JOURNAL OF VIROLOGY, Apr. 1993, p. 2034–2042
0022-538X/93/042034-09$02.00/0

Vol. 67, No. 4

Deletion Analysis of Dengue Virus Type 4 Nonstructural Protein NS2B: Identification of a Domain Required for NS2B-NS3 Protease Activity

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Received 8 October 1992/Accepted 4 January 1993

Most proteolytic cleavages in the nonstructural protein (NS) region of the flavivirus polyprotein are effected by a virus-encoded protease composed of two viral proteins, NS2B and NS3. The N-terminal 180-amino-acid-region of NS3 includes sequences with homology to the active sites of serine proteases, and there is evidence that this portion of NS3 can mediate proteolytic cleavages. In contrast, nothing is known about required sequences in NS2B. We constructed a series of deletion mutations in the NS2B portion of plasmid pTM/NS2B-30%NS3, which expresses dengue virus type 4 (DEN4) cDNA encoding NS2B and the N-terminal 184 residues of NS3 from the T7 RNA polymerase promoter. Mutant or wild-type plasmids were transfected into cells that had been infected with a recombinant vaccinia virus expressing T7 RNA polymerase, and the protease activities of the expressed polyproteins were assayed by examining the extent of self-cleavage at the NS2B-NS3 junction. The results identify a 40-amino-acid segment of NS2B (DEN4 amino acids 1396 to 1435) essential for protease activity. A hydrophobicity profile of DEN4 NS2B predicts this segment constitutes a hydrophilic domain surrounded by hydrophobic regions. Hydrophobicity profiles of the NS2B proteins of other flaviviruses show similar patterns. Amino acid sequence alignment of this domain of DEN4 NS2B with comparable regions of other proteins of flaviviruses indicates significant sequence conservation, especially at the N-terminal end. These observations suggest that the central hydrophilic domain of NS2B of these other flaviviruses will also prove to be essential for protease activity.

The *Flaviviridae* are a family of about 70 viruses which have a positive-strand RNA genome of 10 to 11 kb. Nucleotide sequence analysis has revealed that this RNA contains a single open reading frame spanning more than 95% of the genome (see the references in reference 11). Flavivirus gene expression involves co- and posttranslational proteolytic processing of the large polyprotein translated from genomic RNA. The mature gene products include the three viral structural proteins, the capsid (C), membrane (M), and envelope (E) proteins, and seven nonstructural (NS) proteins. The order of individual proteins in the polyprotein has been determined as NH_2-anchC-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH, where anchC and prM are precursors to C and M, respectively (6, 20, 27).

Processing the polyprotein requires both host and viral proteases. The endoplasmic reticulum enzyme signalase is responsible for the anchC-prM, prM-E, and E-NS1 cleavages and probably the NS4A-NS4B cleavage (6, 18, 20, 27, 29, 31). Cleavage at the NS1-NS2A junction requires the eight C-terminal amino acids of NS1, most of NS2A, and a signal sequence for entry into the exocytic pathway, but the protease has not been identified (10, 15). Similarly, the protease used for the secondary cleavage that converts prM to M is not known, although this processing event is known to occur at a late stage of virus assembly in an acidified vesicular compartment (26). The remaining primary cleavages at the NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5 junctions, and probably the secondary cleavage that converts anchC to C, are effected by a virus-encoded protease activity (for a review, see reference 28). Studies conducted by several other laboratories as well as ours have established that this protease activity requires two viral NS proteins, NS2B and NS3, and it has been suggested that they form a complex (2, 4, 5, 11, 24, 25, 33). Computer alignment of flavivirus NS3 sequences with those of known proteases demonstrated that the N-terminal third of NS3 contains limited similarity to serine proteases (1, 14). Four separate similar regions were identified, three of which include a member of the proposed catalytic triad of Asp, His, and Ser, while the fourth was proposed as a substrate binding domain (1). Mutagenesis experiments have shown that the three proposed catalytic residues are essential for enzymatic activity (5, 33).

In contrast to the current state of knowledge about NS3, little is known about the sequences in NS2B required for protease activity. The only information to date comes from the analysis of chimeric NS2B proteins where the N- and C-terminal portions of NS2B were from different flaviviruses (23). This study showed that protease activity was greatly diminished when NS3 and the C-terminal half of NS2B were mismatched. In the current study, deletion mutations were introduced into the NS2B portion of plasmid pTM/NS2B-30%NS3, a plasmid encoding NS2B, plus 30% of the NS3 protein of dengue virus type 4 (DEN4) under control of the T7 promoter. The resulting plasmids were then introduced into cells that had been infected with a recombinant vaccinia virus expressing T7 polymerase (13), and the effects of the mutations on protease activity were assessed by monitoring cleavage at the NS2B-NS3 junction in the expressed proteins. The results of these analyses identify an essential 40-amino-acid (aa) domain of NS2B.

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TABLE 1. Oligonucleotides used to introduce restriction sites into NS2B

Oligo	Sequence[a]
3077	ATAGTTGGATCCTACTTTCGAAAAATGTCCTCATC
3085	GGGATCCCTCGAGGAATTCAT
3088	CATATGGCTAGCTCTTGGCCTCTTAACGAGGGCATAATGGCTGTAGGCCTGGTTAGTCTC
3089	CCAAGAGCTAGCCATATGGGATCTGGGG
3090	GCTCTTCTTAAGAATGATGTCCCTTTAGCTGGGCCCATGGTGGCAGGAGGC
3091	ATCATTCTTAAGAAGAGCGCTTCCTAAGAGACT
3092	AACCCTACTAGTGAAACTGGCACTGATAACA
3093	AGTTTCACTAGTAGGGTTATCATATTGGTTTCCTCGACGTCGCGAATGGAGAAAGAGCC
3094	AATTCCGGTCACCATGACCTTATGGTACATGTGG
3095	GGTCATGGTGACCGGAATTGCCAAGGGGTAGAG
D154	CACCATGGGCCCAGCTAAAGGGACATCATTCTTAAGAAGAGC
D229	CGAACCCGGGTT
D247	CCAAGAGCTAGCGCTCATGAGGGATCTGGGGGATC

[a] Nucleotides underlined once are restriction sites referred to in the text, and nucleotides underlined twice are base changes introduced into NS2B.

MATERIALS AND METHODS

Cells and viruses. CV-1 cells were grown as monolayers in Eagle's minimal essential medium containing 10% fetal bovine serum in a humidified 37°C incubator. The recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase, has been described elsewhere (13).

