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Cleavage at a Novel Site in the NS4A Region by the Yellow Fever Virus NS2B-3 Proteinase Is a Prerequisite for Processing at the Downstream 4A/4B Signalase Site

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Flavivirus proteins are produced by co- and posttranslational proteolytic processing of a large polyprotein by both host- and virus-encoded proteinases. The viral serine proteinase, which consists of NS2B and NS3, is responsible for cleavage of at least four dibasic sites (2A/2B, 2B/3, 3/4A, and 4B/5) in the nonstructural region. Since the amino acid sequence preceding NS4B shares characteristics with signal peptides used for translocation of nascent polypeptides into the lumen of the endoplasmic reticulum, it has been proposed that cleavage at the 4A/4B site is mediated by a cellular signal peptidase. In this report, cell-free translation and in vivo transient expression assays were used to study processing in the NS4 region of the yellow fever virus polyprotein. With a construct which contained NS4B preceded by 17 residues constituting the putative signal peptide (sig4B), membrane-dependent cleavage at the 4A/4B site was demonstrated in vitro. Surprisingly, processing of NS4A-4B was not observed in cell-free translation studies, and in vivo expression of several yellow fever virus polyproteins revealed that the 4A/4B cleavage occurred only during coexpression of NS2B and the proteinase domain of NS3. Examination of mutant derivatives of the NS3 proteinase domain demonstrated that cleavage at the 4A/4B site correlated with expression of an active NS2B-3 proteinase. From these results, we propose a model in which the signalase cleavage generating the N terminus of NS4B requires a prior NS2B-3 proteinase-mediated cleavage at a novel site (called the 4A/2K site) which is conserved among flaviviruses and located 23 residues upstream of the signalase site. In support of this model, mutations at the 4A/4B signalase site did not eliminate processing in the NS4 region. In contrast, substitutions at the 4A/2K site, which were engineered to block NS2B-3 proteinase-mediated cleavage, eliminated signalase cleavage at the 4A/4B site. In addition, the size of the 3_{502}-4A product generated by *trans* processing of a truncated polyprotein, 3_{502}-5_{356}, was consistent with cleavage at the 4A/2K site rather than at the downstream 4A/4B signalase site.

Yellow fever virus (YF) is the prototype member of the *Flavivirus* genus of the family *Flaviviridae*, which also contains two additional genera, the pestiviruses and hepatitis C virus. Flaviviruses (for reviews, see references 4, 10, 36, and 46) are enveloped, positive-stranded RNA viruses, containing a single-stranded genomic RNA approximately 11 kb in length. Genome-length RNAs, which contain a single long open reading frame capable of encoding a polyprotein of approximately 350 kDa, are the only viral mRNAs found in infected cells. The flavivirus gene order has been established as 5′-anchC-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′. The three structural protein species are encoded in the N-terminal fourth of the polyprotein and include the capsid (C) protein (precursor, anchC), the membrane (M) protein (precursor, prM), and the envelope (E) protein. The remaining portion is processed to produce at least seven nonstructural proteins (NS1 through NS5), which are believed to be structural and enzymatic components of the viral replicase.

Flavivirus proteins are produced by several proteolytic processing events by a combination of virus- and host-encoded proteinases (for reviews, see references 10 and 37). The proteinase responsible for cleavage at the 1/2A site has not been identified, but a conserved sequence of eight residues at the C terminus of NS1 (19) as well as downstream NS2A sequences (14) is required for efficient cleavage. The amino acid residues surrounding the other four nonstructural cleavage sites (2A/2B, 2B/3, 3/4A, and 4B/5) are highly conserved and usually include a pair of basic residues (Arg or Lys) at the P2 and P1 positions and a Gly or Ser residue at the P1′ position of the scissile bond (10, 37). Cleavage at these dibasic sites is catalyzed by a virus-encoded serine proteinase activity which requires NS2B and NS3 (9, 13, 15, 33). The catalytic domain of this proteinase appears to lie within the N-terminal one-third of NS3 (181 residues of YF NS3) and has been defined by sequence alignment with known serine proteinases of the trypsin superfamily (2, 3, 18), by deletion analysis (13, 15, 33, 45), and by site-directed mutagenesis of the residues in the putative catalytic triad (YF polyprotein residues His-1537, Asp-1561, and Ser-1622) (13, 45) or the substrate-binding pocket (31). The hydrophobic amino acid segments preceding the N termini of prM, E, NS1, and NS4B are believed to act as signal sequences for translocation of these proteins into the lumen of the endoplasmic reticulum (ER). Experimental evidence has been obtained to support the host signalase-mediated cleavage, in association with the ER, at all of these sites except for the 4A/4B site (14, 25, 28, 38).

In this study, by using cell-free translation of in vitro-transcribed YF RNAs, evidence has been obtained to support the hypothesis that the N terminus of NS4B is generated by signalase-mediated cleavage. A microsomal membrane-dependent cleavage was observed for an NS4B construct containing 17 additional upstream residues (the putative signal peptide); however, NS4A-4B was not processed,

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suggesting that the hydrophobic residues located upstream of NS4B do not function efficiently as an internal signal sequence. Rather, cleavage at the 4A/4B site of several YF polyproteins was shown to be dependent upon coexpression of an active NS2B-3 proteinase. These results suggest that the signalase-mediated cleavage at the 4A/4B site may depend on prior cleavage at a novel site in the NS4A region (mediated by the NS2B-3 proteinase). Support for this hypothesis has been obtained by site-directed mutagenesis of a putative cleavage site located 23 residues upstream from the N terminus of NS4B and by further characterization of NS4A region cleavage products.

MATERIALS AND METHODS

Cell culture and virus growth. Growth of BHK-21 and BSC-40 cells and the infection of BHK-21 cells with the YF 17D strain have been described before (11). Four vaccinia virus recombinants were used in this study, including vTF7-3, which expresses the T7 DNA-dependent RNA polymerase (17), and vYF2B, vYF3_{181}, and vYF2B-3_{181}, which express the YF NS2B, 3_{181}, and 2B-3_{181} proteins, respectively (1) (see below and Fig. 1 for a description of the nomenclature for YF expression constructs). Large-scale preparations of these vaccinia virus recombinants were grown, and titers of infectious progeny were determined by plaque assay on BSC-40 cells.

Construction and nomenclature of expression plasmids. Standard recombinant DNA techniques (40) were used for construction of the expression plasmids described below and summarized in Fig. 1. For all plasmids, regions of the YF coding sequence amplified by the polymerase chain reaction (39) were verified by DNA sequence analysis (40). Ligation sites which could not be verified by restriction enzyme digestions were also sequenced.

For expression plasmids and the YF resulting protein products, the subscripts indicate the residue at which the particular protein starts or ends, and the NS prefix is not used for truncated products. Substitution mutations are named according to the position of the substituted residue in the full-length YF polyprotein (35). For instance, the mutation in which Arg is substituted with Glu at the P1 position of the 4B/5 site, which blocks cleavage at this site, is called R2506E.

