JOURNAL OF VIROLOGY, Sept. 1990, p. 4364–4374
0022-538X/90/094364-11$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 64, No. 9

In Vitro Processing of Dengue Virus Type 2 Nonstructural Proteins NS2A, NS2B, and NS3

FRANK PREUGSCHAT, CHEN-WEN YAO,† AND JAMES H. STRAUSS*
Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125

Received 19 March 1990/Accepted 4 June 1990

We have tested the hypothesis that the flavivirus nonstructural protein NS3 is a viral proteinase that generates the termini of several nonstructural proteins by using an efficient in vitro expression system and monospecific antisera directed against the nonstructural proteins NS2B and NS3. A series of cDNA constructs was transcribed by using T7 RNA polymerase, and the RNA was translated in reticulocyte lysates. The resulting protein patterns indicated that proteolytic processing occurred in vitro to generate NS2B and NS3. The amino termini of NS2B and NS3 produced in vitro were found to be the same as the termini of NS2B and NS3 isolated from infected cells. Deletion analysis of cDNA constructs localized the protease domain within NS3 to the first 184 amino acids but did not eliminate the possibility that sequences within NS2B were also required for proper cleavage. Kinetic analysis of processing events in vitro and experiments to examine the sensitivity of processing to dilution suggested that an intramolecular cleavage between NS2A and NS2B preceded an intramolecular cleavage between NS2B and NS3. The data from these expression experiments confirm that NS3 is the viral proteinase responsible for cleavage events generating the amino termini of NS2B and NS3 and presumably for cleavages generating the termini of NS4A and NS5 as well.

The dengue viruses belong to the *Flaviviridae*, a family of approximately 70 viruses, most of which are arthropod borne, which can be grouped into eight antigenic complexes (6, 7). There are four serotypes of dengue virus, and all are involved in worldwide epidemics of increasing proportion (18, 24, 45). Our laboratory has been working with the PR159 S1 (candidate vaccine) strain of dengue virus type 2 (1, 2, 17, 27, 46). The PR159 genome is a positive-stranded RNA molecule 10,712 nucleotides in length that is capped at the 5′ terminus with a type 1 cap and has at the 3′ terminus a uridine residue (11, 27, 53). Translation begins at nucleotide 97 and continues for 10,173 bases to produce a 3,391-amino-acid polyprotein that is both co- and posttranslationally processed into at least 10 different polypeptides (47).

Sequence analysis of flavivirus proteins and in vitro expression experiments have implicated host cell signalases in the amino-terminal processing of prM, E, and NS1 and possibly NS2A and NS4B (4, 9, 12, 19, 23, 35, 37, 44, 54). These host cell signalases cleave on the carboxy-terminal side of hydrophobic leader sequences and function in the lumen of the endoplasmic reticulum (51). On the other hand, the cleavages that generate the amino termini of NS2B, NS3, NS4A, and NS5, which follow two basic amino acids and occur in the cytosol, are believed to be due to a viral proteinase (39, 41).

The short half-lives of polyprotein precursors in vivo combined with the poor fidelity of in vitro expression systems have made the study of flaviviral polyprotein processing difficult. Early experiments that translated genomic RNA in vitro did not produce detectable amounts of nonstructural proteins, and correct processing of structural proteins occurred only in the presence of exogenously supplied microsomal membranes (50, 52). In vivo pulse-chase experiments that utilized amino acid analogs or starved cells prior to amino acid labeling enabled the detection of dengue virus-specific higher-molecular-weight proteins, but the lack of specific immune reagents and the inability to chase these putative precursors limited the experimental analysis (10, 38). Recent studies utilizing improved expression systems have made progress in analyzing signalase-mediated cleavage events but have not addressed the cleavages that occur after dibasic amino acids (19, 35, 37, 44).

Recently, two laboratories have proposed models predicting that the amino terminus of NS3 is a trypsin-like protease (3, 20), on the basis of limited sequence similarity to cellular serine proteases and more extensive similarity to the protease domain of the capsid protein of the alphavirus Sindbis virus (25, 26). These molecular modeling studies predict that His-51, Asp-75, and Ser-135 of NS3 form a classic serine protease catalytic triad and that the entire protease domain lies within the first 180 amino acids of this protein. The proteolytic activity of this NS3 enzyme is thought to be responsible for cleavages generating the amino termini of NS2B, NS3, NS4A, and NS5 and possibly the carboxy terminus of the capsid protein. To test this model, we have developed an efficient in vitro expression system for NS2B and NS3 and generated specific immune reagents reactive with these proteins. In this system, the processing events that generate the amino termini of NS2B and NS3 occur faithfully in vitro, mediated by the nonstructural protein NS3. By deletion analysis, the protease domain has been mapped to within the first 184 amino acids of NS3, and from the kinetics of cleavage we propose that an intramolecular cleavage between NS2A and NS2B precedes an intramolecular cleavage between NS2B and NS3.