Recombinant DNA. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs or Bethesda Research Laboratories. Polymerase chain reaction (PCR) was performed for 30 cycles, with each cycle consisting of 20 s at 94°C, 20 s at 55°C, and 1 min at 72°C. The oligonucleotides used are listed in Tables 1 and 2. The D series of oligonucleotides were made on an Applied Biosystems model 351 synthesizer. Dideoxynucleotide sequencing was performed with the Sequenase 2.0 kit (United States Biochemical) and [α-^{35}S]dATP (>1,000 Ci/mmol; Amersham).

Introduction of restriction sites into NS2B. Nucleotide substitutions were introduced into NS2B to create unique restriction sites without altering the encoded amino acids. The new restriction sites and corresponding base changes are as follows, with the nucleotide number (17) listed as a subscript after the old base and before the new base: *Nhe*I, insertion of GCTAGC immediately upstream of T_{4131}; *Stu*I, $G_{4163}A$, $T_{4166}C$, and $T_{4167}C$; *Afl*II, $T_{4194}C$ and $A_{4196}T$; *Apa*I, $C_{4220}G$ and $A_{4223}C$; *Nru*I, $A_{4388}T$ and $G_{4391}C$; *Spe*I, $T_{4421}A$, $T_{4422}C$, and $G_{4424}A$; and *Bst*EII, $A_{4475}G$ and $A_{4481}C$. In addition, there is a naturally occurring *Bgl*II site at nucleotides 4277 to 4282. Four iterations of PCR were done to create these mutations, and the oligonucleotides used are listed in Table 1. Briefly, plasmid pSC11/NS2B-NS3 (11) was used as the template in one PCR with oligonucleotide 3085 (oligo 3085) and oligo 3089 as primers and in another PCR with oligo 3077 and oligo 3088. The flanking oligos 3085 and 3077 contain sequences around the unique *Xho*I site in pSC11 and the unique *Bst*BI site in NS3, respectively. The PCR products were digested with *Nhe*I and ligated together, and the 1.3-kb ligation product was gel purified. This material was used directly as template for another pair of PCRs, using oligos 3085 and 3091 or oligos 3077 and 3090. The products were digested with *Afl*II, ligated together, and gel purified, and the process was repeated with oligos 3085 and 3095 or oligos 3077 and 3094. These PCR products were digested with *Bst*EII, ligated, and purified, and the final pair of reactions was done with oligos 3085 and 3093 or oligos 3077 and 3092. The final PCR products were digested with *Spe*I, ligated together, and then digested with *Xho*I and *Bst*BI. The 1.3-kb fragment was ligated to the 8.8-kb *Xho*I-*Bst*BI fragment of pSC11/NS3 (11), creating pSC11/NS2B-NS3RE*.

The nucleotide sequence of the amplified portion of pSC11/NS2B-NS3RE* was determined, as was the analogous region of pSC11/NS2B-NS3. This analysis demonstrated that the expected novel restriction sites were present in pSC11/NS2B-NS3RE*, as were three unexpected mutations, which were presumably PCR errors: $A_{4200}G$, ΔC_{4231}, and $\Delta CG_{4397-4398}$. Both clones differed from the published sequence (17) at six positions: $C_{4151}T$, $G_{4721}A$, $A_{4925}T$, and $T_{4931}C$ (all silent mutations); $A_{4827}G$ (Arg to Gly); and $A_{4860}G$ (Ser to Gly). All three PCR errors were within oligonucleotide sequences and thus could be simply repaired by two more rounds of PCR and cloning as follows. (i) PCR was done with pSC11/NS2B-NS3RE* as the template and oligos 3090 and 3093 as primers, and the *Apa*I-*Spe*I-digested PCR product was used to replace the defective *Apa*I-*Spe*I fragment; (ii) PCR was done on this new DNA by using oligos D154 and 3085, and the *Apa*I-*Xho*I-digested product was used to replace the defective *Apa*I-*Xho*I fragment, creating pSC11/NS2B-NS3RE. The nucleotide sequence from the 5′ end of NS2B to the *Spe*I site was determined for this clone, proving that the PCR errors had been eliminated.

Cloning into the pTM1 expression vector. PCR was done by using pSC11/NS2B-NS3 as the template and oligos 3085 and D247 as primers. The *Xho*I-*Nhe*I-digested PCR product was used to replace the analogous 0.4-kb *Xho*I-*Nhe*I fragment of pSC11/NS2B-NS3RE, resulting in the introduction of a unique *Bsp*HI site, TCATGA, at the 5′ end of NS2B, where the underlined ATG codon encodes the initiating methionine. Then, an unique *Sma*I site was introduced beyond the protease domain of NS3 by cloning oligo D229 into the *Bst*BI site. The 0.9-kb *Bsp*HI-*Sma*I fragment was cloned into *Nco*I-*Stu*I-digested pTM1 (19), creating plasmid pTM/NS2B-30%NS3 (Fig. 1). In this construct, a T7 RNA polymerase promoter drives expression of a transcript in which the initiating ATG codon of NS2B immediately follows the encephalomyocarditis virus 5′ untranslated region, which permits cap-independent translation (9). This transcript is predicted to encode a polyprotein consisting of four residues before the N terminus of NS2B (Met-Ser-Ala-Ser), followed by all of NS2B plus the N-terminal 184 aa of NS3 (DEN4 aa 1344 to 1657), and two extra residues at the C terminus (Thr-Pro).

