some extent by the yellow fever virus (YF) protease (30), thus suggesting the possibility that, at least in vitro, the RRS site could be used by the WN protease. To examine the possible utilization of both cleavage sites, we introduced mutations at either or both sites (Fig. 1). First we altered the dibasic consensus by introduction of branched amino acids Ser→Ile and Gly→Val in the two sites (see Fig. 1 for details). Another variant of this cassette was prepared by introduction of a third Lys→Thr mutation in the KR/G site. We also considered the possibility that introduction of branched amino acids may change significantly the conformation and/or flexibility of the polypeptide chain in the adjacent region. To eliminate that possibility, another set of mutated cassettes was constructed. In these cassettes, the dibasic consensus was disrupted by introduction of an Arg→Gly mutation in the first site and/or a Lys→Gly mutation in the second site. To evaluate the effects of these mutations on formation of prM in the absence of the viral protease, the W{5′-C→NS1} and W{5′-C→$NS3_{65}$} cassettes bearing these mutations were also prepared.

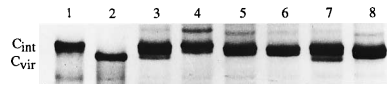

FIG. 2. Cleavage assay of the wild-type C-prM precursor (lane 3) and its IV (lane 4), ITV (lane 5), G_2 (lane 6), G_1 (lane 7), and GG (lane 8) variants. Molecular markers of the C_{int} (lane 1), and C_{vir} (lane 2) proteins were prepared by translation of C_{int}- and C_{vir}-encoding synthetic RNAs in a rabbit reticulocyte lysate as described previously (48).

To investigate directly the effects of these mutations on C_{int}→C_{vir} processing by the viral NS2B-NS3 protease, we constructed W{5′-C-prM} in vitro expression cassettes bearing all the mutations specified above. A Triton X-100 extract was prepared from cells infected with a vaccinia virus recombinant carrying the W{5′-C→$NS3_{243}$} cassette (46, 48).

Processing of mutated C-prM precursors by the viral protease. Using an in vitro assay developed previously (48), we investigated processing of the C-prM precursor bearing the mutations specified above in the C_{int}→C_{vir} processing site by the NS2B-NS_{243} protease. We previously observed formation of C_{vir} upon cotranslation of wild-type C-prM and NS2A→$NS3_{243}$ encoding synthetic RNAs in a rabbit reticulocyte lysate in the presence of microsomal membranes (48). Similarly, C_{vir}, with slightly faster mobility due to reduction in the net positive charge resulting from Lys→Gly replacement, was detected for the G_1 variant of the C-prM precursor (48). In contrast, no C_{vir} was detected for the G_2 and GG mutants of this precursor (48), indicating that disruption of the dibasic context in the KR/G site is not tolerated by the viral protease.

To further demonstrate the specificity of cleavage, we exposed the wild-type and mutated C-prM precursors synthesized in vitro to a Triton X-100 extract from cells infected with a vaccinia virus recombinant carrying the wild-type {5′-C→$NS3_{243}$} cassette as described earlier (48). A similar approach was used to demonstrate processing of a peptide spanning the YF C_{int}→C_{vir} processing site by the YF NS2B-NS3 protease (1). As expected, C_{int}→C_{vir} processing with C_{vir} formation was observed only for the wild-type precursor and for the G_1 variant (Fig. 2, lanes 3 and 7, respectively), not for the IV, ITV, G_2, and GG variants (Fig. 2, lanes 4 to 6 and 8, respectively) confirming the results of in vitro cotranslation assays described above. We were unable to demonstrate C_{int}→C_{vir} processing by in vitro translation of synthetic RNA spanning the whole 5,330-nt {5′-C→$NS3_{243}$} cassette because of the complexity of translation products.

Effect of mutations at the C_{int}→C_{vir} cleavage site on signalase processing of the C-prM precursor. In agreement with

