JOURNAL OF VIROLOGY, Nov. 1995, p. 7232–7243 Vol. 69, No. 11
0022-538X/95/$04.00+0

Evidence that Flavivirus NS1-NS2A Cleavage Is Mediated by a Membrane-Bound Host Protease in the Endoplasmic Reticulum

BARRY FALGOUT[1,2]* AND LEWIS MARKOFF[1,2]†

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892,[1] *and Laboratory of Vector-Borne Viral Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852-1448*[2]

Received 13 June 1995/Accepted 16 August 1995

Previous deletion mutagenesis studies have shown that the flavivirus NS1-NS2A cleavage requires the eight C-terminal residues of NS1, constituting the cleavage recognition sequence, and sequences in NS2A far downstream of the cleavage site. We now demonstrate that replacement of all of NS1 upstream of the cleavage recognition sequence with prM sequences still allows cleavage in vivo. Thus, other than the eight C-terminal residues, NS1 is dispensable for NS1-NS2A cleavage. However, deletion of the N-terminal signal sequence abrogated cleavage, suggesting that entry into the exocytic pathway is required. Cleavage in vivo was not blocked by brefeldin A, and cleavage could occur in vitro in the presence of dog pancreas microsomes, indicating that NS1-NS2A cleavage occurs in the endoplasmic reticulum. Four in-frame deletions in NS2A were cleavage defective in vitro, as were two mutants in which NS4A-NS4B sequences were substituted for NS2A, suggesting that most of NS2A is required. A series of substitution mutants were constructed in which all Asp, Cys, Glu, His, and Ser residues in NS2A were collectively replaced; all standard proteases require at least one of these residues in their active sites. No single mutant was cleavage defective, suggesting that NS2A is not a protease. Fractionation of the microsomes indicated that the lumenal contents were not required for NS1-NS2A cleavage. It seems most likely that NS1-NS2A cleavage is effected by a host membrane-bound endoplasmic reticulum-resident protease, quite possibly signalase, and that NS2A is required to present the cleavage recognition sequence in the correct conformation to the host enzyme for cleavage.

Flaviviruses have a positive-sense RNA genome of 10 to 11 kb, containing a single long open reading frame. Translation of the genome into a polyprotein precursor, coupled with co- and posttranslational proteolytic processing, results in the production of 10 viral gene products: the three structural proteins core (C), membrane (M), and envelope (E) and seven nonstructural proteins (NS). The order of these products in the polyprotein has been established as NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH, where prM is a precursor to M. Work from a number of laboratories has characterized the processing pathway used by flaviviruses to effect gene expression (reviewed in reference 8). At least three proteases are involved. Cleavages at the C-prM, prM-E, E-NS1, and NS4A-NS4B junctions are made by host cell signalase on the lumenal side of the endoplasmic reticulum (ER) membrane. These signalase cleavages occur after a stretch of hydrophobic residues (a signal sequence) followed by a signalase cleavage motif, which typically contains amino acids with small neutral or hydrophobic side chains at the −3 and −1 positions with respect to the cleavage site (39). A viral trypsin-like serine protease composed of NS3 plus NS2B cleaves at the NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5 junctions. Cleavage occurs at a motif with positively charged residues at the −1 and −2 positions and a small side chain amino acid at +1. This protease also cleaves several additional sites: between C and the prM signal sequence, generating the C terminus of C (1, 24, 45, 46); within NS4A near the NS4B signal sequence (22); within NS3 (2, 15); and probably in some flaviviruses within NS2A near the NS2A-NS2B junction (19, 30). Finally, cleavage of prM to M occurs at a late step in virion maturation in an acidic compartment beyond the Golgi in the exocytic pathway (33). This cleavage is presumably mediated by a host enzyme and occurs at a characteristic motif containing multiple positively charged residues.

In contrast, the mechanism of NS1-NS2A cleavage has not been definitively established. Amino-terminal sequencing of NS2A and carboxy-terminal sequencing of NS1 identified the cleavage site (9, 37, 43) and indicated that processing occurs after a sequence that fits the −3,−1 rule for signalase cleavage. However, there is no associated signal sequence. This finding led to speculation that NS1-NS2A processing is an aberrant signalase cleavage (37). Subsequently, a series of studies used vaccinia virus as an expression vector to characterize dengue virus type 4 (DEN4) NS1-NS2A cleavage (13, 14, 18, 32). A vaccinia virus recombinant expressing only NS1-NS2A produced authentic NS1, demonstrating that no other viral proteins are required for NS1-NS2A cleavage. NS2A was shown to be involved in processing, since a series of C-terminal truncations of NS2A progressively reduced NS1-NS2A cleavage, and removal of 68 amino acids (aa) or more blocked cleavage entirely. Since the deleted sequences were more than 145 aa downstream of the NS1-NS2A junction, it is unlikely that sequences required for cleavage site recognition were deleted. Cleavage-defective deletion mutants were not cleaved when wild-type NS1-NS2A was provided in *trans*, implying a *cis*-

* Corresponding author. Mailing address: Laboratory of Vector-Borne Viral Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike (HFM-451), Rockville, MD 20852-1448. Phone: (301) 496-3350. Fax: (301) 496-1810. Electronic mail address: falgout@A1.cber.fda.gov.

† Present address: Laboratory of Vector-Borne Viral Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD 20852-1448.

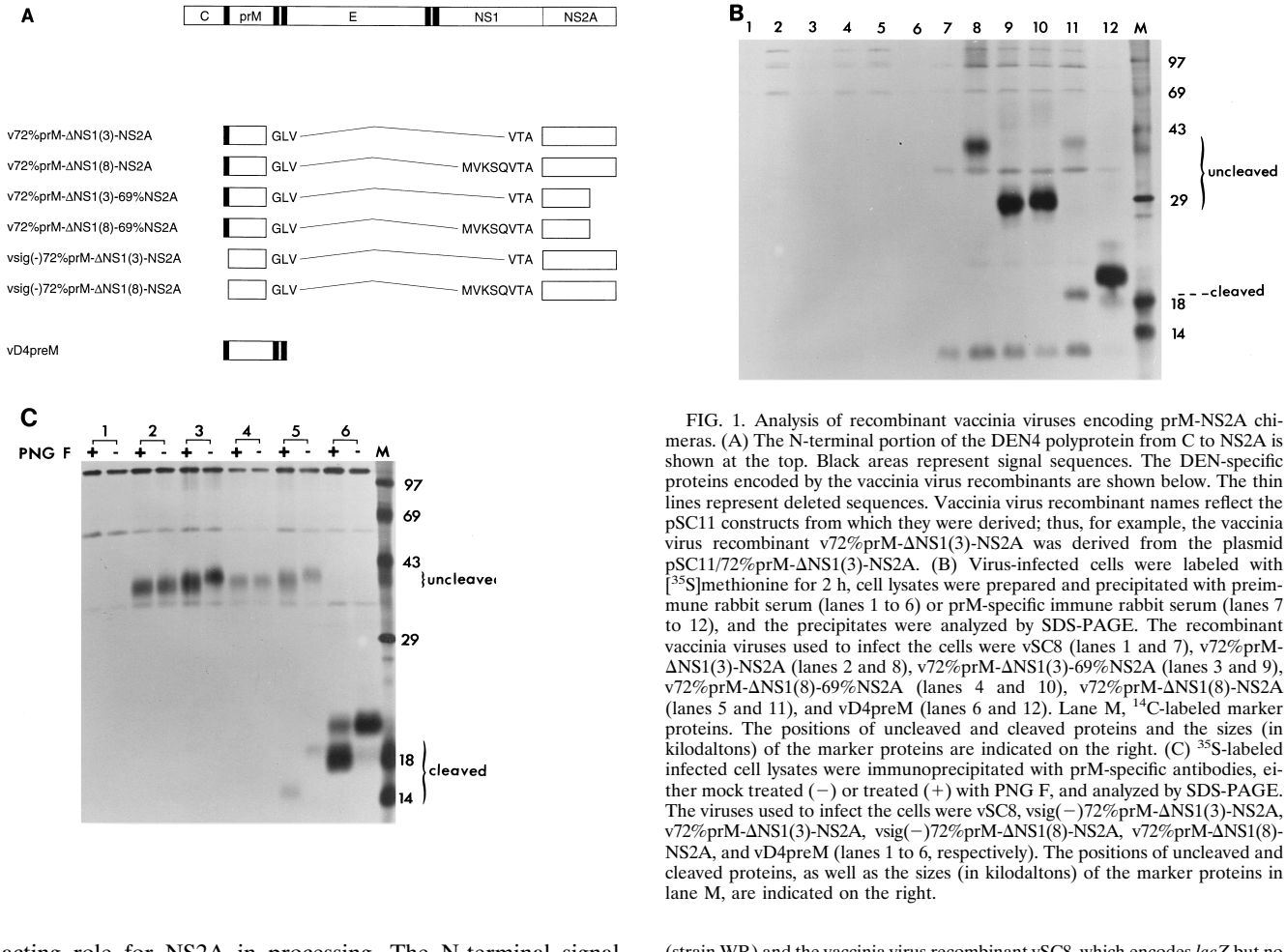


FIG. 1. Analysis of recombinant vaccinia viruses encoding prM-NS2A chimeras. (A) The N-terminal portion of the DEN4 polyprotein from C to NS2A is shown at the top. Black areas represent signal sequences. The DEN-specific proteins encoded by the vaccinia virus recombinants are shown below. The thin lines represent deleted sequences. Vaccinia virus recombinant names reflect the pSC11 constructs from which they were derived; thus, for example, the vaccinia virus recombinant v72%prM-ΔNS1(3)-NS2A was derived from the plasmid pSC11/72%prM-ΔNS1(3)-NS2A. (B) Virus-infected cells were labeled with [^{35}S]methionine for 2 h, cell lysates were prepared and precipitated with preimmune rabbit serum (lanes 1 to 6) or prM-specific immune rabbit serum (lanes 7 to 12), and the precipitates were analyzed by SDS-PAGE. The recombinant vaccinia viruses used to infect the cells were vSC8 (lanes 1 and 7), v72%prM-ΔNS1(3)-NS2A (lanes 2 and 8), v72%prM-ΔNS1(3)-69%NS2A (lanes 3 and 9), v72%prM-ΔNS1(8)-69%NS2A (lanes 4 and 10), v72%prM-ΔNS1(8)-NS2A (lanes 5 and 11), and vD4preM (lanes 6 and 12). Lane M, ^{14}C-labeled marker proteins. The positions of uncleaved and cleaved proteins and the sizes (in kilodaltons) of the marker proteins are indicated on the right. (C) ^{35}S-labeled infected cell lysates were immunoprecipitated with prM-specific antibodies, either mock treated (−) or treated (+) with PNG F, and analyzed by SDS-PAGE. The viruses used to infect the cells were vSC8, vsig(−)72%prM-ΔNS1(3)-NS2A, v72%prM-ΔNS1(3)-NS2A, vsig(−)72%prM-ΔNS1(8)-NS2A, v72%prM-ΔNS1(8)-NS2A, and vD4preM (lanes 1 to 6, respectively). The positions of uncleaved and cleaved proteins, as well as the sizes (in kilodaltons) of the marker proteins in lane M, are indicated on the right.

acting role for NS2A in processing. The N-terminal signal sequence of NS1 appeared to be essential for cleavage, implying that processing requires entry into the exocytic pathway. Finally, analysis of a series of small internal deletions in NS1 near its C terminus indicated that the eight C-terminal residues of NS1 constitute the cleavage recognition site, and subsequent mutational analysis established that the −1, −3, −5, −7, and −8 residues are important for cleavage.