Except as noted, expression constructs were derivatives of pET-BS(+), a phagemid derivative of pET-8c, where expression is under the control of the T7 promoter (44). pET-BS(+)-sig2A-5_{356} (called sig2A-5_{356}) and its derivative, pET-BS(+)-sig2A-5_{356}(R2107E/R2506E) (called sig2A-5_{356}**), have been described before (23). pET-BS(+)-sig2A-5_{356}(S1622A/R2107E/R2506E) (called sig2A-5_{356}***) was constructed by ligation of appropriate fragments from three sig2A-5_{356} constructs containing substitutions S1622A (9), R2107E, and R2506E (23). pET-BS(+)-3-5_{356}(S1622A/R2107E/R2506E) (called 3-5_{356}***) was constructed by a similar strategy, using pET-8c-3-5_{356}(S1622A) (called 3-5_{356}* [9]) instead of sig2A-5_{356}(S1622A). pET-BS(+)-3_{502}-5_{356} was constructed by deleting the sequences between the *Stu*I and *Xba*I sites of sig2A-5_{356} after the protruding ends were filled in with T4 DNA polymerase prior to ligation. This polyprotein initiates with the Met residue at position 502 of NS3.

For several other pET-BS(+) expression constructs, synthetic oligonucleotides and polymerase chain reaction were used to engineer initiation or termination codons as well as convenient restriction sites for subcloning (5′ *Nco*I and 3′ *Bam*HI sites). The sequence encompassing the N terminus of polyproteins initiating with NS4A (4A-5_{356} and NS4A-4B) included a 5′-flanking *Nco*I site containing the initiation codon (boldface) (5′-CC**ATG**GGA-3′) followed by the NS4A sequence (YF codon underlined). For polyproteins initiating with sig4B (sig4B-5_{356} and sig4B), the ATG in the 5′-flanking *Nco*I site is immediately followed by the codon for residue 2240 of the YF polyprotein (5′-CC**ATG**GTG-3′). Hence, this signal sequence consists of the engineered methionine residue followed by the 17 uncharged residues preceding NS4B. Polyproteins initiating with NS4B (4B-5_{356} and NS4B) contain an extra Ala residue betweenthe N-terminal Met and the NS4B sequence (5′-CC**ATG**GCCAAC-3′). Constructs containing a termination codonafter NS4B (NS4A-4B, sig4B, and NS4B) were producedby subcloning a fragment from pET-BS(+)-sig2A-5_{356}(G2507Amb), which contained a substitution creating an amber termination codon at the P1′ position of the 4B/5 cleavage site (24). For construction of plasmids expressing 3_{502}-$4A_{126}$ and 3_{502}-$4A_{149}$, two adjacent termination codons (TAA and TAG) and a 3′ *Bam*HI site were engineered after the codons for YF NS4A residues 126 and 149, respectively.

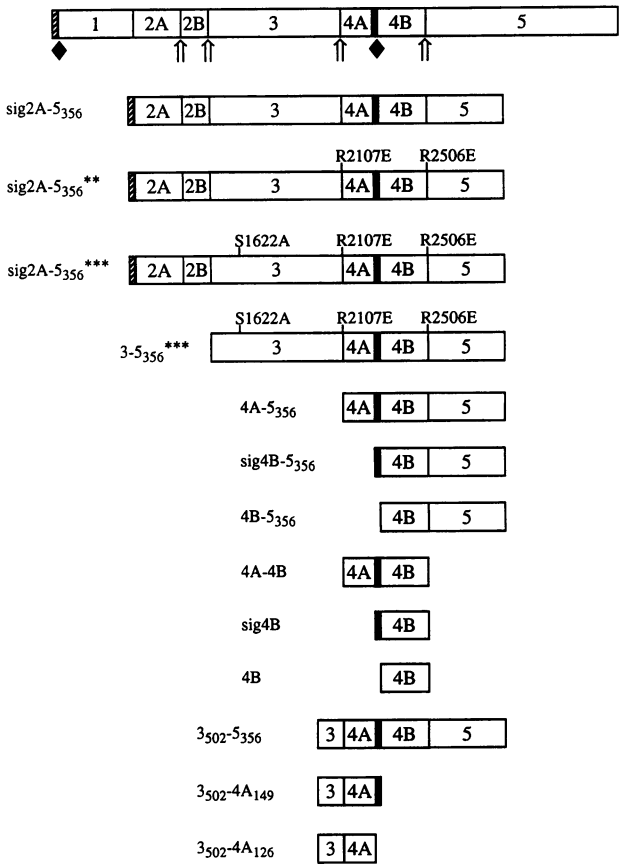


FIG. 1. YF expression constructs. Nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 are indicated by 1, 2A, 2B, 3, 4A, 4B, and 5, respectively. Symbols for cleavage sites: ◆, putative signalase cleavage sites; ⇑, dibasic sites cleaved by the NS2B-3 proteinase. The signal peptide (sig) in sig2A-5_{356}, indicated by the hatched box, is derived from the C-terminal 23 residues of E protein joined to the N-terminal 11 residues of NS1 (9). sig4B-5_{356} and sig4B have the same putative signal peptide, indicated by a black box, consisting of the 17 residues preceding the N terminus of NS4B (see Materials and Methods).

pET-BS(+)-3_{181} derivatives containing substitution H1537A, D1561N, or S1622C were generated by subcloning the appropriate fragments from pET-BS(+)-2B-3_{181} constructs containing the corresponding substitutions (13) into pET-8c-3_{181} (9). The plasmid expressing 3_{181}(S1622A) is a pTM3 derivative (27) and was produced by using the *Dra*III-*Bpu*1102I fragment from pET-BS(+)-2B-3_{181}(S1622A) (13) to replace the corresponding region in pTM3-3_{181} (8).

Site-directed mutagenesis. Single-stranded uridylated template DNA from sig2A-5_{356} was prepared and mutagenesis procedures were carried out as described before (21, 23). For substitutions at YF polyprotein residue 2234 (Ser), the 186-bp *Eco*RV-*Sph*I fragment containing the mutagenized region was subcloned into sig2A-5_{356}. Substitution mutations were identified by sequence analysis, and the sequence of the entire 186-bp region was verified.

Transient-expression assay. Subconfluent monolayers of BHK-21 cells in 35-mm dishes (approximately 10^6 cells) were infected with vTF7-3 for 30 min at 37°C at a multiplicity of infection of 10 PFU per cell in 0.2 ml of minimal essential medium (MEM). For cells coinfected with two or three vaccinia virus recombinants, the multiplicity of infection of each recombinant was 10 PFU per cell. After removal of the inoculum, cells were transfected for 2.5 h at 37°C with a mixture consisting of 1 μg of plasmid DNA and 10 μg of Lipofectin (Bethesda Research Laboratories) in 0.5 ml of MEM. The transfection mixture was then replaced by 1 ml of MEM containing 1/40th the normal concentration of methionine, 2% fetal bovine serum, and 20 μCi of [^{35}S]Translabel (ICN) per ml and incubated for 4 h at 37°C. When two plasmids were used in the same transfection, the amount of each plasmid was 1 μg and the labeling time was 5 h. Authentic YF virus proteins were prepared from YF virus-infected BHK-21 cells which were radiolabeled with [^{35}S] Translabel, beginning at 24 h postinfection, with labeling conditions identical to those used for the transient-expression assays. After labeling, the cell monolayers were washed twice with MEM and lysed with 0.3 ml of sodium dodecyl sulfate (SDS) lysis buffer consisting of 0.5% SDS, 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 20 μg of phenylmethylsulfonyl fluoride per ml (11).