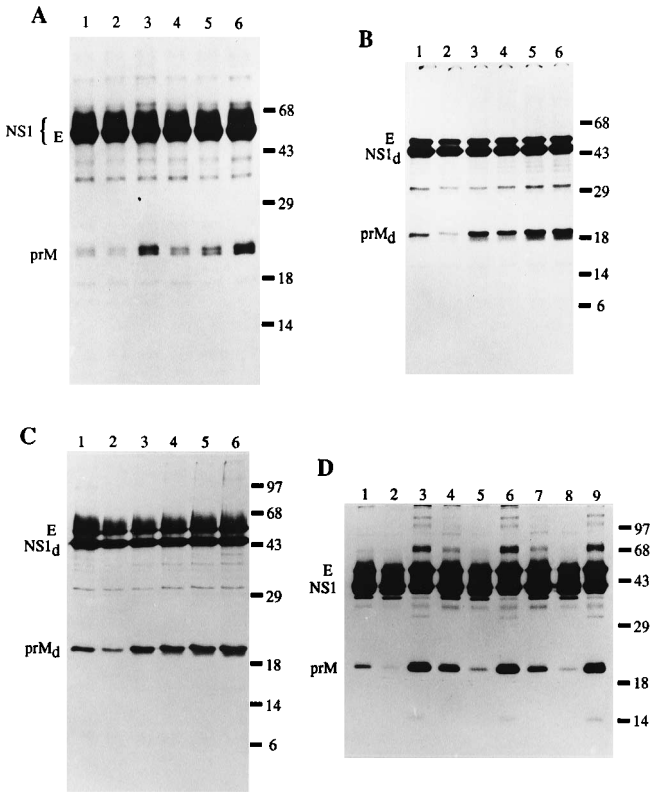

FIG. 3. PAGE analysis of proteins immunoprecipitated from cell lysates by anti-WN hyperimmune serum and protein A-agarose. (A) Transient expression of the wild-type {5′-C→NS1} cassette (lane 1) and its IV (lane 2), ITV (lane 3), G_1 (lane 4), G_2 (lane 5), and GG (lane 6) variants. Individual protein designations are marked on the left. (B) Aliquots of the samples shown in panel A were deglycosylated with PNGase F before PAGE. prM_d and $NS1_d$, deglycosylated prM and NS1. (C) Aliquots of the samples shown in panel A were treated with endo H before PAGE. (D) Comparison of transient expression of the wild-type and mutated {5′-C→NS1} (lanes 1, 4, and 7), {5′-C→$NS3_{65}$} (lanes 2, 5, and 8), and {5′-C→$NS3_{243}$} (lanes 3, 5, and 9) cassettes. Lanes 1 to 3, wild-type cassette; lanes 4 to 6, G_1 mutant; lanes 7 to 9, G_2 mutant. Sizes are indicated in kilodaltons on the right.

previous observations (46), three major proteins were detected by immunoprecipitation of cell lysates upon expression of the wild-type and mutated {5′-C→NS1} cassettes, using the vaccinia virus-T7 polymerase system. The envelope protein, which is not glycosylated in case of WN (5, 31, 44, 46), and glycoprotein NS1 comigrated as a band of approximately 50 kDa (Fig. 3A) which could be resolved either after deglycosylation (Fig. 3B and C) or under nonreducing conditions (Fig. 3D); in the last case, protein NS1 is likely assuming a more compact conformation which exhibits faster mobility. Faint bands in the range of 35 to 40 kDa seen in Fig. 3A may represent a glycosylated C-prM precursor which could be detected with a low efficiency by anti-WN hyperimmune serum (46). This conclusion is apparently supported by the appearance of products with lower molecular masses (of approximately 30 kDa) upon deglycosylation, as demonstrated in Fig. 3B and C, but we did not study these products in detail. The prM protein formed a doublet of approximately 23 to 25 kDa (Fig. 3A) which presumably resulted from oligosaccharide core modification, since deglycosylation yielded a single band of reduced molecular mass (Fig. 3B and C), and only a single band, apparently corresponding to the lower one, was observed after a prolonged chase (Fig. 3D). In contrast, after 5 min of chase, prM was detected as a single band corresponding to the upper band of the doublet (see Fig. 5B, lane 1).

Earlier we have observed inefficient signalase cleavage of the C-prM precursor expressed from the {5′-C→NS1} cassette, resulting in a low but readily detectable level of prM in cell lysates. However, while a low level of prM formation was again observed for the wild-type cassette (Fig. 3A to D, lanes 1) and variant IV (Fig. 3A to C, lanes 2), some increase in the amount of prM was detected for the ITV (Fig. 3A to C, lanes 3), G_1 and G_2 (Fig. 3A to C, lanes 4 and 5, and Fig. 3D, lanes 4 and 7, respectively), and GG (Fig. 3A to C, lanes 6) variants. Mutations in the last four variants resulted in replacement of the positively charged amino acids for noncharged ones, thus suggesting a role of a net positive charge near the end of the C protein in downregulation of cleavage at the signalase site. However, the different effect of the Lys→Thr (in the ITV variant), Arg→Gly (in the G_1 variant), and Lys→Gly (in the G_2 variant) mutations, all of which reduce the positive charge by one, on the level of prM formation suggests that the mutants may have different polypeptide chain conformations or flexibility in this region.