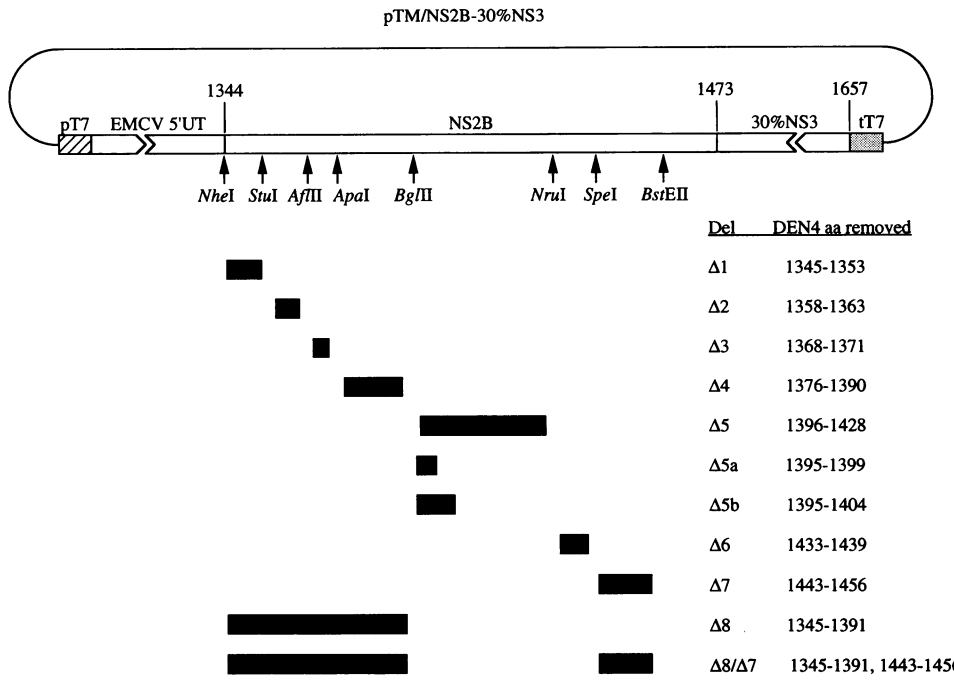

FIG. 1. NS2B deletions. At the top is the structure of expression vector pTM/NS2B-30%NS3, with the T7 RNA polymerase promoter (pT7), encephalomyocarditis virus 5′ untranslated region (EMCV 5′UT), DEN4 NS2B (aa 1344 to 1473), 30%NS3 (aa 1474 to 1657), and T7 RNA polymerase terminator (tT7) indicated. The positions of the restriction sites used in the construction of the deletions are shown underneath NS2B. Below, the solid boxes represent sequences removed in the deletion mutants (Del) indicated to the right.

Construction of deletion mutants. The first set of NS2B deletions, Δ1 to Δ7, were introduced into pTM/NS2B-30%NS3 by using pairs of oligonucleotides to bridge between neighboring pairs of restriction sites (Fig. 1 and Table 2). Digestion of pTM/NS2B-30%NS3 with *Nhe*I and *Stu*I followed by ligation with oligos D230 plus D231 created Δ1. Similarly, Δ2 was created by cloning oligos D232 and D233 between the *Stu*I and *Afl*II sites, Δ3 was created by cloning D234 and D235 between *Afl*II and *Apa*I, Δ4 was created by cloning D308 and D309 between *Apa*I and *Bgl*II, Δ5 was created by cloning D238 and D239 between *Bgl*II and *Nru*I, Δ6 was created by cloning D240 and D241 between *Nru*I and *Spe*I, and Δ7 was created by cloning D242 and D243 between *Spe*I and *Bst*EII. Partial digestions were done for *Apa*I and *Nru*I because of the presence of sites in the pTM1 vector. Also, in order to cleave the *Stu*I site, the pTM/NS2B-30%NS3 template DNA had to be prepared on a *dcm* mutant *Escherichia coli* strain, such as JM110. The double mutant Δ8/Δ7 was made by cloning D531 and D532 into *Nhe*I-*Bgl*II-digested Δ7. The single mutant Δ8 was made by joining the 1.3-kb *Bgl*II-*Xba*I fragment of Δ8/Δ7 to the 4.8-kb *Bgl*II-*Xba*I fragment of pTM/NS2B-30%NS3.

The remaining NS2B deletions were created by PCR. Two small deletions near the *Bgl*II site were made, using pSC11/NS2B-NS3RE as the template, with oligos 3083 and 3077 or 3084 and 3077 as primers (Table 2). Each PCR product was digested with *Bgl*II and *Bst*EII and the 0.2-kb fragments were used to replace the analogous fragment of Δ7, creating Δ5a or Δ5b (Fig. 1). Four shorter versions of Δ6 were made using pTM/NS2B-30%NS3 as the template and oligo 3090 as one primer, with oligo D512, D533, D513, or D514 (Table 2) as the other primer. Each PCR product was digested with *Spe*I and *Bgl*II, and the 0.1-kb fragment was used to replace the small *Spe*I-*Bgl*II fragment of Δ5, thus creating Δ6a, Δ6b, Δ6c, or Δ6d. Five longer versions of Δ4 were made, using Δ4 as the template and oligo D482 as one primer (corresponding to DEN4 nucleotides 4559 to 4539), with oligo D506, D507, D535, D508, or D509 (Table 2) as the other primer. The products were digested with *Nco*I and *Bst*EII, and each 0.2-kb fragment was joined to the 6.0-kb *Nco*I-*Bst*EII fragment of Δ5, creating Δ4a, Δ4b, Δ4c, Δ4d, or Δ4e. The amplified regions of these 11 mutants were sequenced, confirming the structure of all but one of the deletions. Δ4c had an extra C residue immediately after its *Bst*EII site. This frameshift mutation was repaired by replacing the large *Nco*I-*Bst*EII fragment with the analogous fragment of Δ6d. Thus, Δ4c carries an additional deletion of aa 1439 not carried by the other Δ4 deletions.

The pTM/NS2A-NS2B clone used in complementation studies was derived from pSC11/NS2A-NS2B (11) by Hiroshi Kawano.

Analysis of NS2B-NS3 cleavage. CV-1 cells growing in a 35-mm-diameter dish were infected with approximately 5 PFU of vTF7-3 per cell in 0.5 ml of medium, 30 min later the virus inoculum was replaced with 2 ml of fresh medium, and 0.5 ml of calcium phosphate-precipitated plasmid DNA (3 to 5 μg) was added. The cells were incubated at 37°C for 2 h, or until there was noticeable cell rounding, at which point they were rinsed and fed with fresh medium and incubated for 16 to 20 h at 37°C. The cells were starved for 30 to 60 min with 2 ml of methionine-free medium containing 124 mM NaCl (22). The cells were then labeled for 2 h in 0.5 ml of the same medium containing 50 to 100 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham). Cells were lysed in situ with 0.5 ml of RIPA buffer (10), or they were lysed with 0.2 ml of 0.5% sodium dodecyl sulfate (SDS) and then 0.8 ml of 1.25× RIPA buffer without SDS was added. Fractions (50 to 200 μl) were immunoprecipitated with 1-μl portions of DEN4-specific hyperimmune mouse ascitic fluid (HMAF; purchased from