MATERIALS AND METHODS

Construction of *trpE* fusions. cDNA clones of the PR159 strain of dengue virus type 2 were used for all plasmid constructions (27). All plasmids were constructed by using standard recombinant DNA techniques and were purified on CsCl density gradients (34). To create a gene fusion between *trpE* and dengue virus NS2B, the vector pATH3 (49) was

* Corresponding author.
† Present address: Department of Molecular Biology, Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan.

digested with *Bam*HI and the 3′ recessed end was filled in by using the *Escherichia coli* DNA polymerase I Klenow fragment and then digested with *Cla*I. A fragment from *Stu*I to *Hpa*II (from cDNA clone 2, referred to here as pDN2) containing nucleotides 4077 to 4463 of the dengue virus genome was inserted to yield the construct pTNS2.

To create a gene fusion with NS3, the vector pATH3 was digested with *Eco*RI, treated with Klenow fragment, and digested with *Sal*I. A fragment from *Asp*718 (Klenow treated) to *Sal*I (from cDNA clone pDN2) containing nucleotides 4497 to 4944 of the dengue virus genome was inserted to produce pTNS3. Recombinant clones were screened by analysis of protein expression patterns, restriction endonuclease digestion, and DNA sequencing.

Expression of fusion proteins and immunizations. *trpE* fusion proteins were prepared from large-scale induced cultures essentially as described previously (28), with the following modifications. All *trpE* plasmids were propagated in bacterial strain XL1 (Stratagene). After induction, pelleted cells were frozen and thawed (two cycles) in 50 mM Tris (pH 7.5)–0.5 mM EDTA–300 mM NaCl (TEN) buffer. To promote efficient lysis, cells were treated for 15 min in a sonicating water bath (Bransonic 12; Branson Sonic Power Co., Danbury, Conn.) at 4°C. Insoluble inclusion bodies were purified by two successive pelletings through a 25% sucrose–1 mM EDTA cushion and were solubilized directly into sodium dodecyl sulfate (SDS) loading buffer (33) for electrophoresis in preparative 10% polyacrylamide-SDS gels (SDS-PAGE). Proteins were visualized by staining with 250 mM KCl at 4°C. Gels were homogenized in sterile phosphate-buffered saline (pH 7.5) and mixed with MPL adjuvant (RIBI Immunochem) prior to injection. New Zealand White rabbits were injected intramuscularly and subcutaneously on a 3- to 4-week schedule with 100 to 150 μg of fusion protein per injection session and were bled 10 to 14 days after being given a booster dose. Serum samples were stored at −20°C until further use.

Cells and virus stocks. Stocks of the PR159 dengue virus type 2 S1 isolate (1, 2, 46) were prepared on *Aedes albopictus* C6/36 cells essentially as described previously (13). C6/36 cells were propagated at 30°C in Dulbecco modified Eagle medium containing 10% fetal calf serum and supplemented with nonessential amino acids. BHK-21 clone 15 cells, obtained from Joel Dalrymple, U.S. Army Medical Research Institute for Infectious Diseases, Bethesda, Md., were used to plaque purify the virus and to prepare infected cell lysates for immunoprecipitation. BHK cells were propagated at 37°C in minimum essential medium supplemented with nonessential amino acids and containing 5% fetal calf serum. Plaque assays were performed at 34°C on BHK cells by using a 1% low-melting-temperature agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) overlay containing minimum essential medium and 5% fetal calf serum. Plaques were readily visible after 4 days by direct visualization or after staining the monolayer with neutral red.

Labeling of infected cells. Equivalent conditions were used to prepare labeled viral proteins for immunoprecipitation assays and amino-terminal sequencing. BHK cells were infected with dengue virus at a multiplicity of 5 in minimum essential medium. At 30 h postinfection, cells were labeled in minimum essential medium containing 2% dialyzed fetal calf serum, 1/40 the normal concentration of methionine, and 75 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml for 12 h. For preparation of leucine-labeled proteins, cells were incubated in minimum essential medium containing 2% dialyzed serum, 1/100 the normal amount of leucine, and 75 μCi of [^{3}H]leucine (New England Nuclear Corp., Boston, Mass.) per ml for 12 h. Cell lysates were prepared by solubilizing the monolayers in a denaturing lysis buffer containing 0.5% SDS, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 20 μg of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) per ml, 2 μg of aprotinin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml, and 2 μg of leupeptin (Sigma) per ml.

Translation constructs. Vector p5′L213 was constructed to facilitate high-level expression of dengue virus sequences in an in vitro translation system. Briefly, a fragment from *Eco*RI to *Pst*I encoding nucleotides 16 to 213 of the dengue virus type 2 genome (from pDNC3) was cloned into *Eco*RI- and *Pst*I-digested pGEM1 (Promega Biotec, Madison, Wis.) to yield pGem16/213. A double-stranded DNA oligonucleotide that contained a T7 RNA polymerase promoter immediately adjacent to the first 55 nucleotides of the dengue virus genome (the sequence of the first 15 nucleotides was assumed to be the same as for the dengue virus type 2 Jamaica strain [15]) was synthesized and was inserted into *Sac*I-*Pvu*II-digested pGem16/213, yielding vector p5′213. p5′213 and pGEM4 were digested with *Pst*I and *Nhe*I, and polylinker sequences of pGem4 (a fragment from *Pst*I to *Nhe*I) were inserted to yield vector p5′L213. Vector p5′L213 contains several unique restriction sites that allow fusion of heterologous sequences with the dengue virus capsid gene reading frame.