An increase in the amount of the prM protein resulting from signalase cleavage of the C-prM precursor was observed upon expression of the {5′-C→$NS3_{243}$} cassette, which includes the components of the viral protease (compare lanes 1 and 3 in Fig. 3D). This finding is in agreement with our previous results (46) as well as with results reported for Murray Valley encephalitis virus (24) and YF (1), all of which suggest a crucial role of the NS2B-NS3 protease complex in mediating signalase cleavage of the C-prM precursor.

Two alternative models describing NS2B-NS3-mediated upregulation of C-prM processing by signalases have been proposed (Fig. 4). Model A (46) suggests a positive regulatory function of NS2B in the protease complex which interacts either with the prM signal peptide or with the adjacent carboxy terminus of the C protein, thus rendering the signalase cleavage site accessible for cleavage. Thus, C-prM processing results in formation of C_{int} and prM. The alternative hypothesis (1, 24) suggests that the protease first processes the precursor at its C_{int}→C_{vir} dibasic site, resulting in formation of C_{vir} and a (sig)prM precursor (Fig. 4, model B) which is further efficiently processed by signalases to form prM. Both models suggest a negative effect of the capsid sequence on signalase processing of the C-prM precursor which, however, may be detected at some background level in the absence of the protease. From model B, one may expect that mutations abolishing cleavage at the C_{int}→C_{vir} site would either decrease formation of prM or, at least, leave it at the background level observed in the absence of the protease, such as for the {C→NS1} cassette. In contrast, model A suggests that efficient formation of prM may be observed for the mutated cassettes, since signalase cleavage is not directly dependent on processing at the upstream dibasic site in the C-prM precursor.

In addition to the prM, E, and NS1 proteins detected for the {5′-C→NS1} cassettes, two other proteins were detected in cell lysates upon expression of the protease-containing cassettes (Fig. 3D, lanes 3, 6, and 9). The observed bands were found to represent the NS2B and $NS3_{243}$ components of the viral protease (43, 46) which resulted from processing of the NS2A-NS2B-$NS3_{243}$ precursor by the viral protease (3, 7, 10, 14, 16, 33, 43), thus indicating its presence in an active form in cell lysates. In addition to the wild type {5′-C→$NS3_{243}$} cassette, the positive effect of the protease complex on prM formation was also observed for the G_1 and G_2 variants (compare lanes 4 and 7 with lanes 6 and 9 in Fig. 3D). In these experiments, secretion of the NS1 protein was not affected, and we