TABLE 2. Oligonucleotides used to make deletions

Oligo	Sequence[a]	Deletion
Bridging		
D230	CTAGCTCTGTAGG	Δ1 (*Nhe*I-*Stu*I)
D231	CCTACAGAG	
D232	CCTGGTTCTTC	Δ2 (*Stu*I-*Afl*II)
D233	TTAAGAAGAACCAGG	
D234	TTAAGAATGCTGGGCC	Δ3 (*Afl*II-*Apa*I)
D235	CAGCATTC	
D308	CATGTCAGCA	Δ4 (*Apa*I-*Bgl*II)
D309	GATCTGCTGACATGGGCC	
D238	GATCTGTCAATTCG	Δ5 (*Bgl*II-*Nru*I)
D239	CGAATTGACA	
D240	CGACGTCCTA	Δ6 (*Nru*I-*Spe*I)
D241	CTAGTAGGACGTCG	
D242	CTAGTGATTCCG	Δ7 (*Spe*I-*Bst*EII)
D243	GTGACCGGAATCA	
D531	CTAGCTCTGCA	Δ8 (*Nhe*I-*Bgl*II)
D532	GATCTGCAGAG	
PCR		
D482	AGTGGCAGCGGGTGAGGGGAC	Δ4a-Δ4e
D506	GACCGGCCATGGTA GATCTGTCACTAGAGAAGGCC	Δ4a
D507	GACCGGCCATGGTA TCACTAGAGAAGGCCGCCAAT	Δ4b
D535	GACCGGCCATGGTA CTAGAGAAGGCCGCCAATGTG	Δ4c
D508	GACCGGCCATGGTA GAGAAGGCCGCCAATGTGCAG	Δ4d
D509	GACCGGCCATGGTA GCCGCCAATGTCCAGTGGGAT	Δ4e
3083	GTCACTAGATCTC GCCAATGTGCAGTGGGATGAA	Δ5a
3084	CAATGTAGATCTC GATGAAATGGCAGACATAACA	Δ5b
D512	TCAAAGACTAGTAG TTCCTCGACGTCCCGTATGGA	Δ6a
D533	TCAAAGACTAGTAG GGTTTCCTCGACGTCCCGAAT	Δ6b
D513	TCAAAGACTAGTAG ATTGGTTTCCTCGACGTCCCG	Δ6c
D514	TCAAAGACTAGTAG TATCATATTGGTTTCCTCGAC	Δ6d

[a] The gaps shown for PCR oligonucleotides indicate the positions of the deletions.

the American Type Culture Collection) or with 10-μl portions of preimmune or immune NS3-specific rabbit serum raised against a TrpE-NS3 fusion protein expressed in *Escherichia coli*. Antibodies were collected onto Pansorbin beads, which were then washed twice with RIPA buffer or with RIPA buffer containing an additional 2% SDS, as noted in the figure legends. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described previously (10), except in Fig. 7, which was a tricine-buffered 16.5% polyacrylamide gel T and 3% C (29a). Fluorography was done as described previously (10).

RESULTS

Deletion analysis of NS2B. PCR was used to create seven new restriction sites in the NS2B portion of pTM/NS2B-30%NS3. Then, oligonucleotides were used to bridge neighboring pairs of sites, creating Δ1 to Δ7 (Fig. 1). These deletions remove between 4 and 33 amino acids of NS2B. The phenotypes of these deletion mutants were investigated by expressing the mutant and wild-type NS2B-30%NS3 polyproteins in vivo, via the vaccinia virus T7 polymerase system, and analyzing self-cleavage at the NS2B-NS3 junction (Fig. 2). As expected, wild-type NS2B-30%NS3 was predominantly cleaved, as seen from the intense signals of the cleavage products 30%NS3 and NS2B, and the weaker signal of the uncleaved NS2B-30%NS3 polyprotein. The cleavage phenotypes of Δ1 to Δ3 were similar to that of the wild type, except that the deletion mutant NS2B proteins migrated slightly faster than wild-type NS2B, as expected. Δ4 expressed less cleaved 30%NS3 and NS2B proteins than did the wild type, but uncleaved NS2B-30%NS3 polyprotein was undetectable; thus, judging from the precursor/product ratio, Δ4 appeared to cleave the NS2B-NS3 junction at least as efficiently as the wild type did. In other experiments, Δ4 had a pattern of expression very similar to that of the wild type (see Fig. 3). Δ7 was very mildly impaired for cleavage, as judged by a slight reduction in the levels of NS2B and 30%NS3 proteins and a slight increase in the level of NS2B-30%NS3 polyprotein, compared with the wild type. This mild defect in Δ7 is not likely due to the proximity of the deletion to the NS2B-NS3 cleavage site, since we have shown previously (11) that the 10 C-terminal amino acid residues of NS2B are sufficient for cleavage, and Δ7 leaves intact the 16 C-terminal residues. Δ6 had a severe processing

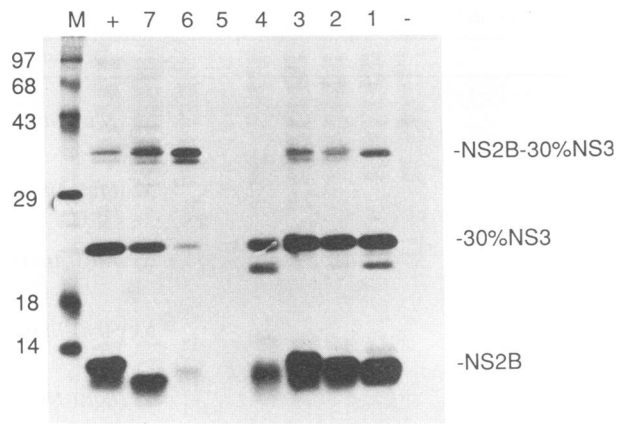

FIG. 2. Analysis of the cleavage phenotypes of Δ1 to Δ7. CV-1 cells were infected with vTF7-3 and transfected with plasmid DNA, and 16 to 20 h later, cells were labeled with ^{35}S for 2 h and lysates were prepared in RIPA buffer. Aliquots were immunoprecipitated with HMAF, and antibodies were collected onto Pansorbin beads, which were then washed with RIPA buffer plus 2% SDS, prior to SDS-PAGE and fluorography. Plasmid DNAs used for transfection: lane -, pTM1 (negative control); lane +, pTM/NS2B-30%NS3 (wild type); lanes 1 to 7, Δ1 to Δ7, respectively. Lane M contains molecular size markers, with the sizes (in kilodaltons) shown on the left. The positions of uncleaved NS2B-30%NS3 polyprotein and of cleaved 30%NS3 and NS2B proteins are indicated to the right.