Vector p5′L213 was digested with *Pst*I, and the 3′ overhang was made blunt by treatment with T4 DNA polymerase and digested with *Eco*RI. An *Ase*I (Klenow treated)- to *Xho*I-digested fragment containing nucleotides 3778 to 5427 of the dengue virus genome, from cDNA clone pDN2, and a fragment from *Xho*I to *Eco*RI containing nucleotides 5428 to 6351 of the dengue virus genome, from cDNA clone pDN5, were inserted to create pT10. This construct contains the 5′ untranslated region of the dengue virus genome and the nucleotides encoding the first 37 amino acids of the dengue virus capsid protein fused in frame to codon 101 of NS2A, followed by the sequences encoding the remainder of NS2A (amino acids 101 to 218), the complete coding sequences of NS2B (amino acids 1 to 129), and the first 610 amino acids of NS3 (the carboxy-terminal 8 amino acids of NS3, amino acids 611 to 618, are not present).

Proteins produced in vitro with the same termini as those in vivo have been designated by the prefix NS, whereas proteins with an altered structure have been designated by the prefix P and a prime superscript. For example, pT10-programmed translations yield the precursor P2A2B3, the processing intermediate P2B3, and the products P2A, NS2B, and P3. Precursors, intermediates, and products (described below) generated by the proteolytically active deletion constructs pT11 and pT12 are designated P2A2B3′, P2A2B3″, P2B3′, P2B3″, P2A, NS2B, P3′, and P3″, respectively.

Plasmid pT10 was used to produce deletion clones to map the boundaries of the protease domain, by using *Xho*I, *Asu*II, *Sal*I, and *Kpn*I (*Asp*718) restriction sites within NS2B and NS3. In each case, pT10 was digested with *Eco*RI and the second enzyme (*Xho*I, *Asu*II, etc.), the 3′-recessed ends were filled in with Klenow, and the plasmid was recircularized by using T4 DNA ligase to create the deleted constructs designated pT11, pT12, pT13, and pT14.

Two additional deletion clones were constructed that together removed almost all the NS2A- and NS2B-coding sequences represented in pT10. pT11 was digested with *Kpn*I, blunt ended with T4 DNA polymerase, and then

digested with *Sna*BI and cyclized to create pT15. pT12 was digested with *Pvu*II and *Sna*BI and then cyclized to create pT16.

In vitro transcription and translation. All plasmids used for in vitro transcription and translation were purified by CsCl density gradient centrifugation. After linearization with appropriate restriction enzymes, templates were digested with proteinase K and phenol extracted. Ethanol-precipitated templates were suspended in diethylpyrocarbonate-treated RNase-free water at a concentration of 0.5 μg/ml. Transcription reaction mixtures containing 0.5 mM ribonucleoside triphosphates (Pharmacia, Inc., Piscataway, N.J.), 0.5 mM cap analog $m^7G(5')ppp(5')G$ (New England BioLabs, Inc., Beverly, Mass.), 50 ng of template per μl, and 2 U of T7 RNA polymerase (Pharmacia) per μl in 1× transcription buffer (42) were incubated at 37°C for 45 min. The quality of synthesized RNA was routinely checked by gel electrophoresis prior to in vitro translation, and the mass of RNA synthesized was quantitated by inclusion of trace amounts of [^{3}H]rGTP in the transcription mix. RNA yields were approximately 5 to 10 μg of RNA per μg of input template. Micrococcal nuclease-treated rabbit reticulocyte lysates containing 0.025 eq of microsomal membranes (Promega) per μl were programmed with in vitro-transcribed RNA at a concentration of 5 to 10 μg/ml. All translations were carried out at 30°C for 90 min unless otherwise noted.

Immunoprecipitation. The amount of antiserum necessary to quantitatively immunoprecipitate radiolabeled viral proteins from cell lysates and in vitro translation mixes was empirically determined. Labeled cell lysates were diluted fivefold with radioimmune precipitation (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris hydrochloride, pH 7.5) prior to immunoprecipitation, and protein A agarose beads (Boehringer Mannheim) were used at a fivefold binding excess over immune sera (31). After a 1-h incubation at room temperature, the immune complexes were washed twice with RIPA buffer containing 1 mg bovine serum albumin per ml and once with RIPA buffer lacking albumin. For immunoprecipitation of in vitro translations, reticulocyte lysates were diluted 25-fold in denaturing lysis buffer and heated to 70°C for 5 min prior to 5-fold dilution with RIPA buffer. Immunoprecipitated proteins were eluted by boiling in SDS-loading buffer. Samples were analyzed by 15% SDS-PAGE, and gels were fluorographed at −80°C (8).

Sequencing of viral proteins. Radiolabeled NS2B was prepared from infected BHK cell lysates by preparative immunoprecipitation. Cells (2×10^7) labeled with [^{35}S] methionine or cells (6×10^7) labeled with [^{3}H]leucine were lysed in 4 ml of denaturing lysis buffer and then diluted in RIPA buffer for immunoprecipitation. Immunoprecipitates were washed three times with RIPA and twice with 10 mM ammonium bicarbonate buffer (pH 7.0) prior to being eluted into 250 μl of 0.1% trifluoroacetic acid and blotted onto glass fiber discs (30). In vitro-processed NS2B was isolated by preparative immunoprecipitation of denatured reticulocyte lysates. Fifty microliters of a pT11-programmed [^{35}S]methionine- or 200 μl of a [^{3}H]leucine-labeled translation mix was denatured and diluted in RIPA buffer prior to immunoprecipitation. For sequencing of P2B3′ precursors, 50 μl of a pT11 [^{35}S]methionine-labeled translation mix was fractionated by SDS-PAGE and then electroblotted onto Immobilon PVDF (Millipore Corp., Bedford, Mass.) membranes by using a carbonate-based buffer system (16, 36).