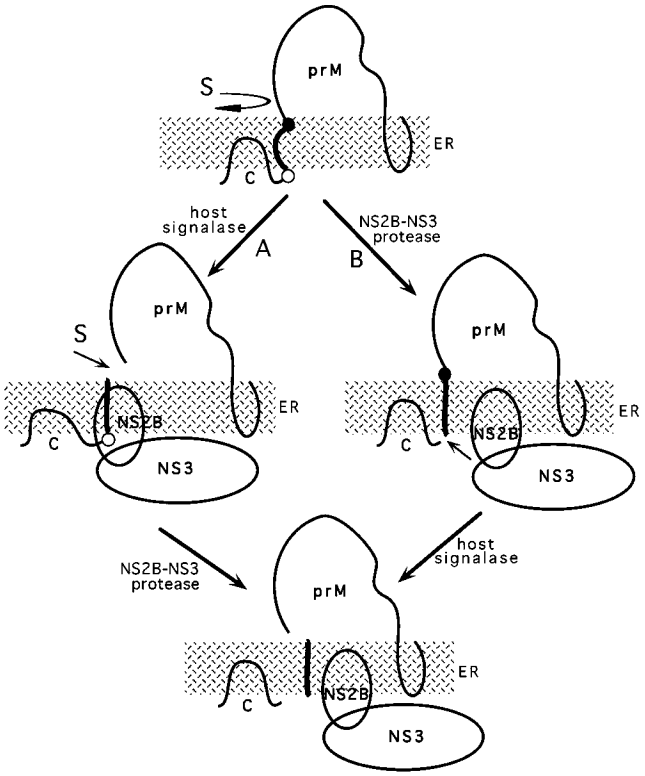

FIG. 4. Schematic representation of two alternative models which have been proposed for processing of the flavivirus C-prM precursor. The ER membrane is depicted by a hatched zone. In the upper part of the figure, the C-prM precursor, produced after cotranslational signalase cleavage at the prM-E junction, is depicted as a continuous molecule with the prM signal peptide shown as the thicker line. The C-prM signalase cleavage site and the C_{int}→C_{vir} dibasic cleavage site are shown as filled and open circles, respectively. In model A (arrow A), the membrane-associated NS2B-NS3 protease complex interacts by its NS2B part either with the prM signal peptide or with the carboxy-terminal region of C, thus rendering the signalase site accessible for cleavage. The cleavage results in formation of prM and C_{int}; the latter is further processed by the NS2B-NS3 protease. In model B (arrow B), the membrane-associated NS2B-NS3 protease complex (two intersecting ovals) first processes the precursor at its C_{int}→C_{vir} dibasic site, yielding C_{vir} and (sig)prM; this releases the signalase site for cleavage. S, host cell signalase.

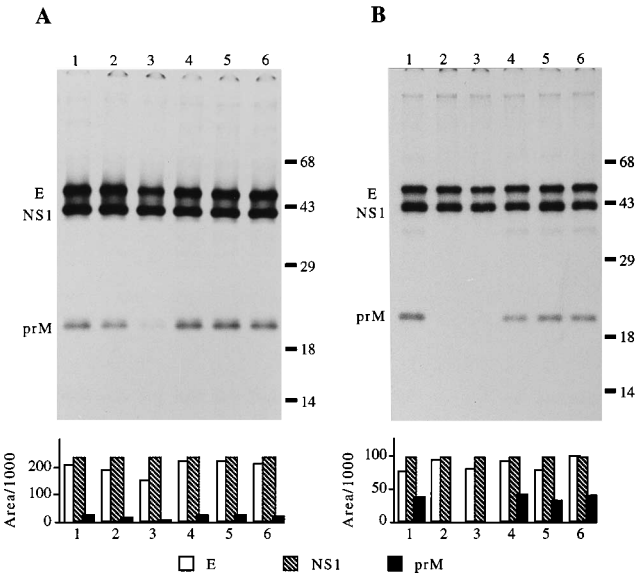

FIG. 5. PAGE analysis of proteins immunoprecipitated from cell lysates upon transient expression of the wild-type {5′-C→$NS3_{243}$} cassette (lanes 1) and the ITV (lanes 2), IV (lanes 3), G_1 (lanes 4), G_2 (lanes 5), and GG (lanes 6) variants. For quantitation, gels were scanned on a PhosphorImager; results were normalized by using amounts of NS1 in the lanes as internal standards. In the graphs below, each lane is represented by a group of three vertical bars depicting the three major products; bar designations are indicated below the graphs. (A) Expression pattern observed after a 1-h pulse and 5-h chase; (B) expression pattern observed after a 5-min pulse and 5-min chase. Individual proteins are designated on the left. Sizes are indicated in kilodaltons on the right.

used it as an internal reference standard for amounts of samples loaded. Model B (Fig. 4) is apparently contradicted by an increase in prM formation for the G_2 cassette, in which the KR/G site was disrupted by Lys→Gly conversion, since this mutation abolishes C_{int}→C_{vir} processing effected by the viral protease (see above and also reference 48). The presence of a NS2A-NS2B-$NS3_{65}$ precursor had a negative effect on prM formation both for wild-type and mutated {5′-C→$NS3_{65}$} cassettes compared with {5′-C→NS1} cassettes (Fig. 3D; compare lanes 2, 5, and 8 with lanes 1, 4, and 7). The precursor produced by the former cassettes remains uncleaved because the active center of the protease (3, 7, 10, 12, 14, 16, 33, 43, 46) is not encoded by these cassettes. Similar observations were reported for Murray Valley encephalitis virus (24) and YF (1) constructs, in which cleavage of NS2A-NS2B-$NS3_x$ precursors abolished as a result of mutations in the catalytic triad decreased prM formation to an undetectable level. Earlier, this observation led us to suggest that the NS2B part of the protease complex is involved in interaction with a region adjacent to the C-prM signalase processing site (46), and this interaction facilitates signalase cleavage of the C-prM precursor when it is expressed as part of the protease-encoding {5′-C→$NS3_{243}$} cassette.