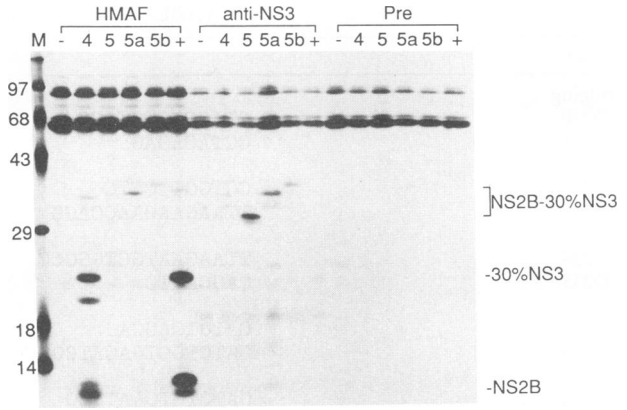

FIG. 3. Anti-NS3 sera identifies the Δ5 polyprotein. Radiolabeled cell lysates were prepared as described in the legend to Fig. 2, and fractions were immunoprecipitated either with HMAF or with preimmune (Pre) or immune (anti-NS3) NS3-specific anti-fusion protein rabbit serum. Pansorbin beads were washed in RIPA buffer and subjected to SDS-PAGE and fluorography. Plasmid DNAs used for transfection: lanes -, pTM1; lanes +, pTM/NS2B-30%NS3; lanes 4, Δ4; lanes 5, Δ5; lanes 5a, Δ5a; lanes 5b, Δ5b. Lane M contains molecular size markers, with the sizes (in kilodaltons) indicated to the left. The positions of NS2B-30%NS3, 30%NS3, and NS2B proteins are shown on the right.

defect, as shown by the greatly reduced levels of the cleaved products and the increased level of the uncleaved polyprotein. The total signal in the Δ6 lane (Fig. 2) was significantly less than that in the other lanes. This result could have been due to a reduced transfection efficiency for this DNA or to reduced stability or immunoprecipitability of the NS2B-30%NS3 polyprotein compared with the cleaved products. No specific products were detected in the Δ5 lane (but see below). It was interesting to note that NS2B-30%NS3 and NS2B proteins were doublets except in the Δ1 lane, with the band of lower mobility being more intense. This result agrees with our previous suggestion (11) that these species are doublets due to internal initiation of translation at the ninth residue of NS2B, because Δ1 removes this residue. There were also two faint bands migrating ahead of the 30%NS3 protein present in variable amounts in all but the Δ5 lane, most easily seen in the Δ1 and Δ4 lanes. We have not investigated the identities of these proteins. In addition, a faint band migrating slightly slower than NS2B was visible in many of the lanes. The mobility of this band seemed to correlate with the size of the deletion, suggesting that this protein resulted from a cleavage within NS3, just downstream of the normal cleavage site.

We were concerned by the failure to detect any products expressed by Δ5. One possible explanation was that the Δ5 deletion interfered with the ability of HMAF to immunoprecipitate the NS2B-30%NS3 polyprotein, presumably by altering the conformation of the molecule so that it was no longer recognized. To test this, we compared the proteins precipitated from a Δ5 lysate by HMAF to those precipitated by an NS3 fusion protein-specific antiserum (Fig. 3). The uncleaved Δ5 NS2B-30%NS3 polyprotein was readily detected by the anti-NS3 serum but was just barely detectable with HMAF. In contrast, the Δ4 and wild-type polyproteins were precipitated slightly more efficiently and the 30%NS3 products were precipitated much more efficiently by HMAF. These data suggest that the Δ5 polyprotein adopts a nonnative conformation, one not readily recognized by HMAF. These data also rule out the possibility that the Δ5 polyprotein degrades itself as a result of a greatly relaxed substrate specificity but raise the prospect that the lack of cleavage of Δ5 was not due to an effect of the deletion on the autoproteolytic activity but rather was due to inherent uncleavability of the misfolded precursor. To address this possibility, two experiments were performed. First, we attempted to complement Δ5 for cleavage by cotransfection with pTM/NS2A-NS2B (Fig. 4). In this experiment, the cells were lysed in 0.5% SDS, as this procedure was found to improve the ability of the NS3-specific antiserum to immunoprecipitate the 30%NS3 protein. The results showed that the Δ5 polyprotein was cleaved when NS2A-NS2B was provided in *trans* (Fig. 4, compare lane 2 with lane 6). Interestingly, not only did the cleaved 30%NS3 product appear upon complementation, but the intensity of the uncleaved polyprotein band also increased (see Discussion). In the second experiment, the deletion mutants Δ5a and Δ5b (Fig. 1) were analyzed. These small deletions overlap the left boundary of Δ5 and remove only 5 or 10 aa. The results (Fig. 3) showed that both Δ5a and Δ5b were defective for NS2B-NS3 cleavage and that the uncleaved polyproteins were detected by HMAF. We do not have an explanation for the aberrantly low mobility of the Δ5b polyprotein. These data suggest that NS2B sequences important for autoproteolytic function have been removed by these deletions, as well as by Δ5.

Fine mapping the essential domain of NS2B. The fact that the two neighboring mutants Δ5 and Δ6 were defective for cleavage suggested that these deletions entered into either side of a domain of NS2B essential for protease activity. We wanted to map the boundaries of this putative domain to the amino acid level. To map the right boundary, mutants Δ6a to Δ6d were constructed to remove progressively fewer amino acids than Δ6 removed (Fig. 5a). Analysis of the cleavage phenotypes of these mutants (Fig. 5b) showed that Δ6a was as defective for cleavage as Δ6 but that Δ6b, which removes

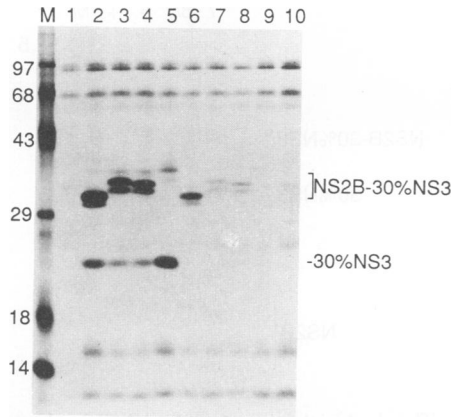

FIG. 4. Complementation of Δ4d, Δ4e, and Δ5. Radiolabeled cells were lysed in 0.5% SDS and adjusted to RIPA buffer, and then immunoprecipitated with preimmune (lane 1) or immune NS3-specific rabbit sera (lanes 2 to 10). Pansorbin beads were washed in RIPA buffer prior to SDS-PAGE and fluorography. DNAs used for transfection: lanes 1 and 4, Δ4d plus pTM/NS2A-NS2B; lane 2, Δ5 plus pTM/NS2A-NS2B; lane 3, Δ4e plus pTM/NS2A-NS2B; lane 5, pTM/NS2B-30%NS3; lane 6, Δ5; lane 7, Δ4e; lane 8, Δ4d; lane 9, pTM/NS2A-NS2B; lane 10, pTM1. Lane M contains molecular size markers, with the sizes (in kilodaltons) shown on the left. The positions of the NS2B-30%NS3 and 30%NS3 proteins are shown on the right.