For sequencing of P3′ produced in vitro, 200 μl of a pT11-programmed [^{3}H]leucine-labeled translation mix or 300 μl of a [^{3}H]valine-labeled translation mix was fractionated by SDS-PAGE and then electroblotted onto Immobilon membranes. Immobilon membranes were autoradiographed to locate [^{35}S]methionine-labeled P3′ markers, and adjacent lanes containing [^{3}H]leucine- or [^{3}H]valine-labeled P3′ were excised. Samples labeled in vivo or in vitro were sequenced on a gas-phase sequenator (model 477A; Applied Biosystems) without phenylthiohydantoin derivatization by using a customized ATZ-1 program. Radioactivity in the eluate of each cycle was determined in a liquid scintillation counter, and ^{3}H-counting efficiency was determined to be 40% by using calibration standards.

RESULTS

Production of fusion proteins and specificity of antisera. We have produced monospecific immune reagents reactive with NS2B and NS3 by constructing plasmids (pTNS2 and pTNS3) which express these moieties as fusion proteins with *trpE*. The locations in the dengue virus genome of the inserts used are shown in Fig. 1, which also illustrates schematically the posttranslational processing of the dengue virus polyprotein to give the mature structural and nonstructural proteins. When bacterial cultures harboring either pTNS3 or pTNS2 were induced and insoluble inclusion bodies were purified and analyzed by SDS-PAGE, fusion proteins of 51 and 54 kilodaltons (kDa), respectively, were found (Fig. 2A), indicating that the fusion proteins were stable when overexpressed. Fusion proteins were purified by preparative SDS-PAGE and used to inject two rabbits each. High-titer antiserum was usually obtained with three injections.

Denatured lysates of dengue virus-infected cells were analyzed by using antisera directed against pTNS2 and pTNS3 fusion proteins. Serum from a rabbit injected with the pTNS2 fusion protein immunoprecipitated a protein of approximately 14 kDa, identified as NS2B from its molecular size, immunoreactivity, and N-terminal sequence (see below), from infected but not from mock-infected cell lysates (Fig. 2B), and the serum was therefore designated αNS2B serum. NS2B was immunoprecipitated from denatured but not from nondenatured lysates, implying that its immunoreactive epitopes are linear in nature or are masked in nondenatured lysates (data not shown). Since pTNS2 contains the last 19 amino acids of NS2A as well as the first 111 amino acids of NS2B (Table 1), antiserum raised against the encoded fusion protein could theoretically have reacted with NS2A sequences. However, no NS2A protein (predicted size, 24 kDa) was detected when either denatured (Fig. 2B) or nondenatured (data not shown) lysates were immunoprecipitated with αNS2B.

Serum from a rabbit injected with the pTNS3 fusion protein precipitated a polypeptide of 69 kDa, identified as NS3, from infected cell lysates (Fig. 2B). In addition, three larger proteins (85, 93, and 130 kDa) were present in significant amounts in the immunoprecipitates. All four species were also immunoprecipitated by a polyvalent anti-dengue virus mouse hyperimmune ascitic fluid (Fig. 2B, lane HIS). The 93-kDa protein appears to be NS5, and the reason it should precipitate with the αNS3 antiserum is obscure. The size and reactivity of the 85-kDa protein are consistent with it being polyprotein NS34A. The identity of the 130-kDa species is unknown; it may be a polyprotein containing NS3.

Since pTNS3 encodes the last 8 amino acids of NS2B as well as the first 141 amino acids of NS3 (Table 1), antiserum raised against the encoded fusion protein could, in theory, also precipitate proteins containing NS2B sequences. αNS3