Cell-free translation, proteinase K protection, and N-terminal sequence analysis. 5′-Capped RNA transcripts were synthesized from linearized cDNA templates with the T7 DNA-dependent RNA polymerase (Epicenter) (34). Cell-free translation mixes with rabbit reticulocyte lysates (Promega) and [^{35}S]methionine ([^{35}S]Met; Amersham) were incubated for 1 h at 30°C essentially according to the manufacturer's instructions. Canine pancreatic microsomal membranes (Promega) were added to the translation reaction mixes as indicated. The translation reactions were terminated by addition of RNase A (Boehringer Mannheim) to 10 μg/ml and incubation for 15 min at 30°C. Before immunoprecipitation, the translation reaction mixes were diluted 10-fold with the SDS lysis buffer described above and heated at 70°C for 10 min prior to addition of YF NS4B-specific antiserum (see below).

Protection of translation products by microsomal membranes was examined by digestion with proteinase K. Following translation, reaction mixes (after incubation with RNase A) were adjusted to 0.5 mg of proteinase K (Boehringer Mannheim) per ml and 10 mM $CaCl_2$ and incubated for 30 min on ice. Proteinase K digestion was terminated by addition of phenylmethylsulfonyl fluoride to 1 mg/ml and continued incubation for 5 min on ice. For some samples, membranes were solubilized by the addition of Triton X-100 to 1% prior to proteinase K digestion. A portion of each reaction mix (generally 4 μl) was mixed with 40 μl of Laemmli sample buffer (22), heated at 95°C for 5 min, and analyzed by tricine-SDS-polyacrylamide gel electrophoresis (PAGE) (41) and fluorography with salicylic acid (7).

For N-terminal sequence analysis, sig4B transcripts were translated in the presence of [^{35}S]Met and saturating amounts of canine microsomal membranes (3.6 U/25-μl reaction mix). Translation products were immunoprecipitated with NS4B-specific antiserum (see below), separated by SDS-PAGE (22), transferred to Immobilon polyvinylidene difluoride membranes (26), and localized by autoradiography. Amino acid sequence analysis was performed essentially as described before (11).

Immunoprecipitation. YF specific proteins in cell lysates or in vitro translation reaction mixes were immunoprecipitated with YF region-specific antisera (11), and the immune complexes were collected by using *Staphylococcus aureus* Cowan strain I (Calbiochem) (11). Washed immunoprecipitates were solubilized and analyzed by SDS-PAGE (22) followed by fluorography with salicylic acid (7).

RESULTS

In vivo studies on NS4 region processing. Previous YF polyprotein processing studies suggested that cleavage at the 4A/4B site may require an active NS2B-3 proteinase (9). It was suggested either that prior cleavage at the 4B/5 site was necessary for cleavage at the 4A/4B signalase site or that interaction of the proteinase with the NS4 region might be required for signalase cleavage. In a previous study, a sig2A-5_{356} polyprotein containing two substitutions, R2107E and R2506E (called sig2A-5_{356}**), which completely abolish cleavage at the 3/4A and 4B/5 dibasic sites, was shown to be capable of processing at the 4A/4B site, as evidenced by the appearance of NS3-4A and 4B-5_{356} (23) (Fig. 2A, lanes DM). This result showed that prior cleavage at the 3/4A and 4B/5 dibasic sites was not necessary for processing at the 4A/4B site. To further characterize the requirements for cleavage at the 4A/4B site, processing of a derivative of sig2A-5_{356}** containing an additional substitution (S1622A) (called sig2A-5_{356}***) previously shown to abolish proteolytic activity of the YF NS2B-3 proteinase (13) was examined. Expression of sig2A-5_{356}*** alone yielded a large protein, presumably 2A-5_{356} (Fig. 2A, lanes TM), which was consistent with lack of processing at any of the nonstructural cleavage sites. However, cleavage at the 4A/4B site of this polyprotein was restored by coexpression with proteinase construct 2B-3_{181} (Fig. 2A).

To determine whether NS2B, 3_{181}, or both proteinase subunits are required for cleavage at the 4A/4B site, a 3-5_{356} polyprotein containing the same three mutations (S1622A, R2107E, and R2506E; called 3-5_{356}***) was used as the substrate in additional coexpression experiments. In the presence of 2B-3_{181}, cleavage at the 4A/4B site of 3-5_{356}*** was observed, as evidenced by the appearance of NS3-4A and 4B-5_{356} (Fig. 2B). When NS2B and 3_{181} were coexpressed with the substrate by using separate vaccinia virus recombinants, cleavage at the 4A/4B site also occurred, albeit with reduced efficiency (Fig. 2B). Coexpression with either NS2B or 3_{181} or with 2B-3_{181} (S1622A) failed to induce any processing of the 150-kDa 3-5_{356}*** polyprotein (Fig. 2B).

Previous studies of dengue virus type 4 (DEN4) suggest that the 4A/4B cleavage may have occurred during expression of DEN4 polyproteins 3-4A-84%4B and 4A-4B-5 (5, 15).

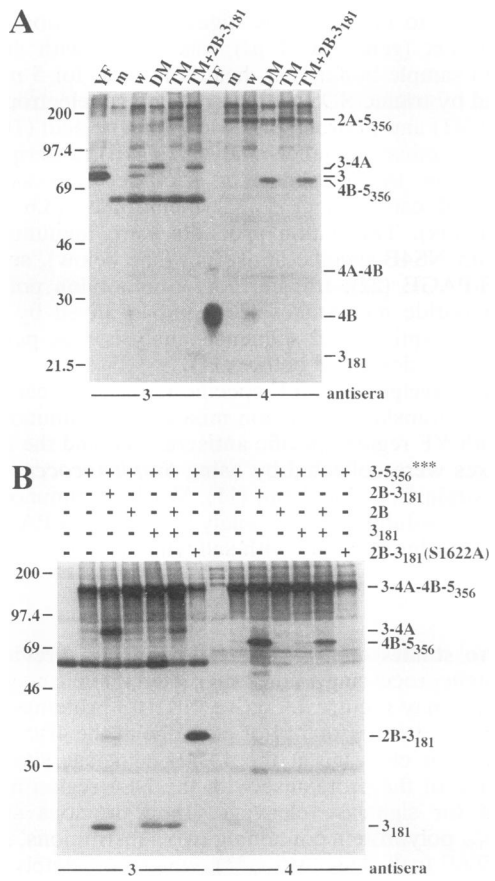


FIG. 2. Processing of polyproteins with blocked 3/4A and 4B/5 cleavage sites. Transient-expression experiments in vTF7-3-infected BHK-21 cells were conducted as described in Materials and Methods. NS3- and NS4B-specific products (as indicated by the numbers at the bottom of each panel) were analyzed by SDS–10% PAGE. (A) Plasmid DNA transfections included the parental sig2A-5_{356} (w), the double mutant with both the 3/4A and 4B/5 cleavage sites blocked, sig2A-5_{356}** (DM), the triple mutant sig2A-5_{356}*** (TM), and cotransfection with 2B-3_{181} (+2B-3_{181}). Control lysates were prepared from YF-infected cells (YF) or from vTF7-3-infected cells (m). It should be noted that a protein species migrating slightly faster than 2A-5_{356} precipitates nonspecifically with the NS4B antiserum. (B) Coexpression of various constructs was used to examine *trans* cleavage of the 3-5_{356}*** substrate. YF constructs 2B-3_{181}(S1622A) and 3-5_{356}*** were delivered by plasmid transfection, whereas vaccinia virus recombinants were used to express NS2B, 3_{181}, and 2B-3_{181}. The presence (+) or absence (−) of a given expression construct (shown at the right) is indicated above each lane. The identities of YF-specific polyproteins and cleavage products are indicated at the right. The molecular masses of ^{14}C-labeled protein standards are indicated at the left (in kilodaltons).