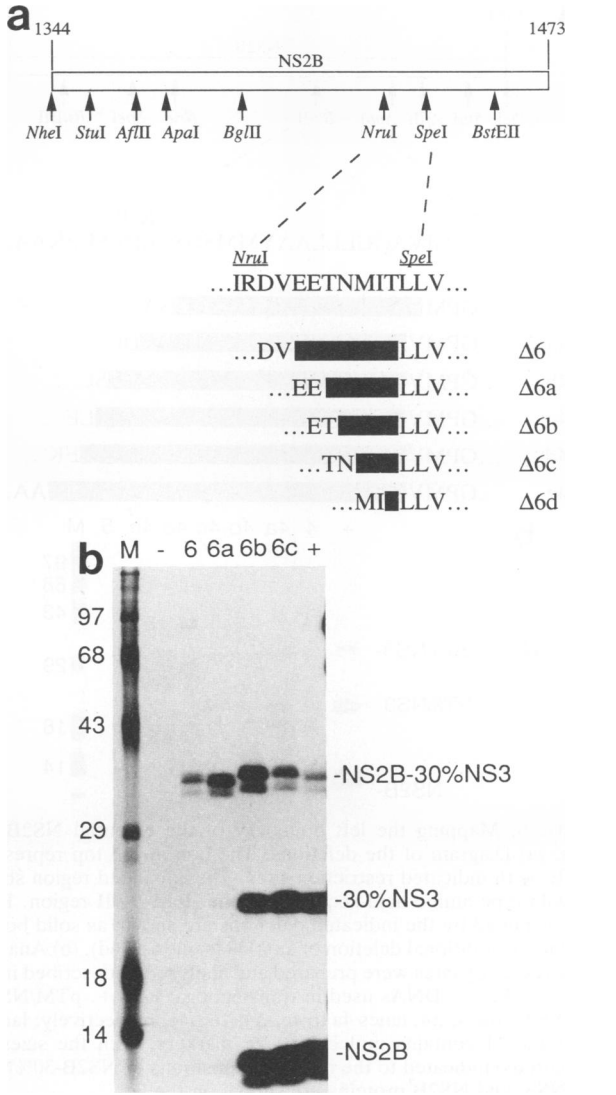

FIG. 5. Mapping the right boundary of the essential NS2B domain. (a) Diagram of the deletions. The box at the top depicts NS2B (aa 1344 to 1473), with various restriction sites indicated. The expanded region shows the wild-type amino acid sequence of the region bounded by the *Nru*I and *Spe*I sites. Residues removed by the indicated deletions are shown as solid boxes. (b) Analysis of cleavage. Radiolabeled lysates were prepared and analyzed as described in the legend to Fig. 2. DNAs used in transfections: lane -, pTM1; lane +, pTM/NS2B-30%NS3; lane 6, Δ6; lanes 6a to 6c, Δ6a to Δ6c, respectively. Lane M contains molecular size markers, with the sizes (in kilodaltons) indicated to the left. The positions of NS2B, 30%NS3, and NS2B-30%NS3 proteins are shown on the right.

1 aa less than Δ6a did, was similar to the wild type. Δ6c (Fig. 5b) and Δ6d (not shown) were also similar to the wild type. The results of this analysis mapped the right boundary to the Thr at DEN4 aa 1435. Similarly, to map the left boundary, mutants Δ4a to Δ4e were made (Fig. 6a). These deletions remove progressively more amino acids than Δ4. The analysis of these mutants (Fig. 6b) showed that mutants Δ4a to Δ4c cleaved the NS2B-NS3 junction as efficiently as Δ4 and the wild type did. On the other hand, the products of Δ4d and Δ4e were not detectable by HMAF. The uncleaved products of Δ4d and Δ4e were weakly detected with the NS3-specific antiserum (Fig. 4, lanes 7 and 8). In addition, Δ4d may have been slightly cleaved, since there is a faint 30%NS3 band in this lane. Upon complementation with pTM/NS2A-NS2B, both Δ4d and Δ4e were clearly cleaved to produce the 30%NS3 protein (Fig. 4, lanes 3 and 4). Thus, these two mutant polyproteins were misfolded and cleavage defective and could be complemented in *trans* by NS2A-NS2B. These data suggest that the left boundary of the essential domain of NS2B is the Leu residue at DEN4 aa 1396.

The data presented above suggested that the 40-aa domain of DEN4 NS2B from residues 1396 to 1435 might be the only region required for protease activity in *cis* other than the residues directly part of the cleavage site. To test this, we constructed deletion mutant Δ8 and the double deletion mutant Δ8/Δ7 (Fig. 1). Δ8 removes most of the NS2B residues upstream of the essential domain, while Δ8/Δ7 removes most of NS2B outside of the essential domain, leaving intact just 5 residues upstream and 24 residues downstream of this domain. The cleavage phenotypes of these mutants are analyzed in Fig. 7. The phenotype of Δ8 was similar to that of Δ4 (Fig. 2), in that the cleavage to produce 30%NS3 and shortened NS2B proteins appeared to be as efficient as that of the wild type, and an additional product was detected just below the 30%NS3 band. The double mutant Δ8/Δ7 was also cleaved, producing the expected 30%NS3 and even smaller NS2B proteins. Interestingly, the additional product seen with Δ8 was not detected with Δ8/Δ7. The identity of this product has not been investigated further. Judging from the ratio of NS2B to NS2B-30%NS3 in this and other experiments (and assuming that these species are equally immunoprecipitable), the cleavage efficiency of Δ8/Δ7 was similar to that of Δ7. This result suggests that little other than the identified 40-aa domain of NS2B is required for NS2B-NS3 self-cleavage.