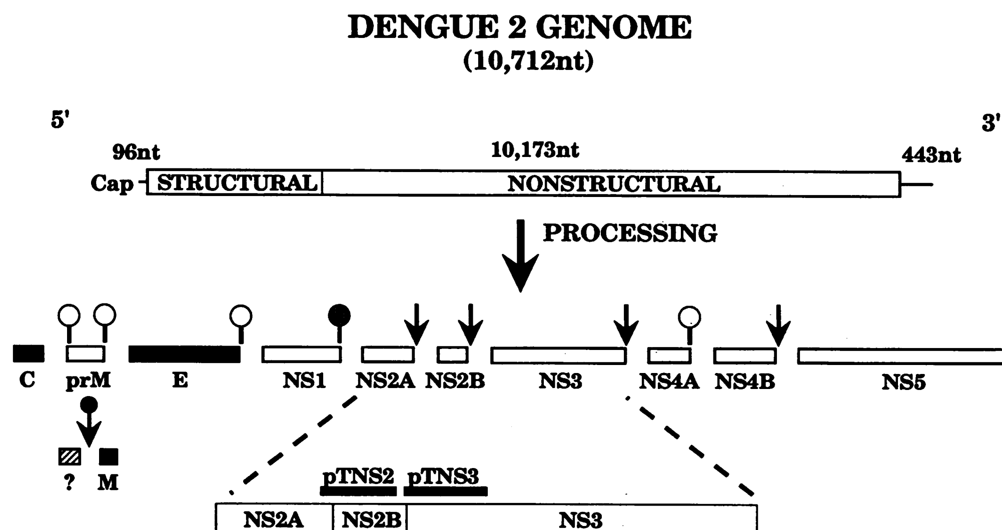

FIG. 1. Genome organization and expression of dengue virus type 2. A schematic diagram aligning the *trpE* cDNA fusions to their corresponding regions of the dengue virus genome is shown. The upper third of the figure details the genome organization of the PR159 strain. The middle third of the figure depicts the processing events generating the structural and nonstructural proteins. Virion structural proteins are designated by shaded boxes, and nonstructural proteins are designated by open boxes. Cleavages after dibasic residues are designated by arrows. Host cell signalase cleavages are designated by open ball-and-stick icons, and a potential viral-encoded signalase-like cleavage is designated by a crosshatched ball-and-stick icon. A late, cell membrane-associated cleavage generating the membrane structural protein is designated by a ball and arrow. The boundaries of the dengue virus inserts in the pTNS2 and pTNS3 cDNA fusions are mapped on the expanded view of NS2A, NS2B, and NS3 shown in the lower third of the figure. Full details of constructs pTNS2 and pTNS3 are given in Table 1. nt, Nucleotides.

serum immunoprecipitated small amounts of NS2B from nondenatured lysates (data not shown) but not from denatured lysates (Fig. 2B). Thus, with denatured lysates, the αNS2B and αNS3 sera described here are essentially monospecific, and denatured lysates were used for all immunoprecipitations shown in the figures.

In vitro translation and processing kinetics. Processing of flavivirus nonstructural proteins occurs very rapidly in vivo, and viral polyproteins can be detected only in pulse-chase experiments under special labeling conditions (10). However, it seemed likely that these processing events could be monitored upon translation in vitro. Our initial expression experiments used RNA transcripts of dengue virus type 2 cDNA clones that were not engineered to have specific 5′ leader sequences or initiation codons in an appropriate context. Only low levels of protein synthesis were obtained in vitro, and variable amounts of specific (i.e., at the 5′ methionine in the RNA) versus nonspecific (internal) initiation of translation were observed. Levels of expression were increased approximately fivefold by inserting the dengue virus 5′ nontranslated region upstream of the region to be translated. The efficiency of translation was increased an additional two- to threefold when the reading frame of interest was fused to that of the dengue virus capsid protein, such that the mRNA translated had the authentic dengue virus type 2 leader and the initiation codon in the normal dengue virus context (data not shown). The yellow fever virus 5′ nontranslated region has also been shown to enhance expression of heterologous coding sequences by reticulocyte lysates (43).

The first construct tested for protease activity was pT10. RNA transcribed from this construct with T7 RNA polymerase was translated into a polyprotein (P2A2B3) containing the first 37 amino acids of the capsid protein, the C-terminal 118 amino acids of NS2A, all of NS2B, and most of NS3 (Fig. 3; Table 2). Protein patterns resulting from different times of translation of this RNA in reticulocyte lysates are shown in Fig. 4A. A protein with the molecular mass predicted for the slightly truncated NS3 (P3) was produced (Fig. 4A), showing that proteolytic processing had occurred in this in vitro system. Proteins with molecular masses predicted for the full-length (unprocessed) translated precursor P2A2B3 and for the processing intermediate P2B3 were also observed (Fig. 4A).

Since the pT10 construct contains two cleavage sites, different processing intermediates would be predicted, depending upon the temporal order in which the cleavages occur. If the cleavage between P2A and NS2B preceded the cleavage between NS2B and P3, intermediate P2B3 would be generated. Conversely, if cleavage between NS2B and P3

TABLE 1. *trpE*/dengue virus cDNA fusions

Construct	Vector[a]	Gene	Insert[b]	Amino acids[b]	Protein[c]
pTNS3	pATH3 (*Eco*(K)/*Sal*)	NS2B3	4497–4944 (*Asp*(K)/*Sal*)	122–129/1–144	NS3
pTNS2	pATH3 (*Bam*(K)/*Cla*)	NS2A2B	4077–4463 (*Stu*/*Hpa*II)	200–218/1–111	NS2B

[a] Vector and insert DNAs were prepared as described in Materials and Methods. (K), 5′ overhangs that were blunted by Klenow treatment.
[b] Nucleotides are numbered relative to the dengue virus genomic sequence, and amino acid numbers of individual gene products are listed.
[c] Dengue virus-specific protein recognized by each antiserum under denaturing conditions.