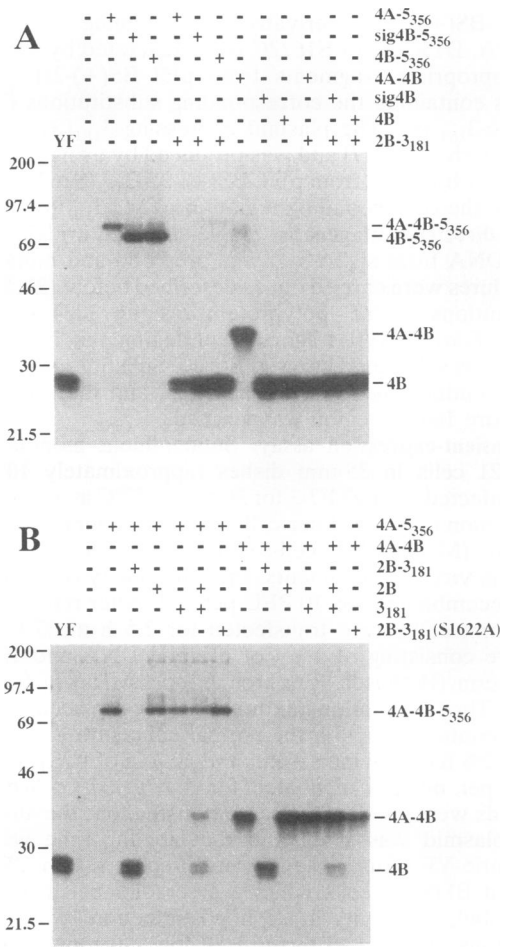


FIG. 3. *trans* processing in the NS4A-4B-5 region. Transient-expression experiments in vTF7-3-infected BHK-21 cells were conducted as described in Materials and Methods. NS4B-specific products were analyzed by SDS–10% PAGE. (A) As described for Fig. 2B, the indicated YF proteins or polyproteins were expressed by transfection of plasmid DNAs. A control lysate was prepared from YF-infected cells (YF). (B) Coexpression of various constructs was used to examine *trans* cleavage of the 4A-5_{356} and NS4A-4B substrates. YF constructs 2B-3_{181}(S1622A), 4A-5_{356}, and NS4A-4B were delivered by plasmid transfection, whereas vaccinia virus recombinants were used to express NS2B, 3_{181}, and 2B-3_{181}.

To clarify whether the YF NS3 or NS5 sequences might be exerting an inhibitory effect on the 4A/4B cleavage, we examined processing of several truncated substrates derived from the YF NS4A-4B-5 region. Expression of polyproteins initiating with the NS4A region, 4A-5_{356} and NS4A-4B, yielded only unprocessed polyproteins of 79 and 36 kDa, respectively (Fig. 3A). In the presence of 2B-3_{181}, cleavage in the NS4A-4B region was almost complete for both polyproteins (as well as at the 4B/5 site for 4A-5_{356}), as evidenced by the predominant NS4B doublet, which comigrated with NS4B produced in YF-infected cells. Expression of 2B-3_{181} allowed essentially complete cleavage at the 4B/5 site in two other polyproteins (sig4B-5_{356} and 4B-5_{356}) (Fig. 3A). In addition, the NS4B-specific products from sig4B and NS4B comigrated, suggesting that the signalase cleavage may have occurred in the sig4B protein (Fig. 3A, and see below).

Additional *trans*-processing experiments with NS2B and 3_{181} were conducted for 4A-5_{356} and NS4A-4B (Fig. 3B) and yielded similar results. Individual proteins NS2B or 3_{181} and the 2B-3_{181} (S1622A) polyprotein failed to induce detectable processing of either substrate. For both substrates, cleavage at the 4A/4B site occurred only in the presence of both NS2B and 3_{181}.

Processing at the 4A/4B site is dependent upon coexpression of an active NS2B-3 proteinase. These experimental results suggest that the NS2B-3 proteinase is somehow involved in the proposed signalase cleavage in the NS4 region. Alternatively, the proteinase-dependent cleavage at the 2B/3 site might be necessary to produce the mature form of NS2B and

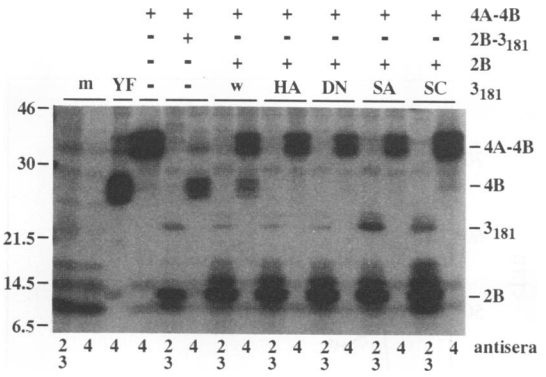


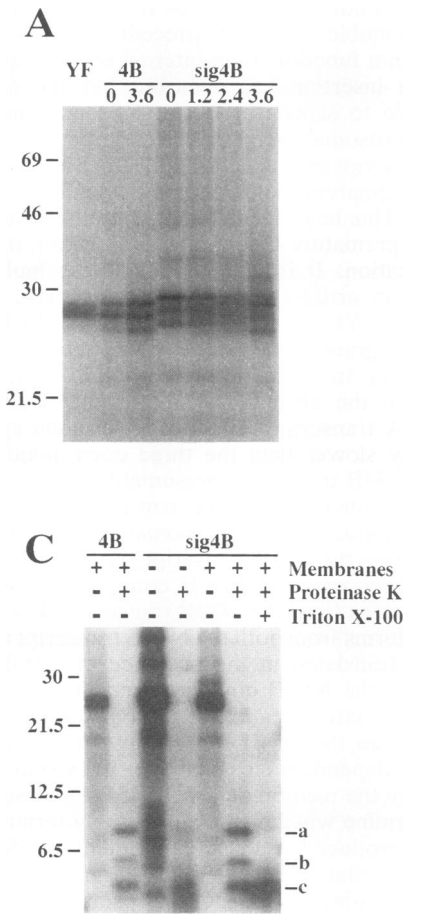
FIG. 4. *trans* processing of the NS4A-4B polyprotein. Coexpression of various constructs was used to examine *trans* cleavage of the NS4A-4B substrate. Parental (w) or mutant 3_{181} derivatives, NS4A-4B, and 2B-3_{181} were delivered by plasmid transfection, whereas vaccinia virus recombinant vYF2B was used to express NS2B. Mutant derivatives of 3_{181} are indicated as follows: 3_{181}(H1537A), HA; 3_{181}(D1561N), DN; 3_{181}(S1622A), SA; and 3_{181}(S1622C), SC. Control lysates were prepared from YF-infected cells (YF) or from vTF7-3-infected cells (m). As indicated by the number(s) under each lane, a portion of each lysate was immunoprecipitated with either NS4B-specific antiserum or a mixture of NS2B- and NS3-specific antisera and analyzed by SDS–14% PAGE.