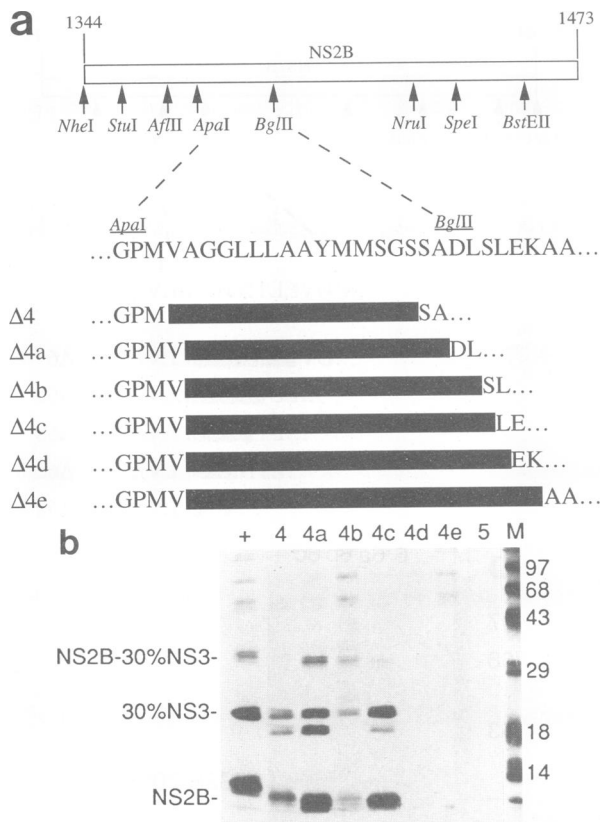

FIG. 6. Mapping the left boundary of the essential NS2B domain. (a) Diagram of the deletions. The box at the top represents NS2B, with indicated restriction sites. The expanded region shows the wild-type amino acid sequence of the *Apa*I-*Bgl*II region. Residues removed by the indicated deletions are shown as solid boxes. Δ4c has an additional deletion of aa 1439 (same as Δ6d). (b) Analysis of cleavage. Lysates were prepared and analyzed as described in the legend to Fig. 2. DNAs used in transfections: lane +, pTM/NS2B-30%NS3; lane 4, Δ4; lanes 4a to 4e, Δ4a to Δ4e, respectively; lane 5, Δ5. Lane M contains molecular size markers, with the sizes (in kilodaltons) indicated to the right. The positions of NS2B-30%NS3, 30%NS3, and NS2B proteins are shown on the left.

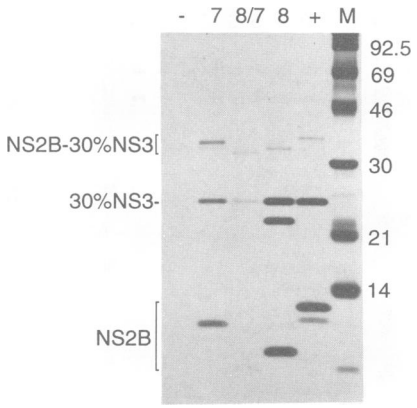

FIG. 7. The double deletion mutant Δ8/Δ7 is cleaved. Lysates were prepared and analyzed as described in the legend to Fig. 2, except that a tricine-buffered 16.5% polyacrylamide gel was used (29a). DNAs used in transfection: lane -, pTM1; lane 7, Δ7; lane 8/7, Δ8 Δ7 double mutant; lane 8, Δ8; lane +, pTM/NS2B-30%NS3. Lane M contains molecular size markers, with the sizes (in kilodaltons) indicated the right. The positions of NS2B-30%NS3, 30%NS3, and NS2B proteins are shown on the left.

DISCUSSION

The virus-encoded protease activity of flaviviruses requires two viral proteins, NS2B and NS3 (2, 4, 5, 11, 24, 25, 33). The amino-terminal domain of NS3 contains sequences with similarity to serine proteases, including a proposed catalytic triad of Asp, His, and Ser (1, 14), suggesting that the NS3 moiety contains the active site of the protease. In order to define regions of DEN4 NS2B important for protease function, we constructed a series of deletions in the NS2B portion of a construct expressing NS2B-30%NS3 polyprotein and analyzed the protease activities of these mutants by monitoring self-cleavage at the NS2B-NS3 site. Deletions which enter either side of a 40-aa region, DEN4 aa 1396 to 1435 (17), eliminated autoproteolytic activity. Some of the deletions upstream of this domain resulted in a large increase in the level of an unidentified alternate cleavage product and thus may have affected the site specificity of the cleavage. Defective mutants could be complemented by supplying NS2B in *trans* to restore cleavage. This result argues against the possibility that these mutant polyproteins are simply uncleavable. In addition, a double deletion mutant (Δ8/Δ7) encoding the identified domain but with most of the remainder of NS2B deleted was still able to cleave. This result suggests that this 40-aa domain is the only portion of NS2B required for cleavage in *cis* at the NS2B-NS3 junction, other than the residues directly involved in the cleavage site. We have not assayed for the ability of any of these NS2B deletion mutants to cleave a substrate in *trans*, and so it is possible that the domain we have identified affects only cleavage in *cis*. However, it has been previously shown that NS2B is required for protease activity in *trans* and in *cis* (2, 4, 11). Interpreted most simply, this suggests that the identified domain in NS2B is also required for the *trans*-acting proteolytic activity of NS2B-NS3.

A hydrophobicity profile of DEN4 NS2B (Fig. 8) reveals that the identified domain (shaded box) is a hydrophilic region surrounded by hydrophobic domains. The NS2B hydrophobicity profiles of nine other flaviviruses (not shown) have very similar overall patterns, with a central hydrophilic domain surrounded by hydrophobic regions. An alignment of the 40-aa hydrophilic domain of DEN4 NS2B with the corresponding regions of nine other mosquito-borne flaviviruses is presented in Fig. 9. Allowing for conservative substitutions, significant homology is evident, particularly near the amino-terminal end of the domain. This degree of homology is evidence that this region of NS2B is also required for protease activity in these other flaviviruses. Indeed, recent deletion mutagenesis experiments confirm this for yellow fever virus NS2B (3a). It is not known if the entire 40-aa domain is required for activity, or if only some of these residues are needed. Site-directed mutagenesis experiments are in progress to address this question.

Given that the active site appears to reside in NS3, how does NS2B affect the protease activity? It was previously suggested that NS2B forms a complex with NS3 (2, 4, 11, 33). It was further suggested that a consequence of association of NS2B and NS3 might be targeting of NS3 to the membrane (33). However, unequivocal evidence for membrane targeting is not yet available. We previously reported (11) that NS3 expressed by a recombinant vaccinia virus encoding NS2B-NS3 polyprotein was completely localized to membrane fractions, whereas NS3 protein expressed