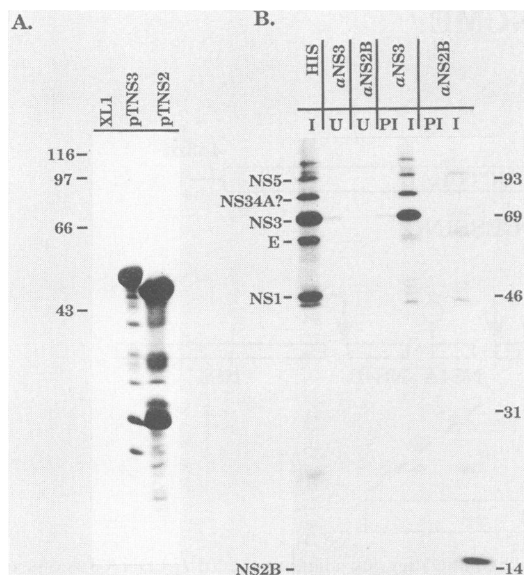

FIG. 2. *trpE*/dengue virus fusion proteins and characterization of antisera. (A) Coomassie blue-stained SDS-PAGE gel with samples of purified inclusion bodies from induced cultures of XL1 alone and XL1 cultures containing plasmids pTNS3 and pTNS2. The positions of protein standards (Bio-Rad Laboratories, Richmond, Calif.) are shown to the left with the molecular masses (kilodaltons) indicated. (B) Immunoprecipitates of [^{35}S]methionine-labeled infected and uninfected BHK cells analyzed by SDS-PAGE. A total of 10^5 infected (I and PI) or mock-infected cells (U) were labeled for 12 h, beginning at 30 h postinfection. Lysates were immunoprecipitated with a polyvalent mouse hyperimmune ascitic fluid (HIS) or monospecific αNS2B and αNS3 immune (I) and preimmune (PI) sera. Locations of bands containing the viral proteins E, NS1, NS2B, NS3, and NS5 and the putative precursor NS34A are indicated. The molecular masses (kilodaltons) of ^{14}C-labeled protein markers (Amersham) are shown.

preceded cleavage between P2A and NS2B, P2A2B rather than P2B3 would be produced. The potential precursor P2A2B was not observed by SDS-PAGE (Fig. 4A) or by immunoprecipitation with αNS2B serum (see below), but P2B3 was readily detected as described above, suggesting that cleavage at the 2A/2B junction occurred first. The amounts and processing kinetics of P2B3 were also consistent with the view that it is the major intermediate in the processing pathway (see below).

Bands containing P2A2B3, P2B3, and P3 (Fig. 4A) were excised from the dried gel, solubilized, and assayed for radioactivity (Fig. 4B). The translations were conducted by

TABLE 2. Deletion constructs

Construct	Gene[a]	Amino acid deletion[b]
pT10	NS3	611–618
pT11	NS3	303–618
pT12	NS3	184–618
pT13	NS3	141–618
pT14	NS2B/NS3	122–129/1–618
pT15	NS2B/NS3	43–121/303–618
pT16	NS2A/NS2B/NS3	146–218/1–42/184–618

[a] Genes affected by deletion.
[b] The amino acids deleted in individual gene products are listed. When more than one gene product contains a deletion, amino acid numbers are separated by a shill.

using an RNA concentration of 10 μg/ml, conditions under which initiation of translation occurs nearly synchronously and the reticulocytes lose their ability to initiate new polypeptide chains after approximately 30 min of translation (14). Thus, the kinetics of accumulation and processing exhibited two phases. All three species accumulated with similar half times during the first 30 min (synthesis phase). After 30 min of translation (postsynthesis phase), there was no further incorporation of label into newly synthesized protein. The label in the full-length P2A2B3 band remained constant between 30 and 90 min, indicating that these molecules were stable and were not processed, whereas P2B3 decayed with a half-life of 42 min and P3 accumulated at the same rate, suggesting a precursor-product relationship between these two species. The fact that P2A2B3 appeared to be processed rapidly in the first 30 min but appeared to be stable thereafter suggests that folding of this protein does not occur optimally during translation in reticulocyte lysates and that those molecules that fold correctly during the initial synthesis phase are processed rapidly, while misfolded molecules are processed slowly or not at all. The fraction of P2A2B3 that was processed varied from experiment to experiment, being sensitive to the lot of reticulocyte lysate used and to other unknown variables.

Cleavage of the dengue virus polyprotein in *cis*. Intermolecular (*trans*) cleavages exhibit second-order kinetics and are concentration dependent, whereas intramolecular (*cis*) cleavages are first order and concentration independent. To determine whether the cleavages at the dengue virus 2A/2B and 2B/3 sites were concentration dependent, we examined the protein products obtained upon translation of different amounts of input RNA, which effectively changes the concentration of the proteinase (Fig. 4C). At the highest concentrations of RNA tested, the components of the translation system were limiting (29) and processing appeared to proceed more rapidly. At lower concentrations of RNA,

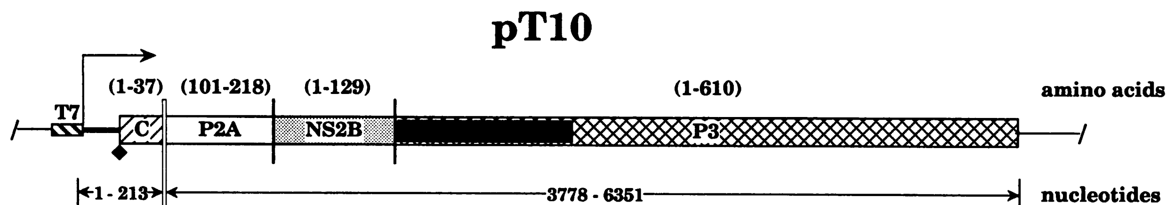

FIG. 3. Schematic diagram of dengue virus type 2 protein and nucleic acid sequences expressed in the translation vector pT10. T7 RNA polymerase transcribes a chimeric RNA which contains nucleotides 1 to 213 fused to nucleotides 3778 to 6351 of the dengue virus genome. An initiator methionine codon at position 96 is designated by a closed diamond. Upon translation, the encoded polyprotein contains amino acids 1 to 37 of the dengue virus capsid protein, amino acids 101 to 218 of NS2A, all of NS2B, and the first 610 amino acids of NS3 (▩). The predicted protease domain of NS3 is indicated (■).