In view of these data, two models for the role of NS2A in NS1-NS2A cleavage have been suggested: NS2A (or NS1-NS2A) may be a *cis*-acting autoprotease that cleaves at the NS1-NS2A junction, or NS2A sequences may be required in some way to permit NS1-NS2A cleavage by a host protease. In the present study, we have continued to investigate DEN4 NS1-NS2A cleavage. We demonstrate that cleavage in vivo does not require any portion of NS1 other than the C-terminal 8 aa but does require a signal sequence. We further show that cleavage in vivo is not blocked by brefeldin A (BFA) and that NS1-NS2A cleavage can occur in vitro only in the presence of dog pancreas microsomes. Analysis of a series of mutations indicates that NS2A itself is not likely to be a protease. These results suggest that NS1-NS2A cleavage occurs in the ER and that NS2A is required to permit a host ER-resident protease, quite possibly signalase, to effect cleavage.

MATERIALS AND METHODS

Cells and viruses. Monkey CV-1 and human TK-143 cells were grown as monolayers in Eagle's minimal essential medium containing 10% fetal bovine serum in a humidified 37°C incubator under 5% CO_2. Wild-type vaccinia virus (strain WR) and the vaccinia virus recombinant vSC8, which encodes *lacZ* but no DEN proteins (7), were originally obtained from Bernard Moss. The recombinants vNS1-NS2A (13) and vD4preM (5) have been described previously. The recombinant vNS1-69%NS2A was previously known as Δ68 (14); this construct is analogous to vNS1-NS2A but has a deletion of the C-terminal 68 aa of NS2A and is completely defective for NS1-NS2A cleavage. Other vaccinia virus recombinants were isolated on TK-143 cells as previously described (7, 13), after homologous recombination between vaccinia virus strain WR and the plasmid pSC11 derivatives whose constructions are described below.

Plasmid constructions. Standard recombinant DNA techniques were used. PCR was done for 30 cycles of 20 s at 94°C, 20 s at 55°C, and 1 min at 72°C with a *Taq* polymerase reaction kit (Perkin-Elmer Cetus), except where noted. Dideoxynucleotide sequencing was performed with the Sequenase 2.0 kit (United States Biochemical). Other enzymes were purchased from New England Biolabs, Life Technologies, or Boehringer Mannheim and used approximately according to the directions of the manufacturers. Brief descriptions of the various plasmids made for this study and their construction are given below; a more detailed account is available upon request. DEN4 nucleotide and amino acid sequence information is from published sources (25, 47).

(i) prM-NS2A chimeras. The unique *Sph*I site of plasmid pSC11/D4preM (5) was converted to a unique *Spe*I site, and the small *Spe*I-*Xho*I fragment of the resulting plasmid was ligated to the large *Spe*I-*Xho*I fragment of plasmid J3 or J8 (18), to create pSC11/72%prM-ΔNS1(3)-NS2A and pSC11/72%prM-ΔNS1(8)-NS2A (Fig. 1A). The recombinant vaccinia viruses derived from these plasmids encode chimeric polyproteins under control of the vaccinia virus p7.5 promoter which initiate at a methionine introduced just prior to DEN4 aa 100 and contain the prM signal sequence (aa 100 to 113), the N-terminal 72% of prM (aa 114 to 232), the sequence GLV (at the *Spe*I site), the C-terminal three or eight residues of NS1 (aa 1123 to 1125 or 1118 to 1125, respectively), all of NS2A (aa 1126 to 1343), and a 27-amino-acid vector-encoded tail. Derivatives of these two plasmids with C-terminal truncations of NS2A were constructed by replacing their homologous fragment with the large *Stu*I-*Xho*I fragment of pSC11/NS1-NS2A Δ68 (14), creating pSC11/72%prM-ΔNS1(3)-69%NS2A and pSC11/72%prM-ΔNS1(8)-69%NS2A (Fig. 1A). The polyproteins encoded by these plasmids are similar to those described above, except that at their C termini they contain only

69% of NS2A (aa 1126 to 1275), followed by a six-residue vector-encoded tail. Derivatives were also made in which the prM signal sequence was deleted. PCR was done, using pSC11/72%prM-ΔNS1(3)-NS2A or pSC11/72%prM-ΔNS1(8)-NS2A as the template. The positive-strand primer annealed to the beginning of prM and introduced a start codon and a *Bgl*II site for cloning, and the negative-strand primer annealed to pSC11 beyond the 3′ end of NS2A, where another *Bgl*II site had been introduced into the unique *Sma*I site of pSC11. Each 1.1-kb PCR product was digested with *Bgl*II and cloned into pSC11[*Bgl*II] (13); a clone of each insert with the sense strand under the control of the p7.5 promoter was designated pSC11/sig(−)72%prM-ΔNS1(3)-NS2A or pSC11/sig(−)72%prM-ΔNS1(8)-NS2A (Fig. 1A). The polyproteins encoded by these two constructs initiate at DEN4 aa 112.

In order to prove that the PCR had not introduced any unexpected mutations in these two signal-deleted clones, the sequence of the amplified regions of both constructs was determined. For comparison, the inserts of pSC11/72%prM-ΔNS1(3)-NS2A and pSC11/72%prM-ΔNS1(8)-NS2A, neither of which had been PCR amplified, were also sequenced. No nucleotide changes specific to the PCR amplification were found. However, the sequences of all four clones differed from the published sequence at six positions: nucleotide (nt) 659, A to T (silent); nt 3609, T to C (silent); nt 3729, A to G (Arg to Gly); nt 3846 and 3847, AG to GA (Ser to Asp); nt 3997, A to T (Asp to Val); and nt 4051, C to T (Ala to Val). The last five of these changes were also found in pSC11/NS1-NS2A when it was partially sequenced. In addition, one substitution was found only in pSC11/72%prM-ΔNS1(3)-NS2A and pSC11/sig(−)72%prM-ΔNS1(3)-NS2A, A to G at nt 3826 (His to Arg). Presumably, this substitution was present in the J3 parent, and we expect that pSC11/72%prM-ΔNS1(3)-69%NS2A has this same substitution, but this has not been proven. The change at nt 3826 was amended for pSC11/72%prM-ΔNS1(3)-NS2A by exchange of *Stu*I-*Xho*I fragments with pSC11/NS1-NS2A (13).

(ii) pGEMNS1-NS2A and derivatives. The *Eco*RI-*Bam*HI insert of pSC11/NS1-NS2A or of pSC11/NS1-NS2A Δ65 or Δ204 (14) was cloned into pGEM 3 (Promega), creating pGEMNS1-NS2A (see Fig. 3A) or pGEMNS1-70%NS2A and pGEMNS1-6%NS2A (see Fig. 5A), respectively. pGEMNS1-NS2A was digested with *Apa*I and recircularized, creating pGEMNS1-NS2A ΔApa (see Fig. 3A), which encodes an NS1 protein that is 17 aa smaller than the wild type. Internal deletions in NS2A were created by digestion of pGEMNS1-NS2A with two restriction enzymes followed by recircularization, using oligonucleotide adaptors designed to maintain the reading frame. The resulting clones are named ΔES, ΔBB, ΔBM, and ΔMA (see Fig. 6A).

PCR was used to make two clones, pGEMNS1-ΔNS2A(1)-81%NS4A-NS4B$_{74}$ and pGEMNS1-ΔNS2A(31)-81%NS4A-NS4B$_{74}$ (see Fig. 6A), in which most of NS2A was replaced with hydrophobic sequences from NS4A and NS4B. In one pair of PCRs, primers were used, which amplified the N-terminal 81% of NS4A (aa 2092 to 2212) while attaching part of NS4B to the 3′ end and attaching sequences from either NS2A or NS1 to the 5′ end. A second reaction amplified a central 74-aa region of NS4B (aa 2301 to 2374), attaching an *Eco*RI site to the 3′ end. This second fragment was joined to each of the first fragments via a *Bgl*I site within NS4B (nt 7020 to 7029), and the ligated PCR products were then cloned into pGEMNS1-NS2A by using the *Eco*RI site and either the *Bsm*I site in NS2A (nt 3537 to 3542) or the *Eag*I site at the NS1-NS2A boundary (nt 3472 to 3477). The amplified regions of pGEMNS1-ΔNS2A(1)-81%NS4A-NS4B$_{74}$ and pGEMNS1-ΔNS2A(31)-81%NS4A-NS4B$_{74}$ were sequenced, and the sequences of both clones differed from the published sequence at two positions: nt 6519, A to T (Thr to Pro), and nt 7138, C to T (Ala to Thr).

PCR was also used to mutate potential protease active site residues in NS2A (see Fig. 7A). First, a *Bgl*II site was created in pGEMNS1-NS2A by changing nt 3944 from A to T, a silent mutation. Next, mutagenic oligonucleotides which created the desired mutations and contained a unique (or two-cut, in the case of *Bgl*I) restriction enzyme site near one end were designed. These oligonucleotides were used as primers for PCR, in combination with either another mutagenic primer or a wild-type primer, with either pGEMNS1-NS2A or its *Bgl*II-containing derivative as the template. The PCR products were digested with the appropriate restriction enzymes, and the resulting mutated restriction fragment was used to replace the homologous wild-type fragment in either pGEMNS1-NS2A or its *Bgl*II-containing derivative. The restriction sites used were *Eag*I, nt 3472 to 3477; *Bcl*I, nt 3586 to 3591; *Bgl*I, nt 3699 to 3709; *Msc*I, nt 3796 to 3801; *Bgl*II, nt 3939 to 3944; and *Avr*II, nt 4070 to 4075. The sequence of the PCR-amplified regions was determined to confirm that no unexpected mutations were introduced.

Clones containing only NS1 or NS2A (pGEMNS1 or pGEMNS2A; see Fig. 3A) were also created by PCR. For NS1, the positive-strand primer annealed at the beginning of the NS1 signal sequence and contained an upstream *Bam*HI site for cloning, while the negative-strand primer annealed to the 3′ end of NS1 and contained a downstream *Eco*RI site for cloning. This PCR was performed with *Pfu* polymerase (Stratagene). The 1.1-kb PCR product was digested with *Eco*RI and *Bam*HI and cloned into pGEM3, creating pGEMNS1. For NS2A, the positive-strand primer annealed to the beginning of NS2A and provided an ATG to initiate translation and a *Sal*I site for cloning, and the negative-strand primer annealed to the T7 RNA polymerase promoter. The 0.7-kb PCR product was digested with *Eco*RI and *Sal*I and cloned into pGEM3, creating pGEMNS2A.

Antisera. Hyperimmune mouse ascitic fluid (HMAF) specific for DEN4 was obtained from the American Type Culture Collection. The peptide FSLSTRD GEPLMIVC, consisting of the N-terminal 14 residues of prM (aa 114 to 127) plus Cys, was coupled to keyhole limpet hemocyanin (26). The product was used to raise prM-specific antibodies in rabbits.

Analysis of recombinant vaccinia virus-infected cell proteins. The procedures for infection of CV-1 cells with recombinant vaccinia virus and metabolic labeling of infected cell proteins with [^{35}S]methionine have been described previously (13). To chase, the labeling medium was removed, and the cells were rinsed once and then incubated with 2 ml of Eagle's minimal essential medium containing 2% fetal bovine serum. In experiments using BFA (Epicentre Technologies), the drug was present in the starve, label, rinse, and chase media at 10 μg/ml. The preparation of lysates and immune precipitation was performed essentially as described previously (13), using 1/100 volume of HMAF or 1/10 to 1/20 volume of antipeptide sera, except that the Pansorbin (Calbiochem) pellets were washed twice with radioimmunoprecipitation assay buffer plus 2% sodium dodecyl sulfate (SDS). For removal of N-linked carbohydrates by using protein N-glycosidase F (PNG F) (Boehringer Mannheim), the Pansorbin pellets were washed a third time in 10 mM Tris-HCl (pH 7.5)–10 mM EDTA, boiled in a small volume of 0.8% SDS, and recentrifuged. Then, the supernatants were mixed with 3 volumes of 1.33× endoglycosidase F buffer (13), divided into two portions, and incubated for 1 h at 37°C with or without the addition of 1 μl (0.2 U) of PNG F. SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography were done as described previously (13).