the 3_{181} domain, which then allows these proteins to interact with the NS4 region in such a way that signalase cleavage at the 4A/4B site can occur. To distinguish between these two possibilities, several 3_{181} mutants, which contain substitutions that either diminish or eliminate the proteolytic activity, were used in *trans*-processing experiments. Sequence alignment with known serine proteinases (2, 3, 18) and site-directed mutagenesis studies (13) suggest that YF polyprotein residues His-1537, Asp-1561, and Ser-1622 constitute the catalytic triad of the NS2B-3 proteinase. It has been shown previously that three mutations (H1537A, D1561N, and S1622A) resulted in complete loss of proteolytic activity, while a fourth mutation (S1622C) allowed diminished activity for cleavage at the 2B/3 site (13). NS4A-4B and NS2B were coexpressed with either 3_{181} or various 3_{181} derivatives containing these mutations (Fig. 4). As observed previously, coexpression with NS2B and 3_{181}, either as individual proteins or as the 2B-3_{181} polyprotein, resulted in cleavage of the NS4A-4B substrate. Of the mutant 3_{181} constructs, only the 3_{181}(S1622C) mutant was able to induce processing of NS4A-4B, albeit inefficiently, as evidenced by a faint NS4B doublet (Fig. 4). These findings strongly suggest that both NS2B and a catalytically active 3_{181} proteinase domain are necessary for processing at the 4A/4B signalase site.

Evidence that signalase mediates the cleavage producing the N terminus of NS4B. Although an active NS2B-3 proteinase appears to be required for processing at the 4A/4B site in the NS4A-4B polyprotein, the sequences preceding the N terminus of NS4B and the residues flanking the 4A/4B cleavage site suggest that signalase, rather than the NS2B-3 proteinase, is responsible for cleavage at this site (11, 42). To examine membrane-dependent processing at the 4A/4B site, RNA transcripts encoding NS4A-4B, sig4B, or NS4B were synthesized in vitro from the corresponding linearized DNA templates. As expected from the results of the in vivo transient-expression assays, only full-length proteins were produced by cell-free translation of the NS4A-4B RNA transcript in the presence or absence of microsomal membranes (data not shown). These results indicate either that the hydrophobic sequence preceding the N terminus of NS4B cannot function as an internal signal sequence or that membrane insertion has occurred but the 4A/4B site is inaccessible to signalase. In the presence and absence of canine microsomal membranes, cell-free translation of the NS4B transcript produced three distinct NS4B-specific proteins with apparent molecular masses of 28, 27, and 26 kDa (Fig. 5A). This heterogeneity could be the result of internal initiation, premature C-terminal termination, or other types of modification. It is unlikely that these multiple species represent an artifact of cell-free translation, since NS4B isolated from YF-infected (9, 11, 12, 23) and DEN2-infected (32) cells migrates as a doublet or sometimes a triplet (see also Fig. 5A). In addition, internal initiation seems unlikely because, in the absence of microsomal membranes, the sig4B RNA transcript yielded three protein species which ran slightly slower than the three corresponding products from the NS4B transcript, presumably because of the presence of the uncleaved signal peptide. In the presence of increasing amounts of microsomal membranes, the three products from the sig4B transcript gradually shifted to three faster-moving species, which comigrated with the three protein species from the NS4B transcript. The more slowly migrating forms from both the NS4B transcript and the sig4B transcript translated in the presence of membranes comigrated with the NS4B doublet found in YF-infected cells. The simplest interpretation of these results is that all three products from the sig4B RNA transcript were undergoing membrane-dependent cleavage after the signal peptide, presumably by the membrane-associated signalase.

To determine whether the authentic N terminus of NS4B had been produced in these experiments, [^{35}S]Met-labeled products translated from the sig4B transcripts in the presence of saturating amounts of microsomal membranes were isolated by SDS-PAGE and subjected to N-terminal sequence analysis. The sample sequenced was the most slowly migrating form. If the sig4B products were not cleaved, the next Met residue after the initiating Met would be recovered at position 23 (or at position 22 if the N-terminal Met was removed by methionine aminopeptidase). Alternatively, if cleavage occurred to produce the authentic NS4B N terminus, the first Met residue would be found at position 5 (11). As shown in Fig. 5B, a prominent peak of ^{35}S-label was released at cycle 5, showing that membrane-dependent cleavage had occurred in vitro at the authentic 4A/4B site.

Membrane association and topology of NS4B. Since signalase cleavage produces the N terminus of NS4B whereas the cytoplasmic NS2B-3 proteinase cleaves at the 4B/5 site, the simplest model for the topology of NS4B would predict a type I transmembrane protein configuration, with the N terminus in the lumen of the ER and the C terminus in the cytoplasm. To examine the topology of membrane-associated NS4B products, proteinase K protection analyses of the in vitro-translated products were performed. In the absence of membranes, a smear of low-molecular-weight degradation products was observed for the sig4B construct (Fig. 5C). When sig4B was translated in the presence of microsomal membranes and digested with proteinase K, three small, distinct polypeptide fragments (called a, b, and c in Fig. 5C) were observed. These results suggest that NS4B is not a simple type I transmembrane protein, with the majority of the protein localized in the ER lumen. Surprisingly, similar proteinase K-resistant products were observed for NS4B translation products made in the presence of membranes (Fig. 5C). Hence, portions of the NS4B protein may be



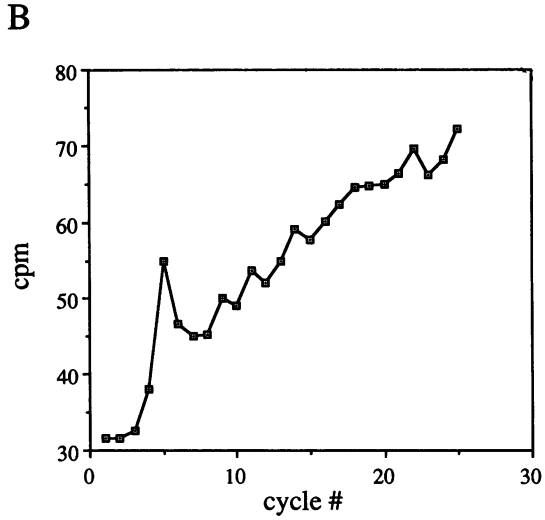


FIG. 5. In vitro signalase cleavage to generate the NS4B N terminus. (A) Microsomal membrane-dependent processing of sig4B. In vitro transcriptions and translation assays were conducted as described in Materials and Methods. NS4B-specific products were analyzed by SDS–14% PAGE. YF indicates lysates from YF-infected BHK-21 cells. NS4B and sig4B indicate in vitro translation reaction mixes containing NS4B and sig4B transcripts, respectively. The amount of canine pancreatic microsomal membranes (in units per 25-μl reaction mix) used in each translation reaction is indicated at the top of each lane. (B) N-terminal sequence analysis of sig4B translation products made in the presence of microsomal membranes. The [^{35}S]Met translation product was isolated and subjected to sequential Edman degradation as described in Materials and Methods. The graph represents uncorrected counts per minute released per sequencing cycle. (C) Digestion of sig4B and NS4B translation products with proteinase K. Cell-free transcription and translation assays and proteinase K digestion were conducted as described in Materials and Methods. NS4B and sig4B indicate in vitro translation assay with NS4B and sig4B transcripts, respectively. The presence (+) or absence (−) of a given component (shown on the right) is indicated above each lane. The digestion products were analyzed by tricine–SDS–14% PAGE, and the three major protected products are indicated as a, b, and c.

capable of insertion or association with the ER membrane even in the absence of an N-terminal signal peptide.