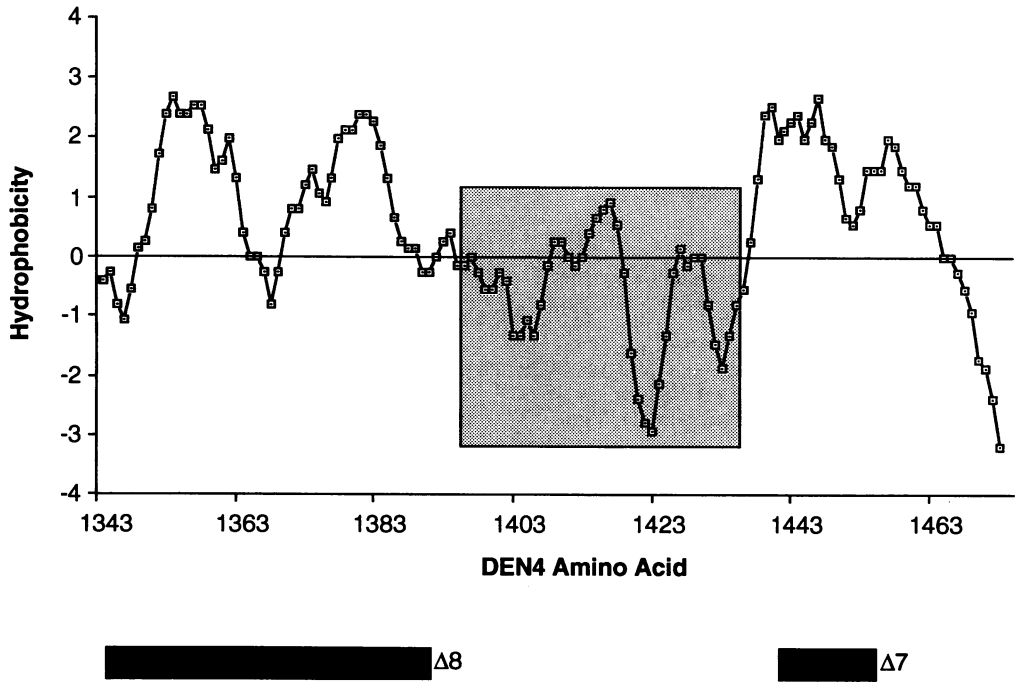

FIG. 8. Hydrophobicity profile of DEN4 NS2B. DEN4 NS2B (17) hydrophobicity was plotted by the method of Kyte and Doolittle (16). The shaded region represents the essential 40-aa domain identified by deletion analysis. The sequences removed by Δ8 and Δ7 are shown below the profile.

alone was 25% soluble. While this indicates that NS2B may have a role in assisting NS3 to the membrane, it also shows that NS3 is mostly membrane-bound in the absence of NS2B. Furthermore, membrane association of NS3 may not be necessary for protease activity, as protease activity has been observed in vitro in the absence of added membranes (5). Evidence for an interaction of NS2B and NS3 comes from complementation experiments such as those in Fig. 4, where NS2B apparently acted in *trans* to partially correct the cleavage defects of Δ4d, Δ4e, and Δ5. This evidence is consistent with an interaction between the wild-type NS2B of the NS2A-NS2B polyprotein and the wild-type 30%NS3 on the Δ4d, Δ4e or Δ5 polyprotein to produce an active protease. Another possible explanation of these results is homologous recombination between the two transfected DNAs to produce wild-type NS2B-30%NS3 polyprotein. However, it seems unlikely that homologous recombination would be efficient enough to produce as much complementation as was observed. Also, in previous experiments, the *trans* activities of NS2B and NS3 were observed under conditions where homologous recombination was not possible (2, 4). Furthermore, in Fig. 4 the amount of precipitated uncleaved mutant NS2B-30%NS3 polyprotein increased upon complementation with NS2A-NS2B polyprotein. While we cannot rule out trivial explanations, such as an increase in transfection efficiency of the mutant DNA upon cotransfection with NS2A-NS2B, this result seems to suggest an interaction of NS2B with NS3 that results in a change in conformation and/or stability of the precursor. Indeed, we have previously observed that both NS2B and NS3 proteins are better immunoprecipitated when coexpressed (11). Also, the fact that the Δ5 precursor is readily detected with NS3-specific anti-fusion protein antibodies but not by HMAF argues that NS3 is misfolded in the absence of functional NS2B. Recently, direct coimmunoprecipitation of NS2B and NS3 proteins has been observed in our laboratory using Japanese encephalitis virus (15a) and by Tom Chambers using yellow fever virus (3a). With yellow fever virus, it was further demonstrated that this association depends on the conserved hydrophilic domain of NS2B. Thus, available evidence suggests a model in which this conserved NS2B domain is required for interaction with the NS3 protein, and this interaction results in a conformational change in NS3, presumably activating the protease activity inherent in the NS3 moiety.

```
DEN4    LEKAAnVqWDemADITGSSpiIeVkqdEDGsfsIrdveet
DEN3    VEKAADVTWEeeAEqTGvShnLmItVdDDGtmrIkdDete
DEN2    LERAADVkWEdqAEISGSSpiLsItIsEDGsmsIknEeee
DEN1    LEKAAEVSWEeeAEhSGAShnIlVeVqDDGtmkIkdEerd
JEV     LERAADISWEmdAaITGSSrrLdVkLdDDGdfhLidDpgv
WNV     IERTADITWEsdAEITGSSerVdVrLdDDGnfqLmnDpga
KUN     IERTADISWEgdAEITGSSerVdVrLdDDGnfqLmnDpga
MVE     LERAADVSWEagAaITGTSerLdVqLdDDGdfhLlnDpgv
SLE     IEKAADITWEqnAEITGTSprLdVdLdshGnfkLlnDpga
YFV     LkKlGEVSWEeeAEISGSSarydVaLsEqGefkLlsEekv
                * *  * *      *
```

FIG. 9. Alignment of the DEN4 essential 40-aa NS2B domain with the corresponding regions of other mosquito-borne flaviviruses. The flavivirus amino acid sequences used for alignment (and their sources) were as follows: DEN4 (17), DEN3 (21), DEN2 (8), DEN1 (12), Japanese encephalitis virus (JEV) (30), West Nile virus (WNV) (3), Kunjin virus (KUN) (6), Murray Valley encephalitis virus (MVE) (7), Saint Louis encephalitis virus (SLE) (32), and yellow fever virus (YFV) (27). Invariant residues are indicated by asterisks. Uppercase type depicts conserved residues at positions where at least 8 of the 10 viruses have identical or conservatively substituted amino acids (e.g., I = L = V, D = E, A = G = S = T, and H = K = R). Lowercase type depicts nonconserved residues at conserved positions and all residues at positions where there is not an 8- of 10-residue consensus.

ACKNOWLEDGMENTS

We thank Bernard Moss for providing pTM1 and vTF7-3, Richard Wolf, Jr., for the gift of *E. coli* JM110, Myron Hill for preparation of some of the oligonucleotides, Annie Cahour for preparation of the NS3-specific antiserum, Tom Chambers for communicating results prior to publication, Terry Popkin for photographic assistance, and Todd Heishman for editorial assistance. Computer-assisted amino acid analysis was through the GenBank On-Line Service.

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