In vitro transcription and translation. pGEM NS1-NS2A and derivatives were linearized at the *Eco*RI site downstream of NS2A or at other restriction sites within NS2A as noted. Linearized DNA (2 μg) was phenol-chloroform extracted, ethanol precipitated, and then transcribed with SP6 RNA polymerase (Life Technologies or New England Biolabs) in a 30-μl reaction volume at 40°C for 2 h. One-tenth of the reaction mixture was analyzed by electrophoresis on a 1% agarose gel containing 0.5 μg of ethidium bromide per ml. The remainder was stored at −70°C.

Translation in vitro was carried out by using nuclease-treated rabbit reticulocyte extract and dog pancreas microsomes (Promega) with [^{35}S]methionine (>800 Ci/mmol; Amersham) according to instructions provided by Promega. *Saccharomyces cerevisiae* α-factor RNA used as a glycosylation control was also obtained from Promega. The reaction volume of 10 μl usually contained 0.5 μl of transcription reaction mixture and 1 μl of microsomes; however, these volumes could vary. For the membrane extraction experiment for which the results are shown in Fig. 8, the microsomes were diluted in pH 7.5 or pH 9.5 buffer and resealed by centrifugation through sucrose as described previously (31). Translation was routinely done at 37°C; similar results could be obtained at 30°C. After translation, usually for 1 to 2 h, samples were taken either directly or after the membranes had been pelleted (27). Protease protection experiments using thermolysin and fractionation of membrane pellets in sodium carbonate (pH 11.5) were performed essentially as described previously (27). Removal of N-linked glycans was done by using 1,000 U of PNG F or endoglycosidase H (Endo H; New England Biolabs) essentially according to directions of the manufacturer. Reactions were carried out for 1 h at 37°C. SDS-PAGE and fluorography were done as described previously (13).

RESULTS

NS1 is dispensable for NS1-NS2A cleavage. A recent study investigated the role of sequences at the C terminus of NS1 in NS1-NS2A cleavage (18). In these experiments, a series of deletions were introduced into a vaccinia virus recombinant expressing DEN4 NS1-NS2A, extending from the unique *Spe*I site in NS1 (aa 1080 and 1081) toward the NS1/NS2A cleavage site (aa 1125/1126). The NS1-NS2A junction was cleaved in deletion mutants which retained eight or more C-terminal residues of NS1 but not in mutants that retained seven or fewer residues, leading to the conclusion that the eight C-terminal residues of NS1 comprise the cleavage recognition site. However, these data do not address whether any of NS1 upstream of the *Spe*I site might be required for cleavage. To investigate this, the vaccinia virus recombinants v72%prM-ΔNS1(3)-NS2A and v72%prM-ΔNS1(8)-NS2A were constructed (Fig. 1A). These viruses encode chimeric proteins consisting of the prM signal sequence plus the N-terminal 72% of prM (aa 100 to 232) fused in frame via the sequence GLV to the C-terminal three (VTA, aa 1123 to 1125) or eight (MVKSQVTA, aa 1118 to 1125) residues of NS1 followed by all of NS2A (aa 1126 to 1343). If the only portion of NS1 that is required for NS1-NS2A cleavage is the eight C-terminal residues, then the product of v72%prM-ΔNS1(8)-NS2A should be cleaved. The product of v72%prM-ΔNS1(3)-NS2A should not be cleaved at the NS1-NS2A junction, since it contains only the three C-terminal residues of NS1. The recombinants v72%prM-ΔNS1(3)-69%NS2A and v72%prM-ΔNS1(8)-

69%NS2A were also made (Fig. 1A). These viruses encode chimeric proteins similar to those discussed above, except that only the N-terminal 69% of NS2A (aa 1126 to 1275) is present. This truncation of NS2A is expected to block authentic NS1-NS2A cleavage (14).

The radiolabeled proteins of cells infected by these viruses were analyzed by immunoprecipitation with rabbit prM-specific antipeptide antibodies, followed by SDS-PAGE and fluorography (Fig. 1B). The recombinant vD4preM (5) was also analyzed, as a control known to express prM. The recombinant v72%prM-ΔNS1(8)-NS2A (lane 11) expressed two prM-specific proteins, one with a mass of about 42 kDa and the other with a mass of about 19 kDa. The size of the larger protein was consistent with that expected for the singly glycosylated uncleaved polyprotein, 43 kDa. The 19-kDa protein was assigned as the glycosylated NS1-NS2A cleavage product 72%prM-ΔNS1(8), with a predicted size of 16 kDa. (See Fig. 1C for a proof of glycosylation.) On the basis of the intensities of the bands, and given that the precursor has 24 Met residues while the cleaved product has only 7, cleavage of this precursor was estimated to be 80 to 85% complete. In contrast, v72%prM-ΔNS1(3)-NS2A (lane 8) expressed only the uncleaved polyprotein, which migrated slightly faster than the uncleaved polyprotein made by v72%prM-ΔNS1(8)-NS2A. Furthermore, v72%prM-ΔNS1(3)-69%NS2A (lane 9) and v72%prM-ΔNS1(8)-69%NS2A (lane 10) expressed only uncleaved polyproteins with sizes of about 29 kDa (predicted sizes, 33 kDa). In addition to the intense 29-kDa proteins, there were faint diffuse protein bands at 50 to 60 kDa in both lanes 9 and 10. The identity of these proteins has not been further investigated. The failure to observe cleavage in v72%prM-ΔNS1(8)-69%NS2A shows that the processing seen in v72%prM-ΔNS1(8)-NS2A is dependent on NS2A, consistent with authentic NS1-NS2A cleavage. Thus, the only portion of NS1 that is essential for NS1-NS2A cleavage is the 8-aa cleavage recognition site.

A signal sequence is required for NS1-NS2A cleavage. It was previously suggested that the N-terminal NS1 signal sequence is required for the C-terminal NS1 cleavage at the NS1-NS2A site (13). However, this suggestion was based only on the lack of observed cleavage products in an NS1-NS2A construct lacking a signal sequence; the uncleaved precursor could not be detected by the antibodies used. To directly demonstrate that NS1-NS2A cleavage requires a signal sequence, the recombinants vsig(−)72%prM-ΔNS1(3)-NS2A and vsig(−)72%prM-ΔNS1(8)-NS2A were constructed (Fig. 1A). These recombinants are similar to v72%prM-ΔNS1(3)-NS2A and v72%prM-ΔNS1(8)-NS2A, but they lack the prM signal sequence. If an N-terminal signal sequence is required for NS1-NS2A cleavage, the products of both of the signal-deleted recombinants should remain uncleaved, whereas if the signal sequence is dispensable, then the product of vsig(−)72%prM-ΔNS1(8)-NS2A should be cleaved.

The prM-specific products of these two recombinants were compared to those of v72%prM-ΔNS1(3)-NS2A and v72%prM-ΔNS1(8)-NS2A (Fig. 1C). vsig(−)72%prM-ΔNS1(3)-NS2A (lanes 2) and vsig(−)72%prM-ΔNS1(8)-NS2A (lanes 4) both made only uncleaved precursor-sized products. Half of these immune precipitated products were digested with PNG F to assay for N-linked glycans. Since N-linked glycosylation occurs on the lumenal side of the ER membrane, susceptibility to PNG F digestion is indicative of translocation into the ER. The uncleaved products of both signal-deleted constructs were unaffected by PNG F, indicating that these proteins had not entered the ER, consistent with the lack of a signal sequence. Both the uncleaved precursor and the cleavage product made by v72%prM-ΔNS1(8)-NS2A (lanes 5) were glycosylated. Similarly, the uncleaved precursor made by v72%prM-ΔNS1(3)-NS2A (lanes 3) was glycosylated. Thus, removal of the signal sequence from v72%prM-ΔNS1(8)-NS2A results in failure of the polyprotein to enter the ER and failure of NS1-NS2A cleavage, suggesting that NS1-NS2A cleavage requires translocation into the ER.

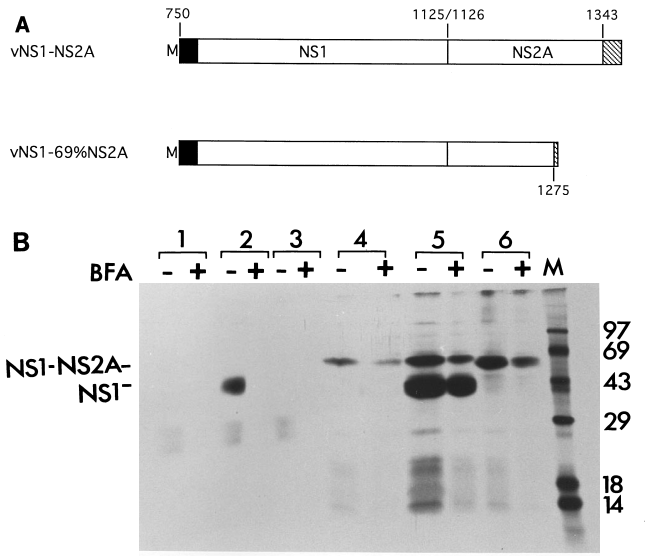


FIG. 2. BFA does not block NS1-NS2A cleavage. (A) The polyproteins encoded by the vaccinia virus recombinants are shown. The black area represents the NS1 signal sequence (aa 750 to 773). M is the initiating Met residue. The striped areas represent the C-terminal vector-encoded residues (27 aa for vNS1-NS2A and 6 aa for vNS1-69%NS2A). The numbers indicate the first and last DEN-specific amino acids in the proteins, as well as the NS1-NS2A cleavage site. (B) Cells infected by vSC8 (lanes 1 and 4), vNS1-NS2A (lanes 2 and 5), or vNS1-69%NS2A (lanes 3 and 6) were radiolabeled for 2 h and then chased for 5 h, in the presence (+) or absence (−) of BFA. The extracellular media (lanes 1 to 3) and the cell lysates (lanes 4 to 6) were immunoprecipitated with HMAF and analyzed by SDS-PAGE. The positions of NS1-NS2A and NS1 are indicated at the left. Lane M, marker proteins, with sizes (in kilodaltons) indicated on the right.

BFA does not block NS1-NS2A cleavage. BFA is an inhibitor of the secretory pathway that blocks membrane traffic from the ER to the Golgi, resulting in the disappearance of the Golgi as a separate organelle and the redistribution of Golgi markers into the ER (reviewed in reference 20). To further characterize the intracellular location of NS1-NS2A cleavage, we investigated the effect of BFA on processing of the products of vNS1-NS2A and vNS1-69%NS2A (Fig. 2A). Since cleaved NS1 is normally secreted slowly (28), cells infected by these recombinants were pulse-labeled and then chased in the presence of BFA, and fractions of the extracellular media and cell lysates were immune precipitated with HMAF and analyzed by SDS-PAGE (Fig. 2B). As expected, vNS1-69%NS2A-infected cell lysates contained only the uncleaved precursor at about 60 kDa (lanes 6), and the extracellular media above these cells contained no specific products (lanes 3). Also, vNS1-NS2A-infected cells in the absence of BFA (lane 5−) contained a prominent NS1 band near 43 kDa, as well as a band slightly larger than the NS1-69%NS2A polyprotein, which was probably a combination of the NS1-NS2A polyprotein and the nonspecific band seen in lane 4. NS2A (predicted M_r, 24,000) was not detected, presumably because the HMAF did not precipitate it, although we cannot rule out the possibility that NS2A is unstable. In the presence of BFA, vNS1-NS2A produced the same relative amounts of the NS1-NS2A and NS1 bands (lane 5+), suggesting that NS1-NS2A cleavage occurs early in the exocytic pathway prior to the BFA block, possibly within the ER. BFA was shown to be active, since secretion of NS1 by vNS1-NS2A was blocked in the presence of BFA (lane 2− versus lane 2+).