In an attempt to localize fragments a, b, and c in the NS4B sequence, a series of RNA transcripts encoding NS4B products with successive C-terminal deletions were translated in the presence of microsomal membranes. Protein products were subjected to protection assays with proteinase K, trypsin, or chymotrypsin (data not shown). The patterns of protected fragments indicate that fragments b and c are present in the middle of the N-terminal half of NS4B, whereas fragment a is located in the C-terminal half. From the hydropathy profile, there are five hydrophobic segments in YF NS4B (residues 40 to 58, 91 to 107, 109 to 124, 171 to 187, and 191 to 208). Although the following model will need to be verified by sequence analysis of protected fragments, we propose that YF NS4B may have the following membrane topology: (i) a short N-terminal domain translocated into the lumen of the ER, (ii) a transmembrane segment (probably residues 40 to 58), (iii) a cytoplasmic loop, (iv) two adjacent hydrophobic membrane-associated segments (residues 91 to 107 and 109 to 124), (v) a second cytoplasmic loop, (vi) two more adjacent hydrophobic membrane-associated segments (residues 171 to 187 and 191 to 208), and (vii) a C-terminal cytoplasmic tail generated by NS2B-3 proteinase cleavage.

Cleavage at a novel site in the NS4A region by the YF NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site: a model. Some of the experimental results described above are seemingly difficult to reconcile; signalase appears to mediate cleavage at the 4A/4B site, yet an active NS2B-3 proteinase is required for this cleavage in NS4A-4B or longer YF polyproteins. These results could be explained if the NS2B-3 proteinase cleaves at a novel site within the NS4A region to release a functional N-terminal signal peptide which is otherwise not accessible for cleavage by the host signal peptidase. From previous mutagenesis studies examining the cleavage site specificity of the YF NS2B-3 proteinase (23), a possible cleavage site (Q-Q-R ↓ S) can be found 23 residues upstream of the 4A/4B signalase site. The amino acid residues flanking this site and the distance from this site to the downstream 4A/4B signalase site are highly conserved among flaviviruses (Fig. 6A). This potential cleavage site is called the 4A/2K site, and the downstream signalase cleavage site is called the 2K/4B site because a polypeptide of 23 amino acids would be produced if cleavage occurred at both sites.

This model predicts that, if there is no further trimming, YF NS4A would be 126 rather than 149 residues in length. Because of the lack of an antiserum specific for YF NS4A, NS4A-specific products can only be identified as part of an NS3-4A polyprotein immunoprecipitated by YF NS3-spe-

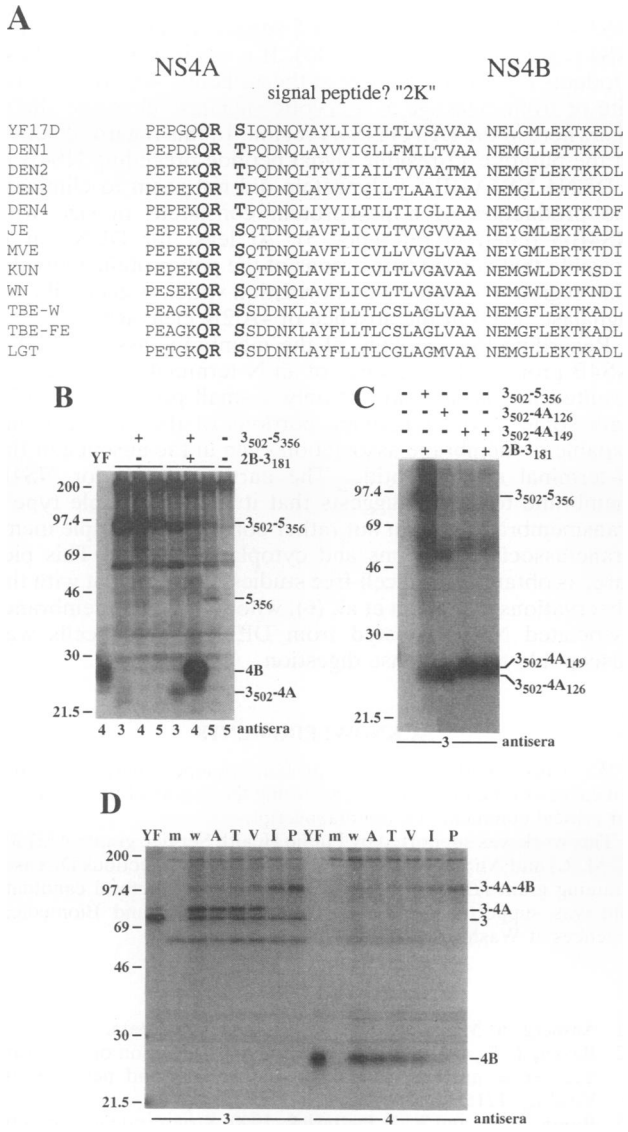


FIG. 6. Proposed 4A/2K cleavage site. (A) The amino acid sequences flanking the putative 4A/2K and 2K/4B cleavage sites are aligned for 12 flaviviruses. The positions of the putative NS2B-3 proteinase 4A/2K and signalase 2K/4B sites are indicated by spaces, and the residues flanking the 4A/2K site shown in boldface type. The 2K/4B signalase site has been defined by N-terminal sequence analysis of NS4B from YF, Kunjin virus (KUN), and DEN2; the rest are tentatively assigned based on amino acid sequence homology. The single-letter amino acid code is used. See the legend to Fig. 3 in reference 10 for primary literature citations, except for the recently published sequences for DEN1 (16), DEN3 (29), tick-borne encephalitis virus, Far Eastern strain (TBE-FE) (30), and langat virus (LGT) (20). Additional abbreviations: JE, Japanese encephalitis virus; MVE, Murray Valley encephalitis virus; WN, West Nile virus; TBE-W, tick-borne encephalitis virus, Western strain. (B and C) *trans* processing of 3_{502}-5_{356} polyprotein. Experimental details and figure labels are the same as for Fig. 3A. As indicated by the number under each lane, a portion of each lysate was immunoprecipitated with either NS3-, NS4B-, or NS5-specific antiserum and analyzed by SDS–14% PAGE. The NS3-specific antiserum used in this experiment does not react with 3_{181} because the immunogen contained only the 48 C-terminal amino acids of NS3 (immunogen NS3-4A [11]). (D) Processing of sig2A-5_{356} polyproteins with substitutions at the P1′ position of the putative 4A/2K cleavage site (Ser-2234). Substitutions are indicated by the single-letter code. NS3- and NS4B-specific products (as indicated at the bottom) were analyzed by SDS–10% PAGE.