Using expression conditions with long chase periods to detect secreted proteins, no distinctive changes in amounts of intracellular prM were observed in case of the IV, ITV, and GG variants of the {5′-C→$NS3_{243}$} cassette compared with these variants of the {5′-C→NS1} cassette. For variant IV, the amount of prM detected by immunoprecipitation remained at the same low level (compare lane 2 in Fig. 3A and lane 3 in Fig. 5A), suggesting that the protease complex is apparently unable to facilitate signalase processing of the C-prM precursor. For the ITV and GG variants, an increased level of prM was observed for the {5′-C→NS1} cassette (i.e., in the absence of the protease complex), thus precluding comparison (compare lanes 3 and 6 in Fig. 3A and lanes 2 and 6 in Fig. 5A). However, while prM for the wild-type version and G_1 and G_2 variants of the {5′-C→$NS3_{243}$} cassette may be readily detected after a 5-min pulse with a 5-min chase (Fig. 5B, lanes 1, 4, 5, and 6, respectively), the amounts of prM for both the ITV and IV variants (Fig. 5B, lanes 2 and 3) were below the detectable level. The data in Fig. 5 were quantitated and normalized by using the levels of NS1 as internal standards (the graphs are shown below the gels), and similar results were obtained. The increased amounts of prM for the ITV cassette observed upon longer chase may reflect an altered conformation of the signal peptide in the endoplasmic reticulum (ER) membrane resulting from the Lys→Thr replacement and thus increased exposure of the cleavage site to the enzyme. The apparent failure of the protease complex to facilitate signalase processing of the C-prM precursor for the IV and ITV variants of the {5′-C→$NS3_{243}$} cassette may be attributed to a significant change (resulting from introduction of two branched amino acids) in the conformation of the prM signal peptide and/or of the adjacent carboxy-terminal region of the capsid protein, thus preventing interaction of NS2B with this region. The observed

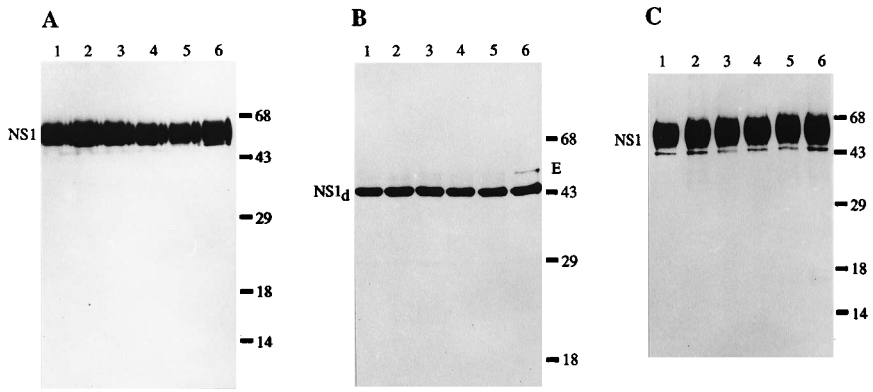

FIG. 6. PAGE analysis of secreted proteins expressed from the wild-type {5′-C→NS1} cassette (lanes 1) and the IV (lanes 2), ITV (lanes 3), G_1 (lanes 4), G_2 (lanes 5), and GG (lanes 6) variants. (A) Samples immunoprecipitated from media; (B) immunoprecipitates after deglycosylation with PNGase F; (C) samples were treated with endo H prior to PAGE. Sizes re indicated in kilodaltons on the right.

differences clearly support model A which suggests that facilitation of C-prM signalase cleavage and C_{int}→C_{vir} processing are two separate events mediated by the NS2B-NS3 protease complex.

Effects of mutations at the C_{int}→C_{vir} cleavage site on secretion of the prM and E proteins. In agreement with previous reports (12, 24, 27, 46), expression of the wild-type {5′-C→NS1} cassette resulted in secretion of heterogeneously glycosylated NS1 (Fig. 6A, lane 1), but no prM or E could be detected in the media, as this could clearly have been seen after deglycosylation with PNGase F (Fig. 6B, lane 1; compare with Fig. 3B, lane 1, for intracellular levels). In contrast to the intracellular form, the secreted form of NS1 acquired resistance to endo H (compare lanes 1 in Fig. 3C and 6C) apparently resulting from oligosaccharide core modification during NS1 transport. Similarly, secretion of NS1 at the same level was observed for the IV, ITV, G_1, G_2, and GG variants (lanes 2 to 6 in Fig. 6A to C).