NS1-NS2A cleavage in vitro. To test the hypothesis that

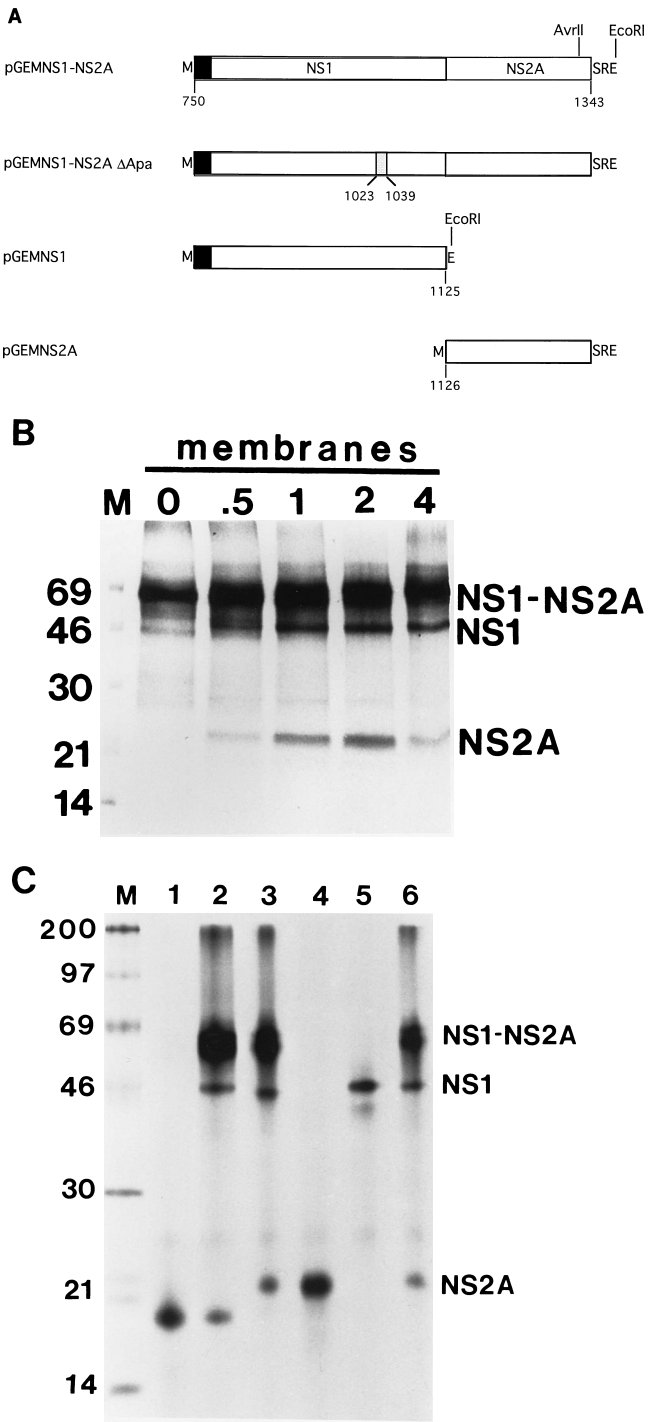


FIG. 3. NS1-NS2A cleavage in vitro. (A) The diagram shows the proteins encoded by transcripts of the listed plasmids. The black region is the NS1 signal sequence. The stippled area represents the 17 aa deleted in the ΔApa construct; the numbers below the deletion show the first and last residues removed. Other numbers are the first and last DEN-specific amino acids in the constructs. M, initiating methionine. SRE and E, C-terminal vector-encoded residues. The positions of the *Eco*RI and *Avr*II sites used to linearize the templates are shown. (B) RNA made from *Eco*RI-linearized pGEMNS1-NS2A was translated in vitro in a 10-μl reaction mixture plus the indicated volume (in microliters) of microsomal membranes, and 1/10 of the products were analyzed by SDS-PAGE. The positions of uncleaved NS1-NS2A as well as cleaved NS1 and NS2A are indicated on the right. M, marker proteins, with sizes (in kilodaltons) indicated on the left. (C) Transcripts prepared from pGEMNS2A (lanes 1 and 4), pGEMNS1-NS2A (lanes 2 and 6), pGEMNS1-NS2AΔApa (lane 3), and pGEMNS1 (lane 5) were in vitro translated in the presence of dog pancreas microsomes and analyzed by SDS-PAGE. *Avr*II-linearized templates were used in lanes 1 and 2; *Eco*RI-linearized templates were used in lanes 3 to 6. The positions of NS1-NS2A, NS1, and NS2A are indicated on the right, and the sizes (in kilodaltons) of the marker proteins in lane M are indicated on the left.

NS1-NS2A cleavage takes place in the ER, NS1-NS2A was translated in vitro in the presence of dog pancreas microsomes. These microsomes contain membrane organelles with properties of the ER, including signal peptidase and N-linked glycosylation activities (41). The plasmid pGEMNS1-NS2A was constructed, and linearization of this plasmid with *Eco*RI followed by in vitro transcription with SP6 RNA polymerase produced RNA which encoded the NS1 signal sequence followed by NS1-NS2A plus three vector-encoded amino acids (Fig. 3A). Figure 3B shows the results of in vitro translation of a constant amount of this RNA in the presence of increasing amounts of dog pancreas microsomes. In the absence of membranes, a major product with a size of about 69 kDa was observed, along with several minor, lower-M_r (between about 55,000 and 28,000) species. The major product was identified as full-length NS1-NS2A, on the basis of size. The minor species at about 46 kDa could have been NS1. However, there was not a corresponding band for NS2A, and a careful comparison of this 46-kDa band to deglycosylated NS1 showed that they did not comigrate (data not shown). We conclude that this protein was not authentic NS1 and presume that all of the minor products resulted from internal initiation or premature termination of translation. As increasing amounts of microsomes were added from 0.5 to 2 μl, an increasing proportion of the 69-kDa band attained a slightly higher M_r, the minor species mostly faded away, and two new species, with sizes of about 47 and 23 kDa, appeared in increasing amounts. On the basis of size, these bands were tentatively identified as the authentic cleavage products NS1 and NS2A. The increase in M_r of the NS1-NS2A polyprotein was presumably due to glycosylation on entry into the microsomes; there are two N-linked glycosylation sites in NS1 but none in NS2A. The addition of 4 μl of microsomes further increased the proportion of the higher M_r precursor but led to a reduction in the intensities of all bands. Experience indicated that it was important to titrate each lot of microsomes to obtain good NS1-NS2A cleavage without drastic inhibition of translation.

To verify the identification of the 47- and 23-kDa proteins as NS1 and NS2A, respectively, the plasmids pGEMNS1, pGEMNS2A, and pGEMNS1-NS2AΔApa were constructed (Fig. 3A). These plasmids encode, respectively, NS1, NS2A, and NS1-NS2A with a 17-aa in-frame deletion in NS1. RNA transcripts of these three plasmids and pGEMNS1-NS2A were translated in vitro in the presence of microsomes and analyzed by SDS-PAGE (Fig. 3C). As expected, pGEMNS1-NS2A (lane 6) produced a doublet at 69 kDa, which is assigned as uncleaved NS1-NS2A with and without N-linked glycans, and apparent cleavage products at 47 and 23 kDa. The 47-kDa protein comigrated with the major product of pGEMNS1 (lane 5), while the 23-kDa protein comigrated with the product of pGEMNS2A (lane 4). The minor, faster-migrating protein made by pGEMNS1 was unglycosylated NS1 (data not shown). In addition, pGEMNS1-NS2AΔApa (lane 3) gave rise to a cleavage product that comigrated with NS2A, but its other product was slightly smaller than 47 kDa, consistent with the deletion of 17 aa in the NS1 portion of this construct. Next, transcripts from pGEMNS1-NS2A and pGEMNS2A templates that had been linearized with *Avr*II were translated in vitro, and the products were analyzed. Digestion with *Avr*II

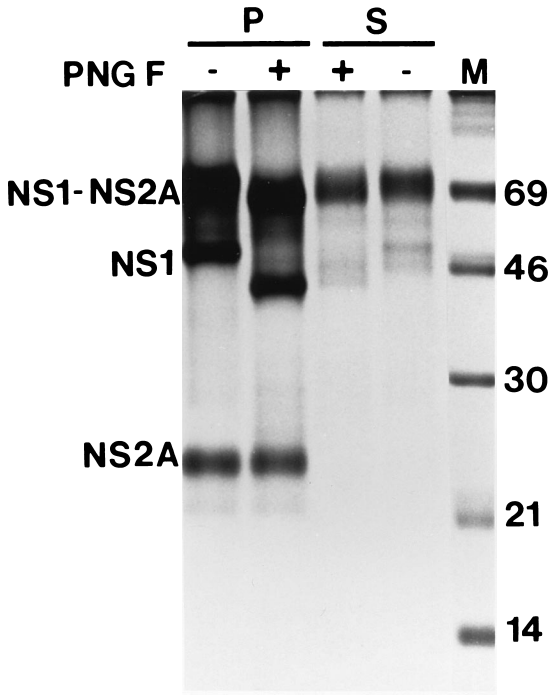


FIG. 4. Membrane association of in vitro translation products. RNA made from *Eco*RI-linearized pGEMNS1-NS2A was translated in vitro, and the reaction mixture was centrifuged to produce membrane pellet (P) and supernatant (S) fractions. These were either treated with PNG F (+) or mock treated (−) before SDS-PAGE. The positions of NS1-NS2A, NS1, and NS2A are indicated on the left. M, marker proteins, with sizes (in kilodaltons) indicated on the right.