cific antiserum. Since NS3-4A has an apparent molecular mass of 80 kDa, the difference in the mobility of 3-$4A_{126}$ and 3-$4A_{149}$ is too small to be detected by SDS-PAGE (data not shown). Therefore, a truncated construct was made which initiates with NS3 residue 502 (Met) and extends through the N-terminal 356 residues of NS5 (called 3_{502}-5_{356}; Fig. 1). When coexpressed with 2B-3_{181}, three cleavage products were immunoprecipitated by antiserum specific for YF NS3, NS4B, and NS5 (11, 12) and identified as 3_{502}-4A, NS4B, and 5_{356}, respectively (Fig. 6B). An additional protein, which migrated slightly faster than 3_{502}-5_{356}, was precipitated nonspecifically by the NS3 antiserum. As shown in Fig. 6C, the cleavage product 3_{502}-4A comigrated with 3_{502}-$4A_{126}$, which resolved from the more slowly migrating 3_{502}-$4A_{149}$ marker. In the presence of 2B-3_{181}, the mobility of 3_{502}-$4A_{149}$ was unchanged, indicating that 3_{502}-$4A_{149}$ was not further processed by the 2B-3_{181} proteinase. These results suggest that the C terminus of 3_{502}-4A is produced by cleavage at or near the 4A/2K site rather than at the 2K/4B signalase site.

Additional support for this model was obtained by site-directed mutagenesis of the putative 4A/2K cleavage site. If the NS2B-3 proteinase-mediated 4A/2K cleavage is a prerequisite for signalase cleavage at the downstream 2K/4B site, then mutations blocking the 4A/2K cleavage should also eliminate the 2K/4B processing. Previous studies indicate that Gly, Ser, and Ala are allowable at the P1′ position of the NS2B-3 proteinase dibasic cleavage sites (23). Five substitutions were introduced at the 4A/2K P1′ position (Ser) (Fig. 6D). Substitutions with Ala or Thr resulted in a pattern of NS3- and NS4B-specific products similar to that obtained from the parental sig2A-5_{356} polyprotein, whereas substitution with Val yielded reduced levels of NS3-4A and NS4B, with a corresponding increase in the level of NS3-4A-4B. Mutants with Ile or Pro substitutions generated NS3, NS4A-4B, and NS3-4A-4B but very little, if any, NS3-4A or NS4B, indicating that processing between NS4A and NS4B had been largely blocked.

DISCUSSION

The N-terminal sequences of NS4B proteins produced in YF-, Kunjin virus-, and DEN2-infected cells have been determined (11, 32, 42). The amino acid sequences immediately preceding these N termini share characteristics with signal peptides, leading to the proposal that cleavage at the 4A/4B site is mediated by a host signal peptidase in association with the ER (11, 42). This proposal suggests that during translation of the flavivirus polyprotein, the hydrophobic sequence preceding NS4B should function as an internal signal peptide and that the resulting cleavage products will probably be integral membrane proteins. In support of this model, this report has shown that the N-terminal signal peptide in a sig4B protein can be cleaved by signalase in association with ER membranes. However, cleavage at the 4A/4B site of several NS4A-4B-containing polyproteins occurred only in the presence of an active YF NS2B-3 proteinase. From these observations, we have proposed and tested a model for NS4 region processing in which NS2B-3 proteinase-mediated cleavage at a novel site (4A/2K) in the NS4A region is required for processing at the downstream signalase site (2K/4B).

Evidence from two experimental approaches supports this model. First, it was shown that the size of the 3_{502}-4A product generated by *trans* processing of a truncated polyprotein, 3_{502}-5_{356}, was consistent with cleavage at or near the 4A/2K site rather than at the downstream signalase site.

Second, some substitutions at the P1′ position of the putative 4A/2K site eliminated processing in the NS4A-4B region, presumably by blocking cleavage at the 4A/2K site. The lack of NS4 region processing in these mutant polyproteins parallels the results obtained for unaltered polyproteins expressed in the absence of an active NS2B-3 proteinase. In addition, the effects of individual substitutions at the 4A/2K site on processing were generally consistent with the results obtained in mutagenesis studies on the 3/4A and 4B/5 dibasic sites (23). The previous mutagenesis results (23) and the conservation of residues flanking NS2B-3 proteinase cleavage sites (10) suggest that Gly, Ser, Ala, and Thr are likely to be tolerated at the P1′ position, which is consistent with the results reported here, in which Ala and Thr substitutions had no effect on processing. In contrast, substitution of the P1′ Ser with either Ile or Pro eliminated processing. It was surprising, however, that substitution with Val only reduced 4A/2K cleavage, since the same substitution at the P1′ position of the 4B/5 site abolished cleavage. This difference may indicate that the YF NS2B-3 proteinase has distinct structural requirements at different cleavage sites. It remains possible, however, that substitutions at the putative 4A/2K site had an indirect effect on processing by altering the conformation of the polyprotein substrate. This possibility seems unlikely, since a series of double-substitution mutations, designed to eliminate signalase cleavage at the 2K/4B site, did not eliminate processing in the NS4 region (see below).

While these indirect experiments strongly support the model, N- or C-terminal sequence data are required to prove that cleavage actually occurs at the proposed YF 4A/2K site. Thus far, attempts to isolate a 2K-4B precursor resulting from cleavage at the putative 4A/2K site in sufficient quantities for N-terminal sequence analysis have been unsuccessful. In one strategy, mutations were engineered in the residues preceding the 2K/4B signalase site to block signalase cleavage and facilitate isolation of the 2K-4B precursor. Six pairs of double substitutions were introduced at the P4 (Ala) and P3 (Val) positions of the 2K/4B site. As expected from the model, these mutations did not eliminate processing in the NS4 region. However, a 2K-4B precursor resolved from NS4B could not be identified for any of the mutants. We have also attempted isolation of the 2K-4B species by cell-free translation of radiolabeled NS4A-4B-containing substrates and *trans* cleavage by a detergent extract containing the YF 2B-3_{181} proteinase. However, despite analysis of several substrates, sufficient cleavage has not been obtained in vitro. C-terminal sequence analysis of NS4A or NS3-4A could also be used to obtain direct evidence for utilization of the proposed 4A/2K site. Such data exist only for Kunjin virus NS4A but do not distinguish between cleavage at the 4A/2K and the 2K/4B site, since carboxypeptidase B and NS4A metabolically labeled with [^{3}H]lysine were used for this analysis (43) (Fig. 6A).