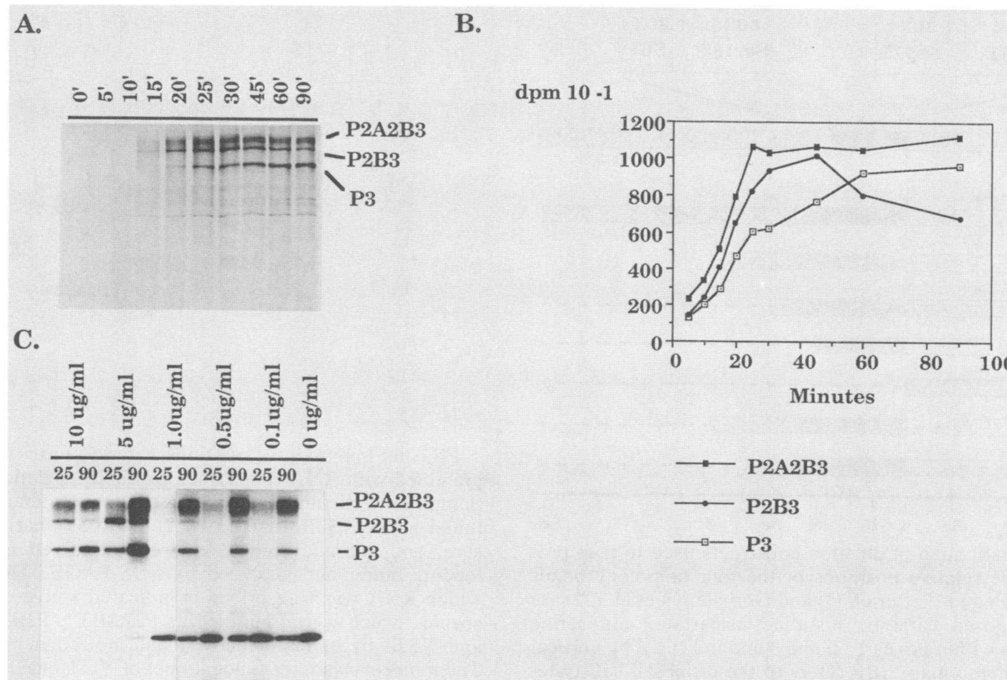

FIG. 4. Processing of proteins during in vitro translation of construct pT10. (A) Samples of a pT10-programmed translation were removed at the designated times (min) after initiation of translation and analyzed by SDS-PAGE. Precursors P2A2B3 and P2B3 as well as mature P3 are indicated. (B) Bands containing P2A2B3, P2B3, and P3 were excised from gels like that shown in panel A and quantitated as described in the text. (C) Samples of RNA transcribed from pT10 were serially diluted and translated in vitro. Samples of the translation mixtures were analyzed by SDS-PAGE. In order to equalize for [^{35}S]methionine incorporation, various amounts of lysate were loaded in each lane. Lanes 1 to 4 contained 1 μl of lysate; lanes 5, 6, 8, and 10 contained 2 μl of lysate; and lanes 7, 9, and 11 contained 4 μl of lysate. Mass of RNA per milliliter and the length of the translation incubation (min) are indicated. Locations of bands containing nonstructural protein precursors P2A2B3 and P2B3 as well as mature P3 are indicated.

total incorporation appeared to increase between 25 and 90 min of translation and proportionately more label was present in a 46-kDa reticulocyte-specific band. However, the ratio of P2A2B3 to P3 after 90 min of translation at 30°C appeared to be independent of the concentration of input RNA (Fig. 4C), suggesting that cleavage occurs in *cis*, although extremely efficient *trans* cleavage by the dengue virus proteinase cannot be ruled out.

We have used a second approach to examine whether the dengue virus polyprotein can be cleaved in *trans*. Polyproteins produced by pT13- and pT14-programmed translations contain an inactive protease, but the potential cleavage sites are intact (see below). These polyproteins were used as substrates for the active proteinase produced by a pT10-programmed translation, and no detectable cleavage of these polyproteins occurred (data not shown). The lack of detectable *trans* cleavage in these experiments is consistent with the results of the kinetic and dilution sensitivity experiments which suggested that cleavage occurred at the 2A/2B and 2B/3 junctions only in *cis*.