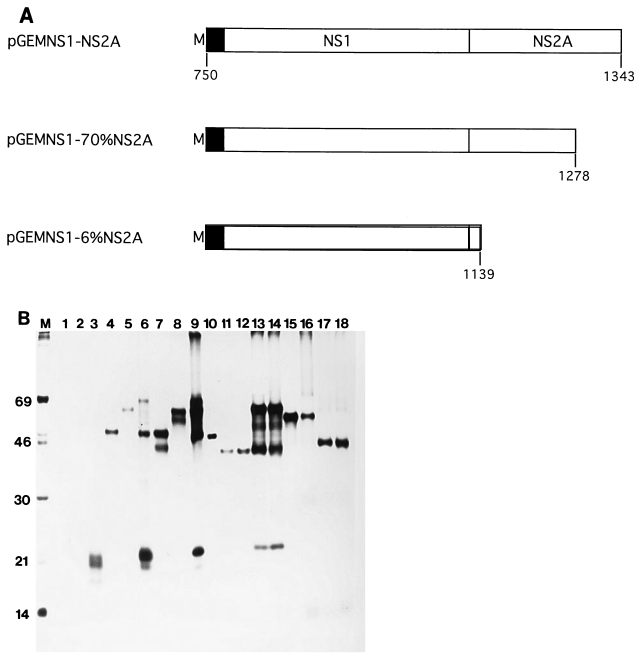
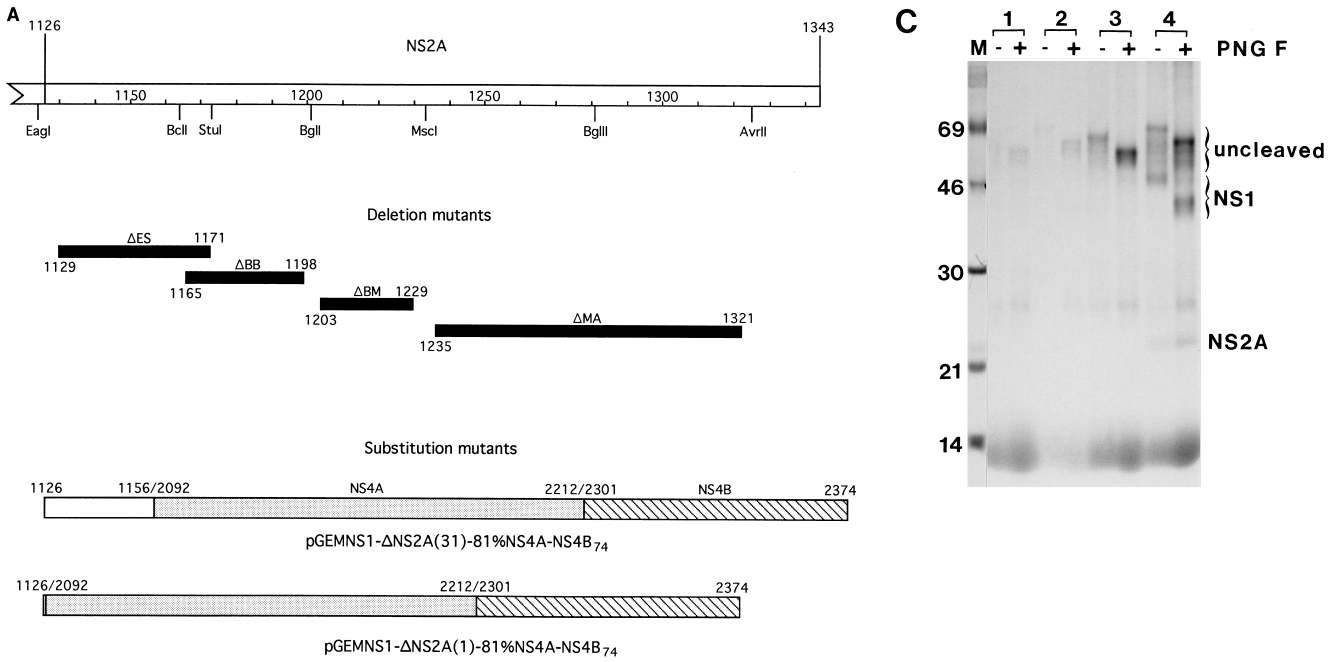


FIG. 5. Protease protection of translation products and dependence of in vitro cleavage on NS2A sequences. (A) Diagram of DEN-specific proteins encoded by the plasmids listed. M is the initiating methionine. The black region represents the NS1 signal sequence. Numbers indicate the first and last encoded DEN amino acids of templates linearized by *Eco*RI. (B) Transcripts were in vitro translated and then either digested with thermolysin in the presence of detergent (lanes 1 to 3), with thermolysin alone (lanes 4 to 6), with Endo H (lanes 13, 15, and 17), with PNG F (lanes 14, 16, and 18) or analyzed without further treatment (lanes 7 to 9). The templates used were pGEMNS1-6%NS2A (lanes 1, 4, 7, 17 and 18), pGEMNS1-70%NS2A (lanes 2, 5, 8, 15, and 16), and pGEMNS1-NS2A (lanes 3, 6, 9, 13, and 14). Lanes 10 to 12, HMAF-precipitated NS1 made in vivo by vNS1-NS2A and analyzed untreated (lane 10) or after digestion with Endo H (lane 11) or PNG F (lane 12). Lane M, marker proteins, with sizes (in kilodaltons) indicated on the left.

removes the C-terminal 19 aa of NS2A, which is not expected to affect cleavage efficiency but should noticeably reduce the size of the NS2A protein. For both pGEMNS2A (lane 1) and pGEMNS1-NS2A (lane 2), linearization with *Avr*II resulted in a faster-migrating NS2A product. Furthermore, it can be seen in Fig. 5B that the in vitro NS1 cleavage product (lane 9) comigrated with authentic in vivo NS1 (lane 10), and this was true even after deglycosylation with PNG F or Endo H (compare lanes 11 and 12 to lanes 13 and 14). Taken together, these data confirm that the 47-kDa protein represents authentic NS1, while the 23-kDa protein is NS2A. The dependence of the in vitro production of NS1 and NS2A on added microsomal membranes is consistent with the in vivo requirement for a signal sequence and indicates that NS1-NS2A cleavage occurs in the ER.

Several attempts were made to directly demonstrate a precursor-product relationship between the 69-kDa NS1-NS2A polyprotein and the 47-kDa NS1 and 23-kDa NS2A proteins. Such a relationship was previously suggested from in vivo pulse-chase experiments (10). NS1-NS2A RNA was translated in vitro for 5 to 7 min, an excess of unlabeled methionine was added, and the incubation at 37°C was continued for 1.5 h, with samples taken periodically (data not shown). The intensity of the 69-kDa NS1-NS2A band diminished over the course of the chase, and the 47-kDa NS1 and 23-kDa NS2A proteins accumulated during the early part of the chase. However, the levels of NS1 and NS2A soon reached a plateau, and the large protein that remained at the interface between the stacking and resolving gels accumulated throughout the chase. Numerous unsuccessful attempts were made to change conditions so as to eliminate this large band, including varying the incubation temperature, varying the denaturation temperature prior to gel loading, and adding additional 2-mercaptoethanol to the denaturation buffer. The apparent conversion of NS1-NS2A and/or NS1 and NS2A into a large aggregate confounded the direct demonstration of a precursor-product relationship. Nevertheless, the results for the early part of the chase were consistent with the notion that the NS1-NS2A polyprotein is the direct precursor of the NS1 and NS2A products.

Membrane association of in vitro products. To investigate the membrane association of the NS1-NS2A polyprotein and the NS1 and NS2A cleavage products, pGEMNS1-NS2A was translated in vitro, the membranes were pelleted, and the pellet and supernatant fractions were examined with and without PNG F treatment (Fig. 4). The pellet fraction contained both NS1-NS2A precursor species as well as most of the NS1 and all of the NS2A product. The supernatant contained mainly the smaller NS1-NS2A band and a small fraction of the NS1. The majority of the NS1-NS2A in the pellet was glycosylated, as was NS1 in both fractions, while NS2A in the pellet and NS1-NS2A in the supernatant were not glycosylated. This confirms that the increase in size of NS1-NS2A on the addition of microsomes was due to glycosylation and indicates that the glycosylated precursor and both products were membrane associated, while much of the unglycosylated precursor was not membrane associated. The glycosylated NS1 in the supernatant perhaps indicated some membrane leakiness. Next, membrane pellets were treated with sodium carbonate (pH 11.5) and recentrifuged, generating carbonate pellet and superna-



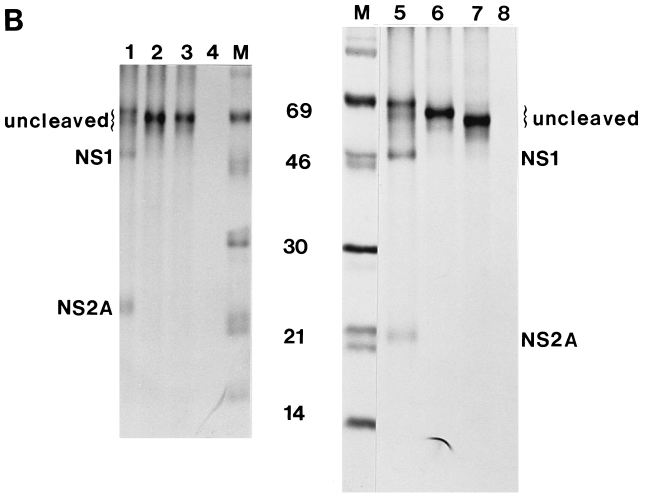


FIG. 6. Analysis of NS2A deletion and substitution mutants. (A) The extreme C-terminal portion of NS1 and all of NS2A (aa 1126 to 1343) are diagrammed at the top. Specific restriction sites are indicated, and tick marks occur at 10-aa intervals in NS2A, with residue numbers at 50-aa intervals. The amino acids missing in the four deletion mutants (ΔES, ΔBB, ΔBM, and ΔMA) are shown as black bars in the middle. Sequences between pairs of restriction sites were removed, as indicated by the two letters in the names of the mutants. The numbers indicate the first and last amino acids removed. At the bottom is a diagram of the sequences beyond NS1 in two substitution mutants, pGEMNS1-ΔNS2A(31)-81%NS4A-NS4B$_{74}$ and pGEMNS1-ΔNS2A(1)-81%NS4A-NS4B$_{74}$. The open regions represent NS2A sequences (aa 1126 to 1156 or aa 1126 only), the stippled regions denote NS4A sequences (aa 2092 to 2212), and the striped regions are NS4B sequences (aa 2301 to 2374). (B) Analysis of translation products of NS2A deletion mutants. RNA transcripts were translated in vitro, and the membrane-associated products were analyzed by SDS-PAGE. The templates used were pGEMNS1-NS2A (lanes 1 and 5), ΔBB (lane 2), ΔBM (lane 3), ΔES (lane 6), and ΔMA (lane 7). Lanes 4 and 8 are the products of translation without RNA. The positions of uncleaved precursor and cleaved NS1 and NS2A are indicated. Lanes M, marker proteins, with sizes (in kilodaltons) indicated. (C) Analysis of translation products of NS2A substitution mutants. Transcripts were in vitro translated, and the products were then either treated with PNG F (+) or mock treated (−) before SDS-PAGE. Lanes: 1, no RNA control; 2, pGEMNS1-ΔNS2A(31)-81%NS4A-NS4B$_{74}$; 3, pGEMNS1-ΔNS2A(1)-81%NS4A-NS4B$_{74}$; 4, pGEMNS1-NS2A. The positions of uncleaved precursor and cleaved NS1 and NS2A are indicated, as are the sizes (in kilodaltons) of the marker proteins in lane M.

tant fractions. Integral membrane proteins are expected to remain in the carbonate pellet, whereas soluble intralumenal and peripheral membrane proteins should fractionate into the supernatant (16). NS1-NS2A and NS2A fractionated predominantly in the carbonate pellet, whereas NS1 was almost equally distributed between the carbonate pellet and supernatant (data not shown). These results suggest that NS1 is soluble in the ER lumen or is peripherally associated with membranes, while NS1-NS2A and NS2A are membrane anchored.

To confirm that the glycosylated NS1-NS2A precursor and its cleavage products were primarily localized inside the membranes, a protease protection experiment was done. Proteins inside the membranes are expected to be resistant to added protease in the absence of detergent. An RNA transcript of pGEMNS1-NS2A was in vitro translated, and fractions were untreated, digested with thermolysin in the presence or absence of detergent, or digested with PNG F or Endo H (Fig. 5B). The untreated sample (lane 9) was overloaded, but it could be seen to contain the expected NS1-NS2A doublet and the cleavage products NS1 and NS2A. Thermolysin completely digested the unglycosylated NS1-NS2A but not the glycosylated NS1-NS2A, NS1, or NS2A (lane 6). On thermolysin digestion in the presence of detergent (lane 3), both precursor forms and NS1 were totally digested, confirming their sensitivity to thermolysin. However, there was a band in this lane near 21 kDa, which may indicate that NS2A was at least partially thermolysin resistant; thus, its location cannot be assayed by this method. The results indicate that the unglycosylated precursor is completely external to the membranes and suggest that the major portions of glycosylated NS1-NS2A and NS1 are localized inside the membranes. We suspect that the partial digestion of the glycosylated precursor and NS1 was due to membrane leakiness, but we cannot rule out the possibility that some portions of these proteins were external to the membranes.