The strict conservation of sequences around the 4A/2K site and the distance between this site and the downstream signalase site (Fig. 6A) suggest that this processing strategy may be common among flaviviruses. Like YF, DEN4 may also require an active NS2B-3 proteinase for cleavage in the NS4 region, since expression of DEN4 NS2B-5 but not NS3-5 allowed processing at the 4A/4B site to occur (5). In the case of DEN2, NS4A-4B expressed alone was not processed, but the requirement for the NS2B-3 proteinase has not been tested (32). However, different results have been obtained in experiments with other truncated DEN4 polyproteins. The products obtained by expression of DEN4 NS3-4A-84%4B and NS4A-4B-5 suggest that cleavage in the NS4 region has occurred (5, 15). It is unclear whether these products represent cleavage at the authentic 4A/4B signalase site or from cleavage at a cryptic signalase cleavage site(s) elsewhere in the DEN4 NS4A region. In this regard, deletion of the putative 17-residue signal peptide preceding NS4B in a DEN4 NS4A-4B-5 polyprotein did not seem to eliminate the production of a polyprotein consistent in size with NS4B-5 (cited in reference 5). At least for DEN4, it is possible that certain truncations of the polyprotein allow the NS4 region to assume a conformation which is accessible to signalase, even in the absence of 4A/2K cleavage.

Regarding the topology of the membrane-associated YF NS4B protein, the presence of an N-terminal signal peptide resulted in translocation of only a small portion of NS4B, with some of the remaining portions of the protein being capable of membrane association even in the absence of the N-terminal signal peptide. The current model for NS4B membrane topology suggests that it is not a simple type I transmembrane protein but rather consists of multiple membrane-associated regions and cytoplasmic loops. This picture, as obtained from cell-free studies, is consistent with the observations of Cauchi et al. (6), who found that membrane-associated NS4B isolated from DEN2-infected cells was susceptible to proteinase digestion.

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REFERENCES

1. **Amberg, S. M., and C. M. Rice.** Unpublished data.
2. **Bazan, J. F., and R. J. Fletterick.** 1989. Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology **171**:637–639.
3. **Bazan, J. F., and R. J. Fletterick.** 1990. Structural and catalytic models of trypsin-like viral proteases. Semin. Virol. **1**:311–322.
4. **Brinton, M. A.** 1986. Replication of flaviviruses, p. 327–365. *In* S. Schlesinger and M. J. Schlesinger (ed.), The Togaviridae and Flaviviridae. Plenum Press, New York.
5. **Cahour, A., B. Falgout, and C.-J. Lai.** 1992. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J. Virol. **66**:1535–1542.
6. **Cauchi, M. R., E. A. Henchal, and P. J. Wright.** 1991. The sensitivity of cell-associated dengue virus proteins to trypsin and the detection of trypsin-resistant fragments of the nonstructural protein NS1. Virology **180**:659–667.
7. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98**:132–135.
8. **Chambers, T. J.** Unpublished data.
9. **Chambers, T. J., A. Grakoui, and C. M. Rice.** 1991. Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J. Virol. **65**:6042–6050.
10. **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.
11. **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.

12. **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.
13. **Chambers, T. J., R. C. Weir, A. Grakoui, D. W. McCourt, J. F. Bazan, R. J. Fletterick, and C. M. Rice.** 1990. Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. Proc. Natl. Acad. Sci. USA **87:**8898–8902.
14. **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.
15. **Falgout, B., M. Pethel, Y. 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. **Fu, J., B.-H. Tan, E.-H. Yap, Y.-C. Chan, and Y. H. Tan.** 1992. Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). Virology **188:**953–958.
17. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
18. **Gorbalenya, A. E., A. P. Donchenko, E. V. Koonin, and V. M. Blinov.** 1989. N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. Nucleic Acids Res. **17:**3889–3897.
19. **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.
20. **Iacono-Conners, L. C., and C. S. Schmaljohn.** 1992. Cloning and sequence analysis of the genes encoding the nonstructural proteins of langat virus and comparative analysis with other flaviviruses. Virology **188:**875–880.
21. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Lin, C., T. J. Chambers, and C. M. Rice.** 1993. Mutagenesis of conserved residues at the yellow fever virus 3/4A and 4B/5 dibasic cleavage sites: effects on cleavage efficiency and polyprotein processing. Virology **192:**596–604.
24. **Lin, C., and C. M. Rice.** Unpublished observations.
25. **Markoff, L.** 1989. In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein. J. Virol. **63:** 3345–3352.
26. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
27. **Moss, B., O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst.** 1990. New mammalian expression vectors. Nature (London) **348:**91–92.
28. **Nowak, T., P. M. Farber, G. Wengler, and G. Wengler.** 1989. Analyses of the terminal sequences of West Nile virus structural proteins and of the *in vitro* translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. Virology **169:**365–376.
29. **Osatomi, K., and H. Sumiyoshi.** 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176:** 643–647.
30. **Pletnev, A. G., V. F. Yamshchikov, and V. M. Blinov.** 1990. Nucleotide sequence of the genome and complete amino acid sequence of the polyprotein of tick-borne encephalitis virus. Virology **174:**250–263.
31. **Preugschat, F., E. M. Lenches, and J. H. Strauss.** 1991. Flavivirus enzyme-substrate interactions studied with chimeric proteinases: identification of an intragenic locus important for substrate recognition. J. Virol. **65:**4749–4758.
32. **Preugschat, F., and J. H. Strauss.** 1991. Processing of nonstructural proteins NS4A and NS4B of dengue 2 virus *in vitro* and *in vivo*. Virology **185:**689–697.
33. **Preugschat, F., C.-W. Yao, and J. H. Strauss.** 1990. In vitro processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. J. Virol. **64:**4364–4374.
34. **Rice, C. M., C. A. Franke, J. H. Strauss, and D. E. Hruby.** 1985. Expression of Sindbis virus structural proteins via recombinant vaccinia virus: synthesis, processing, and incorporation into mature Sindbis virions. J. Virol. **56:**227–239.
35. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science **229:**726–733.
36. **Rice, C. M., E. G. Strauss, and J. H. Strauss.** 1986. Structure of the flavivirus genome, p. 279–326. *In* S. Schlesinger and M. J. Schlesinger (ed.), The Togaviridae and Flaviviridae. Plenum Press, New York.
37. **Rice, C. M., and J. H. Strauss.** 1990. Production of flavivirus polypeptides by proteolytic processing. Semin. Virol. **1:**357–367.
38. **Ruiz-Linares, A., A. Cahour, P. Despres, M. Girard, and M. Bouloy.** 1989. Processing of yellow fever virus polyprotein: role of cellular proteases in maturation of the structural proteins. J. Virol. **63:**4199–4209.
39. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. Anal. Biochem. **166:**368–379.
42. **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.
43. **Speight, G., and E. G. Westaway.** 1989. Carboxy-terminal analysis of nine proteins specified by the flavivirus Kunjin: evidence that only the intracellular core protein is truncated. J. Gen. Virol. **70:**2209–2214.
44. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
45. **Wengler, G., G. Czaya, P. M. Farder, and J. H. Hegemann.** 1991. *In vitro* synthesis of West Nile virus proteins indicates that the amino terminal segment of the NS3 protein contains the active centre of the protease which cleaves the viral polyprotein after multiple basic amino acids. J. Gen. Virol. **72:**851–858.
46. **Westaway, E. G.** 1987. Flavivirus replication strategy. Adv. Virus Res. **33:**45–90.