Previously (46), formation of prM-E heterodimers and their secretion in the form of empty viral envelopes were reported to be dependent on the presence of the viral NS2B-NS3 protease. Subsequently, similar observations were made for Murray Valley encephalitis virus (24) and YF (1). Secretion of the prM-E heterodimers was observed to be accompanied by late prM cleavage (reviewed in reference 8), thus apparently reproducing the late events in flavivirus virion formation and release. In concordance with previous reports (24, 29, 35, 46), expression of the wild-type {5′-C→$NS3_{243}$} cassette resulted in the appearance of protein E and the cleaved glycosylated amino terminal part of prM [often referred to as pr(M)] in the media (Fig. 7A, lane 1). Both secreted NS1 and pr(M) acquired resistance to endo H (Fig. 7C, lane 1) but not to PNGase F (Fig. 7B, lane 1) treatment, indicating that their oligosaccharide cores were modified along the secretion pathway. Earlier (46) we have observed incomplete cleavage of the prM protein during transport. In this study we found that the level of prM cleavage during secretion depends on the time of infection. Almost complete cleavage was observed when transfection, labeling, and chase did not exceed 10 h in total. Cleavage of prM ceases later postinfection, and at approximately 20 h postinfection no cleavage could be detected. Similar phenomena were also observed for cleavage of the human immunodeficiency virus glycoprotein gp160 and the fowl plague virus hemagglutinin upon expression of these proteins from recombinant vaccinia viruses (19) and may be attributed to an inhibitory effect of vaccinia virus infection on activity of the participating host protease.

On the basis of the models proposed earlier (1, 24, 46), we expected that mutations abolishing C_{int}→C_{vir} processing would prevent formation and secretion of the prM-E-containing viral

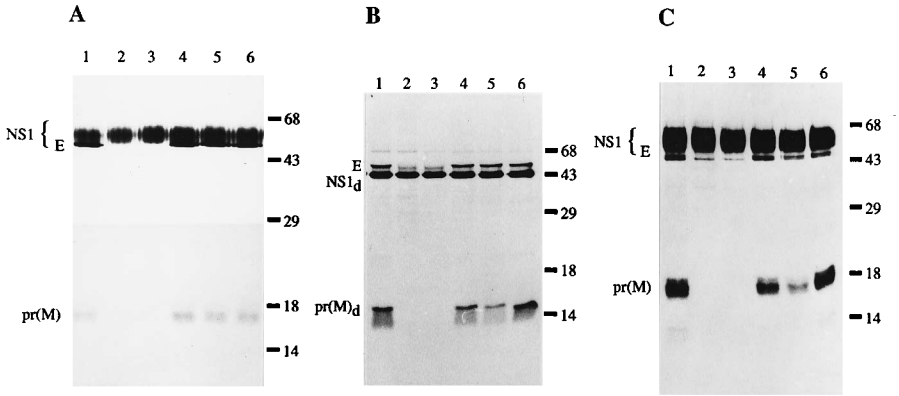

FIG. 7. PAGE analysis of secreted proteins expressed from the wild-type {5′-C→$NS3_{243}$} cassette (lanes 1) and the IV (lanes 2), ITV (lanes 3), G_1 (lanes 4), G_2 (lanes 5), and GG (lanes 6) variants. pr(M), cleaved glycosylated amino-terminal part of the prM protein (see text); $pr(M)_d$ and $NS1_d$, deglycosylated pr(M) and NS1. (A) Samples immunoprecipitated from the media. The upper half of the autoradiograph was reproduced with lower exposure to demonstrate the presence of the E protein. (B) Immunoprecipitates after deglycosylation with PNGase F. (C) Samples treated with endo H prior to PAGE. Sizes are indicated in kilodaltons on the right.

envelopes. Indeed, both the IV and ITV variants of the {5′-C→$NS3_{243}$} cassette failed to produce prM and E in a secreted form (Fig. 7A to C, lanes 2 and 3, respectively) despite the presence of the active viral protease in infected cells (see above); in contrast, secretion of NS1 was not found to be changed. As expected, the Arg→Gly mutation in the RRS site had also no effect on prM-E secretion, because this mutation did not involve the actual C_{int}→C_{vir} processing site KR/G located downstream (Fig. 7A to C, lanes 4). However, surprisingly, disruption of the KR/G site by the Lys→Gly mutation in the G_2 variant also did not prevent secretion of the prM-E heterodimers (Fig. 7A to C, lanes 7). The possibility that in the last mutant alternative C_{int}→C_{vir} processing at the upstream RRS site may account for prM-E secretion was ruled out by using the double GG mutant, which exhibited the same behavior (Fig. 7A to C, lanes 6). It is interesting that upon longer exposures, trace amounts of E and pr(M) were detected for the GG variant of the {5′-C→NS1} cassette (Fig. 6A to C, lanes 6), which does not encode either of the protease components.