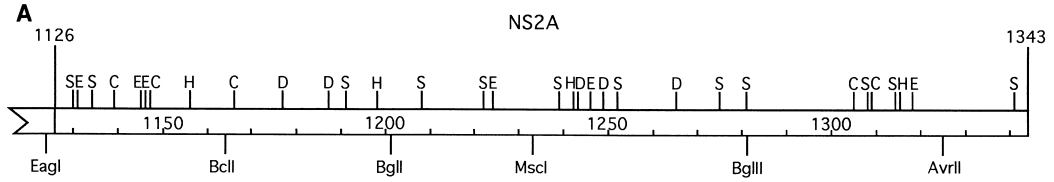
NS2A sequences are required for in vitro NS1-NS2A cleavage. In vivo, NS1-NS2A cleavage is dependent on NS2A, as incremental deletion of C-terminal NS2A sequences progressively reduces cleavage efficiency, and deletion of 68 residues or more completely blocks cleavage (14). To find out if NS2A sequences are also required for in vitro NS1-NS2A cleavage, pGEMNS1-70%NS2A and pGEMNS1-6%NS2A were constructed (Fig. 5A). These plasmids encode NS1 plus C terminally truncated NS2A, such that 65 or 204 residues of the NS2A are deleted. Comparison of the in vitro translation products derived from these two plasmids with those from pGEMNS1-NS2A is shown in Fig. 5B. pGEMNS1-70%NS2A (lane 8) expressed a doublet consistent in size with the uncleaved NS1-70%NS2A precursor. There was no evidence of cleaved NS1 or 70%NS2A (expected size, 17 kDa). Deglycosylation converted the larger species into the smaller one (lanes 15 and 16), while thermolysin treatment completely digested the smaller band but not the larger one (lane 5). Thus, the smaller member of the doublet was the unglycosylated, untranslocated form of the precursor, and the larger protein was the glycosylated, translocated form. Similarly, pGEMNS1-6%NS2A (lane 7) produced two bands. Neither of these bands comigrated with NS1 before or after deglycosylation (compare lane 7 with lanes 9 and 10 and lanes 17 and 18 with lanes 11 to 14), and thus they were assigned as being uncleaved NS1-6%NS2A. Note that the predicted 6%NS2A cleavage product (1.7 kDa) would not have been visible on this gel. On the basis of the susceptibility to endoglycosidases and thermolysin (lanes 1 and 4), the larger band appeared to be translocated and glycosylated, while the smaller band was untranslocated and unglycosylated. These results indicate that in vitro NS1-NS2A cleavage is blocked by C-terminal truncation of NS2A. This was confirmed by an alternate method of truncating NS2A by using restriction enzymes. Transcripts were prepared from pGEMNS1-NS2A templates linearized with one of several enzymes that cut within NS2A, and these were then translated in vitro and compared with the full-length NS2A made from *Eco*RI transcripts. The restriction enzymes used and the number of amino acids removed were *Avr*II, 19; *Bgl*II, 63; *Msc*I, 111; *Bgl*I, 141; *Stu*I, 172; and *Bcl*I, 180 (Fig. 6A). All polyprotein products of truncated transcripts, except for *Avr*II, were completely defective for NS1-NS2A cleavage (data not shown); the polyprotein encoded by *Avr*II transcripts cleaved normally (Fig. 3C). This suggests that NS2A sequences somewhere between the *Avr*II and *Bgl*II sites are required for NS1-NS2A cleavage in vitro. The requirement for NS2A in vitro may be even more stringent than that in vivo, since a deletion that removes 65 aa of NS2A (NS1-70%NS2A) was cleaved inefficiently in vivo but not at all in vitro.

One possible interpretation of the preceding NS2A deletion data is that a single domain near the C terminus of NS2A is required for NS1-NS2A cleavage. To investigate this possibility, four internal in-frame deletion mutants of pGEMNS1-NS2A were constructed: ΔES, ΔBB, ΔBM, and ΔMA (Fig. 6A). These deletions range in size from 27 to 87 aa, and together they remove almost all of NS2A. Transcripts made from these mutants were translated in vitro, and the membrane-associated products were analyzed (Fig. 6B). The supernatant fractions were also analyzed, and they had a similar, though less intense, pattern of bands (data not shown). All four mutants (lanes 2, 3, 6, and 7) were completely defective for NS1-NS2A cleavage, as shown by the absence of the product bands ΔNS2A (expected size range from 14.5 to 21 kDa) and NS1. Thus, most (if not all) of NS2A is required for NS1-NS2A cleavage. However, it cannot be excluded that deletions which do not directly remove important sequences nonetheless affect cleavage by altering the conformation of the whole molecule.

Another possible interpretation of the foregoing deletion data is that NS2A must provide some minimal length of amino acids beyond the NS1-NS2A junction, but the actual sequence of NS2A is not important. To test this possibility, NS2A sequences in pGEMNS1-NS2A were replaced with portions of NS4A and NS4B. Since NS2A is predominantly hydrophobic, the chosen regions of NS4A and NS4B were selected to approximately match the hydrophobicity profile of NS2A (data not shown). Two such constructs were made: pGEMNS1-ΔNS2A(31)-81%NS4A-NS4B$_{74}$ and pGEMNS1-ΔNS1(1)-81% NS4A-NS4B$_{74}$ (Fig. 6A). In the first construct, the NS4A and NS4B sequences were fused in frame after the N-terminal 31 aa of NS2A, while in the second construct only 1 aa of NS2A is retained. The longer construct thus has 227 aa beyond the NS1-NS2A junction, while the shorter construct has 197. For comparison, *Avr*II-truncated NS2A, which cleaves normally, has 199 residues beyond NS1. Transcripts from these substitution mutants were translated in vitro and compared with the wild type for NS1-NS2A cleavage (Fig. 6C). The only specific products made by these two mutants were their respective glycosylated precursors (lanes 2 and 3); there was no evidence for cleaved NS1 and 81%NS4A-NS4B$_{74}$, even with prolonged exposure of the gels. Thus, NS2A sequences are specifically required for NS1-NS2A cleavage and cannot be replaced by a similar length of hydrophobic residues.

Mutagenesis of potential protease catalytic site residues in NS2A. Given that NS2A is required for NS1-NS2A cleavage, an obvious possibility is that NS2A itself is an endoprotease. Comparison of the DEN4 NS2A amino acid sequence to protein sequences in GenBank failed to reveal significant homology to known proteases (data not shown). However, to pursue this possibility further, we decided to mutagenize all potential protease catalytic site residues in NS2A. Proteases can be sorted into four groups based on their catalytic mechanisms: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases (reviewed in reference 12). Protease catalytic sites contain characteristic amino acids which are involved in catalysis. Thus, serine proteases contain a catalytic triad of H, D, and S; cysteine proteases have a catalytic dyad of H and C; aspartic proteases utilize a dyad of D residues; and metalloproteases use conserved H and E residues. Recently, it has been suggested that signalases are an unusual type of serine protease, requiring conserved S and D residues but not requiring an H (3, 38). For any protease, mutagenesis of a catalytic site residue should eliminate protease activity. A set of nine mutant derivatives of pGEMNS1-NS2A were constructed, among which every C, D, E, H, and S residue in NS2A before the *Avr*II site is altered (Fig. 7A, set 1). To try to minimize any wholesale conformational effects, D residues were mutated to N, E residues were changed to Q, and C, H, and S residues were changed to A, except for one case in which S was changed to P.

Initially, the in vitro cleavage phenotypes of these nine mutants were compared with the wild type (Fig. 7B). Judging from the intensities of the precursor and product bands, the cleavage efficiencies of mutants a, b, c, e, and h (lanes 3, 4, 5, 7, and 10, respectively) were roughly the same as that of the wild type. Mutants f, g, and i (lanes 8, 9, and 11, respectively) were cleaved noticeably less efficiently than the wild type but still obviously made cleavage products. In contrast, mutant d was very defective for NS1-NS2A cleavage, producing almost no visible cleavage products (lane 6). Mutant d had eight amino acid changes from the wild type. To further define which of these mutations were responsible for the defect in NS1-NS2A



	Mutant	Cleavage	Amino acid substitutions
SET 1	a	+++	E1131Q, C1139A, E1145Q, E1146Q, C1147A, H1156A
	b	+++	C1166A, D1177N, D1187N, H1198A
	c	++++	E1224Q
	d	+/-	H1242A, D1243N, E1246Q, D1249N, C1305A, C1309A, H1315A, E1318Q
	e	+++	S1130A, S1134A, S1191A
	f	+	S1208A, S1222A
	g	+	S1239A, S1252A, D1265N, S1275A
	h	+++	S1308A, S1314A
	i	+	S1281P
SET 2	d.1234	+/-	H1242A, D1243N, E1246Q, D1249N
	d.5678	++++	C1305A, C1309A, H1351A, E1381Q
	d.1	++++	H1242A
	d.2	+++	D1243N
	d.3	++++	E1246Q
	d.4	++	D1249N
	d.234	+	D1243N, E1246Q, D1249N
	d.134	+	H1242A, E1246Q, D1249N
	d.124	++	H1242A, D1243N, D1249N
	d.123	+++	H1242A, D1243N, E1246Q

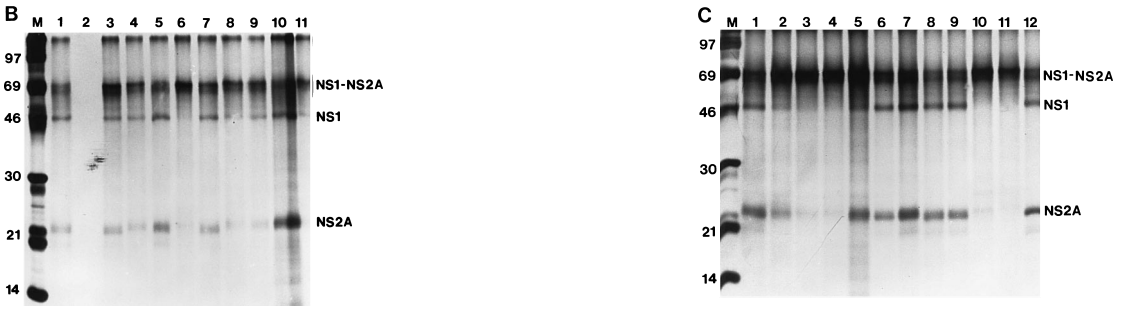


FIG. 7. Mutagenic analysis of potential protease catalytic site residues in NS2A. (A) A diagram of the extreme C terminus of NS1 and all of NS2A is shown at the top. Specific restriction sites used in the construction of the mutants are indicated. Tick marks occur in NS2A at 10-aa intervals, with residue numbers at 50-aa intervals. The positions of all C, D, E, H, and S residues in our NS2A sequence are indicated. Set 1, amino acid substitutions introduced into the first set of nine mutants. Set 2, amino acid substitutions introduced into the second set of 10 mutants. The amino acid substitution designations list the wild-type amino acid first, then the residue number, and then the amino acid present in the mutant: for example, in mutant f the S residue at aa 1208 is changed to A, as is the S residue at aa 1222. The extent of cleavage of these mutants (estimated by inspection of the gels) is also listed, on a scale with wild-type cleavage designated as ++++. Each mutant was analyzed multiple times, at least two independent transcription reaction mixtures were in vitro translated at least twice each, and qualitatively similar results were obtained. (B) RNA transcripts prepared from wild-type or set 1 mutant templates were translated in vitro, and the membrane-associated products were analyzed by SDS-PAGE. Lanes: 1, pGEMNS1-NS2A; 2, no RNA; 3 to 11, mutants a to i, respectively. The positions of NS1-NS2A, NS1, and NS2A are indicated on the right, and the sizes (in kilodaltons) of the markers in lane M are indicated on the left. (C) RNA transcripts from wild-type or set 2 mutant templates were translated and analyzed as described above for set 1 mutants. Lanes: 1, d.123; 2, d.124; 3, d.134; 4, d.234; 5, d.4; 6, d.3; 7, d.2; 8, d.1; 9, pGEMNS1-NS2A; 10, d; 11, d.1234; 12, d.5678; M, marker proteins, with sizes (in kilodaltons) indicated on the left).