These data cannot be explained by alternative model B (1, 24) in which NS2B-NS3-mediated C_{int}→C_{vir} processing occurs prior to formation of prM. However, model A (46) suggesting association of the viral protease with some region adjacent to the C-prM processing site seems to be more compatible with the observed results. The failure of both IV and ITV variants, which possess branched amino acids in the vicinity of the C_{int}→C_{vir} processing site and the prM signal peptide, to produce secreted prM and E may be also explained by an inappropriate (C-prM)-(NS2B-$NS3_{243}$) association, as was suggested from the data discussed in the previous section.

DISCUSSION

Previously, we have reported (46) that both efficient processing of the C-prM precursor by host cell signalases yielding the prM protein and secretion of prM-E heterodimers in the form of empty viral envelopes (27) are mediated by the active viral protease comprised of NS2B and the $NS3_{243}$ protease domain. A second important observation was that the uncleaved NS2A-NS2B-$NS3_{65}$ precursor which resulted from expression of the {5′-C→$NS3_{65}$} cassette significantly decreased the amount of prM compared with the amount of prM detected upon expression of the {5′-C→NS1} cassette. On the basis of these data and other evidence, we have proposed a model (46) in which the NS2B part of the viral protease complex was suggested to facilitate signalase processing of the C-prM precursor by interaction either with the prM signal peptide or with some region adjacent to the C carboxy terminus (Fig. 4, model A). Thus, model A implies that mediation of C-prM signalase cleavage and processing of the intracellular form C_{int} of the viral capsid protein are two distinct and apparently independent functions of the viral NS2B-NS3 protease complex. Recently, the alternative model was proposed (1, 24) which suggests that cleavage of the C-prM precursor at the C_{int}→C_{vir} dibasic site by the viral protease occurs prior to signalase cleavage, thus upregulating its efficiency (Fig. 4, model B). In both of these reports, the conclusion relied upon the observation that inactivation of the protease by point mutagenesis of the protease active center decreased formation of the prM protein to an undetectable level. However, these results do not contradict model A, since inactivation of the protease results in formation of NS2A-NS2B-$NS3_x$ precursors (1, 7, 10, 12, 14, 24, 33, 43) which could affect signalase cleavage of C-prM in a similar way as we observed for the NS2A-NS2B-$NS3_{65}$ precursor. Thus, observations based on inactivation of the protease cannot provide conclusive evidence for a particular model. For this reason, in order to distinguish between these two alternate hypotheses, this study used another approach in which we abolished processing at the C_{int}→C_{vir} site by altering its dibasic consensus site (reviewed in reference 8). Model A predicts that abolished processing at this site will have little or no effect on the efficiency of signalase cleavage of C-prM in the presence of the active protease, since the positive function of NS2B may be expected to be unaffected. In model B, however, a significant decrease in the level of prM would be expected, since the efficient processing at the signalase site should depend on prior cleavage at the dibasic site.

We found that point mutations introduced into the C_{int} processing site KR/G and into the adjacent sequence RRS affected the amount of detectable prM produced both in the presence and in the absence of the active viral protease. Results obtained upon expression of the wild-type {5′-C→NS1} cassette and its mutated versions indicated that a net positive charge at the end of C_{int} plays a role in downregulation of cleavage at the upstream signalase site. Signalase processing may be affected both by amino-terminal sequences of a signal peptide and by its length (reviewed in reference 11). The carboxy terminus of C is located on the cytoplasmic side of the ER membrane (reviewed in reference 8) and, probably, its net positive charge restricts perpendicular movement of the prM signal peptide in the ER membrane, thus preventing the cleavage site from exposure to signalases. Reduction in the net positive charge resulting from replacements of Arg or Lys by noncharged residues increased the amount of detectable prM in cell lysates (the ITV, G_1, G_2, and GG variants), thus supporting this suggestion. A similar conclusion was reported by Lobigs (24). Previously, we have observed (46) that the level of intracellular prM detected upon expression of a {prM→NS1} cassette was not detectably affected by the presence or absence of a KR sequence at the beginning of the prM signal peptide. From this evidence, we conclude that in the C-prM precursor, the positive charge at the amino terminus of the prM signal peptide (or at the carboxy terminus of C_{int}) may not be accounted for solely by downregulation of signalase cleavage. The different effects of the ITV, G_1, and G_2 mutations, all of which reduce the positive charge by one, on the level of prM formation may indicate that the polypeptide chain conformation (or flexibility) in this region also plays a certain role.