cleavage, another set of 10 mutants was created, each of which contains a subset of the changes in mutant d (Fig. 7A, set 2). The phenotypes of these mutants were compared with those of mutant d and the wild type (Fig. 7C). Mutants d.1234 and d.5678 each contain half of the mutations present in mutant d; mutant d.5678 cleaved normally (lane 12), while mutant d.1234 was defective (lane 11). Thus, the cleavage defect is due to some subset of the four mutations present in mutant d.1234. Each of these four changes was present singly in the series of mutants d.1 to d.4, while the series d.234 to d.123 represented the four possible mutants with three of these four changes. Of the single mutants, d.1, d.2, and d.3 were cleaved like the wild type (lanes 8, 7, and 6), while d.4 was perhaps cleaved slightly less efficiently than wild type (lane 5). The fact that no single amino acid change eliminated NS1-NS2A cleavage suggests that NS2A is not a protease, at least not one that uses any of the previously characterized catalytic mechanisms. In the case of the triple mutants, d.123 was cleaved nearly normally (lane 1), d.124 was cleaved somewhat inefficiently (lane 2), and d.134 and d.234 were cleaved poorly. Evidently, mutation of the D residue at aa 1249 (D-1249) coupled with mutation of E-1246 (both are mutated in d.134 and d.234 but not in d.123 or d.124) led to the defect in cleavage. It is possible that an additional mutation at either H-1242 or D-1243 is also required for the defective phenotype. These multiple mutations may alter the gross conformation of NS2A, resulting in defective NS1-NS2A cleavage.

Fractionation of microsomal membranes. Other than NS2A and the 8-aa cleavage recognition site at the C terminus of NS1, no viral sequences are required for NS1-NS2A cleavage. If NS2A is not a protease, then the cleavage would have to be made by an ER-resident host enzyme. The amino acid se-

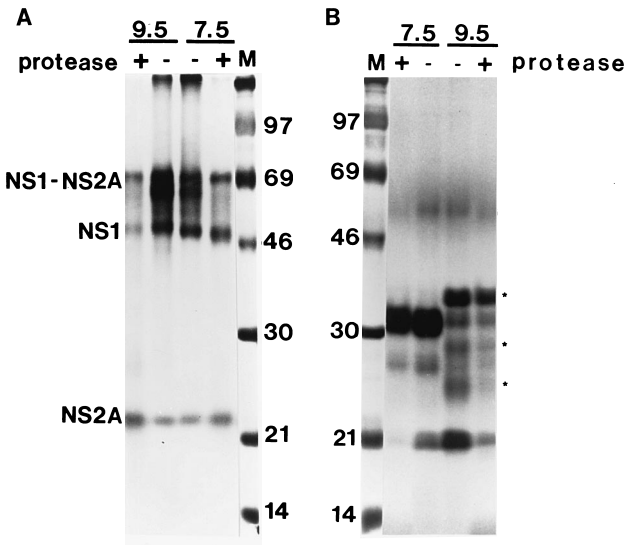


FIG. 8. Fractionation of microsomal membranes. Microsomal membranes were diluted into pH 9.5 buffer, which destroys their closed vesicular structure and allows the lumenal contents to leak out, and then resealed by centrifugation through neutral sucrose (31). Control membranes were diluted into pH 7.5 buffer, which does not affect their structure, and then centrifuged through neutral sucrose. Treated membranes were used for in vitro translation of pGEMNS1-NS2A RNA (A) or yeast α-factor RNA (B), and the products were then digested with thermolysin (+) or mock digested (−) and analyzed by SDS-PAGE. The positions of NS1-NS2A, NS1, and NS2A are shown in panel A. The asterisks in panel B denote species referred to in the text where the N-linked glycans have not been trimmed by glucosidase II. Lanes M, marker proteins, with sizes (in kilodaltons) indicated.

quence at the NS1-NS2A junction fits the −3,−1 rule for cleavage by signalase, but there is no associated signal sequence, which would normally be required to target a signalase-sensitive site for cleavage. Signalase is a membrane-bound, ER-resident enzyme (23). To determine if the putative ER-resident host enzyme responsible for NS1-NS2A cleavage is membrane-bound, a membrane fractionation experiment was performed. Dog pancreas microsomes were diluted into pH 9.5 buffer to disrupt their closed vesicular structure and then resealed by centrifugation through neutral sucrose (31). Nicchita et al. demonstrated that this procedure removes most of the lumenal contents of the microsomes but retains the membrane-bound proteins. Control membranes were diluted into pH 7.5 buffer, which does not affect their structure. Resealed vesicles were then used for in vitro translation of NS1-NS2A RNA (Fig. 8A). Clearly, the pH 9.5 pretreatment of the microsomes did not impair their ability to effect NS1-NS2A cleavage. The protease resistance of glycosylated NS1-NS2A and NS1 indicated that the microsomes had been resealed. Protease treatment in the presence of detergent completely digested NS1-NS2A and NS1, as before (data not shown). Digestion with PNG F confirmed that the larger NS1-NS2A band and NS1 were glycosylated (data not shown).

As a control for the removal of lumenal contents, these membranes were also used to in vitro translate yeast α-factor RNA (Fig. 8B). The enzyme glucosidase II, which trims the terminal glucose residues of high-mannose N-linked glycans, is a lumenal ER-resident enzyme that is removed by the above-described fractionation procedure (31). The α-factor has three N-linked glycosylation sites, and the size of each attached N-linked glycan should be larger in the absence of glucosidase II. When control (pH 7.5 pretreated) membranes were used, the predominant α-factor product had an M_r of about 31,000 as expected for the triply glycosylated protein. There was also a small amount of a protein at about 28 kDa, which is assigned as doubly glycosylated protein, and at 21 kDa, the position of unglycosylated α-factor in these gels. Only the glycosylated species were resistant to protease. In contrast, when pH 9.5 pretreated membranes were used, the pattern was quite different. The most abundant glycosylated product (top*) was slightly larger than 31 kDa, as expected for an untrimmed, triply glycosylated α-factor. There was some trimmed, triply glycosylated protein as well, indicating that not all of the glucosidase II was removed by the fractionation procedure. Similarly, there was untrimmed doubly glycosylated protein (middle*) and significantly less that was trimmed, and there was apparently some untrimmed singly glycosylated protein (bottom*) as well. There was also a significant amount of unglycosylated protein. The pH 9.5 treatment affected the overall efficiency of glycosylation, as a reduced proportion of α-factor was triply glycosylated compared with that achieved by using control membranes. As before, the glycosylated species were more protease resistant than the unglycosylated protein. Treatment with PNG F converted all the larger bands to 21 kDa, confirming their identification as glycosylated species (data not shown). The pH 9.5 treatment reduced the trimming activity of glucosidase II by about 80%, judging from the relative intensities of trimmed and untrimmed bands. It is therefore likely that 80% of other lumenal proteins were also removed. Since this treatment did not affect the NS1-NS2A cleavage activity, the data are consistent with a membrane-bound localization for a putative host protease that cleaves NS1-NS2A.

DISCUSSION

Flavivirus polyprotein processing is effected by a combination of viral and host proteases. It has previously been shown that all of the primary cleavages, with one exception, are performed by either the viral NS2B-NS3 protease or by the host enzyme signalase (8). The one exception is the NS1-NS2A cleavage. The amino acid sequence at the NS1-NS2A cleavage site is known to fit the −3,−1 rule for signalase cleavage but lacks a signal sequence (37). Previous work in vivo with vaccinia virus recombinants showed that NS1-NS2A cleavage occurs in the absence of other viral proteins, requires an 8-aa sequence at the C terminus of NS1 as the cleavage recognition site, possibly requires a signal sequence, and requires sequences near the C terminus of NS2A in *cis* which are 150 aa downstream of the cleavage site (13, 14, 18). In this study, we demonstrated that NS1-NS2A cleavage in vivo does not require any part of NS1 other than the C-terminal 8 aa but does require a signal sequence. Cleavage in vivo was not blocked by BFA, suggesting that it occurs early in the exocytic pathway. NS1-NS2A processing occurred in vitro in the presence of dog pancreas microsomes, suggesting that cleavage in vivo occurs in the ER. In vitro, the NS1-NS2A precursor is targeted to the microsomal membranes, presumably via the N-terminal signal sequence, and is first N glycosylated and then cleaved to produce glycosylated NS1 and unglycosylated NS2A. The glycosylated precursor and both products are resistant to protease and are thus localized inside the membranes. The NS1-NS2A precursor and NS2A behave as integral membrane proteins in a carbonate buffer membrane fractionation, and they are presumably anchored in the membrane by the hydrophobic NS2A sequences. In contrast, NS1 behaves as if it is not an integral membrane protein, which is consistent with previous data that monomeric NS1 is not at all membrane associated, while dimeric NS1 behaves as a peripheral membrane protein (42). Truncation of NS2A blocks cleavage, demonstrating that C-terminal NS2A sequences are required for NS1-NS2A cleavage in vitro, just as in vivo. The requirement for NS2A se-

quences was further analyzed with four in-frame deletion mutations, and no large region of NS2A was found to be dispensable for cleavage.

The foregoing results suggest two possible models for the role of NS2A in NS1-NS2A processing: (i) NS2A is a *cis*-acting protease that cleaves itself from NS1, or (ii) NS2A is required in some fashion to permit an ER-resident host protease to cleave NS1-NS2A. To address the first possibility, all potential standard protease active site residues in NS2A were mutagenized. No single mutant was drastically different from the wild type for NS1-NS2A cleavage, indicating that NS2A is not a standard type of protease. These data do not rule out the possibility that NS2A is a nonstandard protease that utilizes a novel catalytic mechanism that does not require any C, D, E, H, or S residues. One such example of a nonstandard protease was recently identified in the proteasome of *Thermoplasma acidophilum*, where the catalytic mechanism used by the β subunit involves the side chain hydroxyl group of the N-terminal T apparently acting in concert with the free amino group of the same residue plus perhaps the side chain amino group of an internal K (36). There are 20 T (and 5 K) residues in NS2A that could conceivably be involved in a similar proteolytic mechanism, but the catalytic T could not be at the N terminus as it is in the proteasome. Another example of a nonstandard protease is the 16-aa 2A protein of foot-and-mouth disease virus, which cleaves at its own C terminus by an entirely unknown mechanism (34, 35). While the involvement of a catalytic T residue in NS1-NS2A cleavage could in principle be tested by making more mutants, it is not as simple to test unknown mechanisms through mutagenesis experiments.

It is most likely that NS2A plays an indirect role to allow an ER-resident host protease to cleave NS1-NS2A. Recently, a study of DEN2 NS1 expression in *Spodoptera frugiperda* cells using a baculovirus vector found that a construct encoding NS1 plus only 26 aa of NS2A appeared to be partially cleaved at the NS1-NS2A junction (21). If this cleavage in insect cells truly represents authentic NS1-NS2A processing, it would eliminate the possibility that NS2A is a nonstandard protease with novel active center chemistry and would strongly suggest that NS1-NS2A processing is performed by a host enzyme. Evidently, the insect cell enzyme would have less of a requirement for NS2A sequences to effect NS1-NS2A cleavage than the mammalian counterpart. Fractionation of the microsomal membranes suggests that the putative host protease is membrane bound. Given that the NS1-NS2A cleavage site residues fit the −3,−1 rule for signalase cleavage, it is possible that NS1-NS2A processing is done by signalase, which is known to be a membrane-bound resident of the ER. If NS1-NS2A cleavage is a signalase-processing event it would be unique, since there is no signal sequence present immediately upstream of the cleavage site to target the site for cleavage. During normal signalase processing, the signal sequence enters the ER membrane and becomes anchored with its C-terminal end in the lumen, such that the cleavage site at the C terminus of the signal is localized near the intralumenal face of the ER membrane, where the signalase active site is thought to be located (reviewed in references 11 and 29). Perhaps NS2A somehow fulfills the role, normally carried out by a signal sequence, of presenting the cleavage site to signalase for processing. Such a model would suggest that the NS1-NS2A junction is completely translocated into the ER concomitant with translation and membrane insertion of NS2A and that NS2A then adopts a conformation which allows presentation of the NS1-NS2A cleavage site to signalase.