The presence of the active protease resulted in an increased level of prM detected in cell lysates not only for the wild-type {5′-C→$NS3_{243}$} cassette but also for its mutated G_1, G_2, and GG versions. Since processing at the C_{int}→C_{vir} site by the viral protease was not detected for the C-prM precursors with the last two mutations, these data appear to contradict alternative model B, which suggests that signalase processing of the C-prM precursor depends on prior cleavage at the downstream dibasic site. The evidence appears to be more compatible with model A, in which the NS2B part of the viral protease complex is implicated for the positive upregulation of the signalase cleavage. Within this model, the apparent inability of the protease complex to promote efficient signalase processing of the C-prM precursor for the IV and ITV variants could be explained as result of steric restrictions imposed by the presence of the branched amino acids.

We have also suggested (46) that processing of C_{int} by the viral protease triggers formation of the viral envelope. Upon expression of the {5′-C→$NS3_{243}$} cassette, this process results in secretion of membrane-associated prM-E heterodimers. However, secretion of prM and E proteins, which was accompanied by late prM cleavage, was detected for the G_2 and GG variants at the same level as for the wild-type cassette, despite abolished processing at the dibasic site. Thus, these data ap-

parently contradict this part of the model. The evidence presented above suggests that association of C-prM with NS2B-$NS3_{243}$ itself can promote not only efficient C-prM processing at the signalase site but also secretion of the prM-E heterodimers. Such a scenario implies the existence of some mechanism regulating association of C-prM with the NS2B-NS3 complex to prevent formation and secretion of the empty envelopes early in infection (42), when genomic RNA for encapsidation is not yet available. However, it explains formation of the slowly sedimenting noninfectious hemagglutinin released later in flavivirus infection (39, 42), which was found to consist of empty viral envelopes (27).

Recently, it has been found that cleavage at the NS3/NS4A site requires the presence of mature NS3 to occur efficiently (23); on the other hand, cleavage at this site was found to depend on conformation of the entire NS1→NS5 precursor (49). In addition, alternative cleavages mediated by the NS2B-NS3 protease were reported within NS3 (2, 34), NS2A (30), and NS4A (22), with the latter a prerequisite for signalase cleavage at the NS4A/NS4B site (22). These processing events may be a part of the mechanism regulating formation of the NS2B-NS3 complex or its ability to interact with the C-prM precursor in connection with RNA replication. The immediate effect of the NS2B-$NS3_{243}$ protease complex on signalase processing of the C-prM precursor observed for the {5′-C→$NS3_{243}$} cassettes may be explained by the absence of downstream nonstructural proteins. Indeed, we observed less efficient secretion of prM, E, and NS1 proteins for a WN {5′-C→$NS5_{598}$} cassette (47) compared with the {5′-C→$NS3_{243}$} cassette, although we did not investigated this phenomenon further.

The present data have not identified regions involved in interaction of the C-prM precursor and NS2B-NS3 protease complex. Given the negative effect of the uncleaved NS2A-NS2B-$NS3_{65}$ precursor on processing of C-prM at the signalase site, we suggested that NS2B and the prM signal peptide are involved in this interaction. In this regard, an interesting observation was reported by Chambers et al. (9). While mutations in the hydrophobic regions of NS2B preceding a highly conserved region crucial for its interaction with NS3 (13) were found to have little or no effect on formation and activity of the viral protease in expression experiments, no virus was recovered after transfection of full-length transcripts bearing these mutations. It would be of interest to investigate the effects of such mutations on the positive regulatory function of the protease complex toward signalase processing of the C-prM precursor and secretion of the prM-E heterodimers. On the other hand, recruitment of the C_{int}→C_{vir} dibasic site into the substrate pocket of the NS2B-NS3 protease may itself cause conformational rearrangement of the C-prM precursor, releasing its signalase site for cleavage. While the exact timing of the events involved in processing of the C-prM precursor or formation and secretion of the viral envelopes is not known, these events are likely to occur either simultaneously or within a very short time interval and may be controlled by the mechanism connected with replication of the viral genomic RNA.

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