Another way in which NS1-NS2A processing differs from ordinary signalase cleavage relates to the cleavage recognition site. DEN4 NS1-NS2A cleavage apparently uses an 8-aa cleavage recognition site (positions −8 to −1, MVKSQVTA) and site-directed mutagenesis experiments have demonstrated that the amino acids at −8, −7, −5, −3, and −1 are important for cleavage (32). The typical signal sequence of 15 to 25 aa has a tripartite structure consisting of a positively charged N-terminal domain of 1 to 5 residues, a central hydrophobic core of 7 to 15 residues, and a more polar C-terminal region of 3 to 7 residues (for recent reviews, see references 11, 29, and 40). The α-helical hydrophobic core is often separated from the C-terminal region by a helix-breaking residue. Statistical and mutagenic analyses of typical signal sequences have not revealed any specific sequence requirements for signalase cleavage other than the need for small neutral or hydrophobic residues within the C-terminal region at positions −3 and −1 with respect to cleavage (the −3,−1 rule). Some mutations in the N-terminal region and hydrophobic core can affect cleavage, but this is thought to occur by alteration of the positioning of the signal in the membrane which reduces the exposure of the cleavage site on the lumenal side of the membrane. In addition, other studies have demonstrated that a pentapeptide analogous to residues −3 to +2 could be cleaved, albeit inefficiently, by purified signalase in vitro, which rules out an obligate requirement for any sequences beyond −3. Thus, the sequence requirements at −1 and −3 for NS1-NS2A processing fit the −3,−1 rule, but the required conserved residues at −5, −7, and −8 for NS1-NS2A cleavage has no analog in typical signalase cleavage. It is possible that these residues are required for some other reason, perhaps to interact with NS2A.

It will be difficult, if not impossible, to genetically distinguish between the two possibilities that NS2A is a novel protease or that NS2A is a helper for a host enzyme, possibly signalase, since any mutation in NS2A which affected cleavage would support either model. Currently, there are no specific chemical inhibitors of signalase, so it has not been possible to find out if inhibition of signalase blocks NS1-NS2A cleavage. With the recent report of the ability to simulate translocation into the ER and signalase cleavage by using purified proteins and phospholipids (17), it may now be possible to directly address this question by reconstituting artificial membranes with and without signalase and then testing whether NS1-NS2A cleavage can occur in this artificial in vitro system in the presence and absence of signalase. Alternatively, if NS1-NS2A processing can occur in reconstituted proteoliposomes derived from yeast microsomal proteins (6), it may be possible to test whether signalase function is required by using proteins derived from *sec*11 yeast cells, which are deficient in signalase activity (4, 44).

ACKNOWLEDGMENTS

We thank Randolph Clark for technical assistance, Richard Houghton for peptide synthesis, Jeff Smith and Mike Klutch for oligonucleotide synthesis, Roger Miller for help with the NS2A sequence homology search, and Steve Feinstone for providing the T7 promoter primer. We also thank Rosaura Valle, Phil Krause, and Dennis Trent for critical review of the manuscript.

Many of the experiments with recombinant vaccinia viruses were performed while we were members of the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

1. **Amberg, S. M., A. Nestorowicz, D. M. McCourt, and C. M. Rice.** 1994. NS2B-3 mediated processing in the yellow fever virus structural region: in vitro and in vivo studies. J. Virol. **68:**3794–3802.
2. **Arias, C. F., F. Preugschat, and J. H. Strauss.** 1993. Dengue virus NS2B and

NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology **193:**888–899.
3. **Black, M. T., J. G. Munn, and A. E. Allsop.** 1992. On the catalytic mechanism of prokaryotic leader peptidase 1. Biochem. J. **282:**539–543.
4. **Böhni, P. C., R. J. Deshaies, and R. W. Schekman.** 1988. *SEC*11 is required for signal peptide processing and yeast cell growth. J. Cell Biol. **106:**1035–1042.
5. **Bray, M., and C.-J. Lai.** 1991. Dengue virus premembrane and membrane proteins elicit a protective immune response. Virology **185:**505–508.
6. **Brodsky, J. L., S. Hamamoto, D. Feldheim, and R. W. Schekman.** 1993. Reconstitution of protein translocation from solubilized yeast membranes reveals topologically distinct roles for BiP and cytosolic hsc70. J. Cell Biol. **120:**95–102.
7. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5:**3403–3409.
8. **Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice.** 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44:**649–688.
9. **Chambers, T. J., D. W. McCourt, and C. M. Rice.** 1989. Yellow fever virus proteins NS2A, NS2B, and NS4B: identification and partial N-terminal amino acid sequence analysis. Virology **169:**100–109.
10. **Chambers, T. J., D. W. McCourt, and C. M. Rice.** 1990. Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. Virology **177:**159–174.
11. **Dalbey, R. E., and G. von Heijne.** 1992. Signal peptidases in prokaryotes and eukaryotes—a novel protease family. Trends Biochem. Sci. **17:**474–478.
12. **Dougherty, W. G., and B. L. Semler.** 1993. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. Microbiol. Rev. **57:**781–822.
13. **Falgout, B., R. Chanock, and C.-J. Lai.** 1989. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J. Virol. **63:**1852–1860.
14. **Falgout, B., and C.-J. Lai.** 1990. Synthesis of dengue virus nonstructural protein NS1 requires the N-terminal signal sequence and the downstream nonstructural protein NS2A, p. 192–195. *In* M. A. Brinton and F. X. Heinz (ed.), New aspects of positive-strand RNA viruses. American Society for Microbiology, Washington, D.C.
15. **Falgout, B., M. Pethel, Y.-M. Zhang, and C.-J. Lai.** 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J. Virol. **65:**2467–2475.
16. **Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow.** 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. **93:**97–102.
17. **Görlich, D., and T. A. Rapoport.** 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell **75:**615–630.
18. **Hori, H., and C.-J. Lai.** 1990. Cleavage of dengue virus NS1-NS2A requires an octapeptide sequence at the C terminus of NS1. J. Virol. **64:**4573–4577.
19. **Jan L.-R., C.-S. Yang, D. W. Trent, B. Falgout, and C.-J. Lai.** 1995. Processing of Japanese encephalitis virus non-structural proteins: NS2B-NS3 complex and heterologous proteases. J. Gen. Virol. **76:**573–580.
20. **Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz.** 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. J. Cell Biol. **116:**1071–1080.
21. **Leblois, H., and P. R. Young.** 1995. Maturation of the dengue-2 virus NS1 protein in insect cells: effects of downstream NS2A sequences on baculovirus-expressed gene constructs. J. Gen. Virol. **76:**979–984.
22. **Lin, C., S. M. Amberg, T. J. Chambers, and C. M. Rice.** 1993. Cleavage of a novel site in the NS4A region by the yellow fever virus NS2B-NS3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site. J. Virol. **67:**2327–2335.
23. **Lively, M. O., and K. A. Walsh.** 1983. Hen oviduct signal peptidase is an integral membrane protein. J. Biol. Chem. **258:**9488–9495.
24. **Lobigs, M.** 1993. Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc. Natl. Acad. Sci. USA **90:**6218–6222.
25. **Mackow, E., Y. Makino, B. Zhao, Y.-M. Zhang, L. Markoff, A. Buckler-White, R. Chanock, and C.-J. Lai.** 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. Virology **159:**217–228.
26. **Maloy, W. L., J. E. Coligan, and Y. Paterson.** 1991. Production of antipeptide sera, p. 9.4.1–9.4.11. *In* J. E. Coligan, A. M. Kruisbeck, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley & Sons, New York.
27. **Markoff, L., A. Chang, and B. Falgout.** 1994. Processing of flavivirus structural glycoproteins: stable membrane insertion of premembrane requires the envelope signal peptide. Virology **204:**526–540.
28. **Mason, P. W.** 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology **169:**354–364.
29. **Müller, M.** 1992. Proteolysis in protein import and export: signal peptide processing in eu- and prokaryotes. Experientia **48:**118–129.
30. **Nestorowicz, A., T. J. Chambers, and C. M. Rice.** 1994. Mutagenesis of the yellow fever virus NS2A/2B cleavage site: effects on proteolytic processing, viral replication, and evidence for alternative processing of the NS2A protein. Virology **199:**114–123.
31. **Nicchitta, C. V., and G. Blobel.** 1993. Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. Cell **73:**989–998.
32. **Pethel, M., B. Falgout, and C.-J. Lai.** 1992. Mutational analysis of the octapeptide sequence motif at the NS1-NS2A cleavage junction of dengue type 4 virus. J. Virol. **65:**4749–4758.
33. **Randolph, V. B., G. Winkler, and V. Stollar.** 1990. Acidotropic amines inhibit proteolytic processing of flavivirus prM protein. Virology **174:**450–458.
34. **Ryan, M. D., and J. Drew.** 1994. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO J. **13:**928–933.
35. **Ryan, M. D., A. M. Q. King, and G. P. Thomas.** 1991. Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. J. Gen. Virol. **72:**2727–2732.
36. **Seemüller, E., A. Lupas, D. Stock, J. Löwe, R. Huber, and W. Baumeister.** 1995. Proteasome from *Thermoplasma acidophilum*: a threonine protease. Science **268:**579–582.
37. **Speight, G., G. Coia, M. D. Parker, and E. G. Westaway.** 1988. Gene mapping and positive identification of the non-structural proteins NS2A, NS2B, NS3, NS4B and NS5 of the flavivirus Kunjin and their cleavage sites. J. Gen. Virol. **69:**23–34.
38. **Sung, M., and R. E. Dalbey.** 1992. Identification of the potential active-site residues in the *Escherichia coli* leader peptidase. J. Biol. Chem. **267:**13154–13159.
39. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
40. **von Heijne, G.** 1990. The signal peptide. J. Membr. Biol. **115:**195–201.
41. **Walter, P., and G. Blobel.** 1983. Preparation of microsomal membranes for cotranslational protein translocation. Methods Enzymol. **96:**84–93.
42. **Winkler, G., S. E. Maxwell, C. Ruemmler, and V. Stollar.** 1989. Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. Virology **171:**302–305.
43. **Wright, P. J., M. R. Cauchi, and M. L. Ng.** 1989. Definition of the carboxy termini of the three glycoproteins specified by dengue virus type 2. Virology **171:**61–67.
44. **YaDeau, J. T., C. Klein, and G. Blobel.** 1991. Yeast signal peptidase contains a glycoprotein and the *Sec*11 gene product. Proc. Natl. Acad. Sci. USA **88:**517–521.
45. **Yamshchikov, V. F., and R. W. Compans.** 1993. Regulation of the late events in flavivirus protein processing and maturation. Virology **192:**38–51.
46. **Yamshchikov, V. F., and R. W. Compans.** 1994. Processing of the intracellular form of the West Nile virus capsid protein by the viral NS2B-NS3 protease: an in vitro study. J. Virol. **68:**5765–5771.
47. **Zhao, B., E. Mackow, A. Buckler-White, L. Markoff, R. M. Chanock, C.-J. Lai, and Y. Makino.** 1986. Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology **155:**77–88.