Deletion mapping of the dengue virus protease. On the basis of molecular modeling studies, both Bazan and Fletterick (3) and Gorbalenya et al. (21) have predicted the precise boundaries of the flavivirus protease domain. This predicted domain is illustrated schematically in Fig. 5 in relation to the dengue virus polyprotein. The entire domain is hypothesized to span the first 180 amino acids of NS3 (solid boxes) and to contain four subregions or boxes of homology with serine proteases, the first three of which contain the three elements of the catalytic triad and the fourth of which is involved in substrate binding. To test these predictions, we constructed a series of deletion constructs that together span the entire length of NS3 and that delete large regions of P2A and NS2B (Fig. 5; Table 2); translation mixes were programmed with RNA transcribed from these constructs, and the resulting protein products were immunoprecipitated with αNS2B and αNS3 sera and analyzed by SDS-PAGE (Fig. 6).

Constructs pT10, pT11, and pT12 contain the entire proposed protease domain (Fig. 5), and from these constructs polypeptides with the molecular masses and immunoreactivities of mature NS2B and of the truncated forms of NS3 (P3, P3', and P3'') were detected (Fig. 6A). In contrast, pT13-programmed translations did not produce any (truncated) NS3 or NS2B (Fig. 6B), indicating that this deletion abolished activity, presumably by invading the protease domain (Fig. 5). Thus, the protease domain encompasses a maximum of 184 amino acids at the N terminus of NS3. Polypeptides consistent in molecular mass and immune reactivity with a P2B3 processing intermediate could also be detected in pT10-, pT11-, and pT12-programmed translations but not in pT13-programmed translations (Fig. 4 and 6).

Translation of RNA transcribed from pT14 yielded a polypeptide which was consistent in molecular mass and immunoreactivity with that predicted for P2A2B'. Processing did not occur at the 2A/2B site. The location of the band and the amount expressed from pT14 were such that if this precursor had been present upon translation of RNA from constructs such as pT10, it would have been readily detected. This supports the earlier conclusions that P2A2B was never generated during processing in vitro.

To examine the N-terminal boundary of the protease domain, that is, whether sequences in P2A or NS2B are

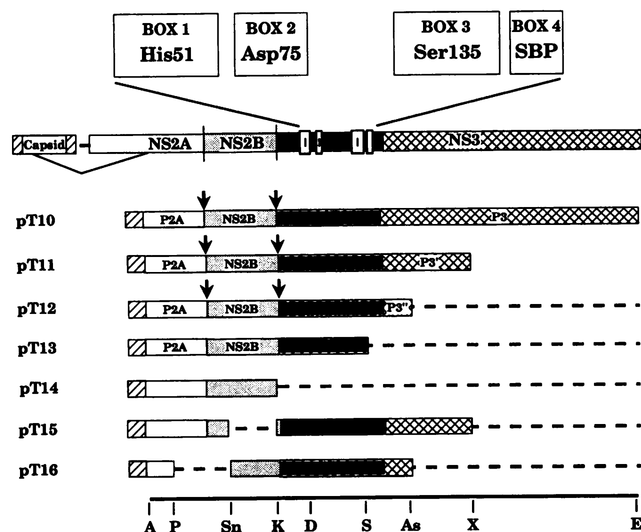

FIG. 5. Schematic map of deletion constructs used to map protease activity. The relative positions of the four homology boxes proposed by Bazan and Fletterick (3) and Gorbalenya et al. (21) are shown. The proposed catalytic histidine, aspartate, and serine residues are listed within boxes 1, 2, and 3 and indicated by vertical lines on the schematic map. SBP refers to the proposed substrate-binding pocket. Gene products P2A and NS2B are designated by open and stippled boxes, respectively. Capsid protein is indicated by hatched lines. The putative protease and helicase domains of NS3 are designated by solid and crosshatched boxes, respectively. The structures of deletion clones are mapped below. The abbreviations for restriction sites used to generate deletion constructs are indicated as follows: A, *Ase*I; P, *Pvu*II; Sn, *Sna*BI; K, *Kpn*I (Asp718); D, *Dra*III; S, *Sal*I; As, *Asu*II; X, *Xho*I; and E, *Eco*RI. Cleavages at the 2A/2B or 2B/3 junctions are indicated by arrows. A detailed description of each construct is given in Table 2.

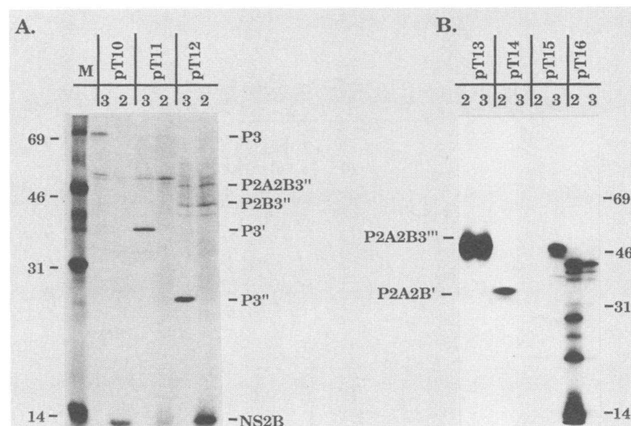

FIG. 6. Immunoprecipitations of in vitro translations programmed with RNA transcribed from NS3 deletion constructs. (A) Samples of reticulocyte lysates were denatured and immunoprecipitated with αNS2B (marked by 2) or αNS3 (marked by 3) immune sera, and immune complexes were dissolved in SDS-containing loading buffer and analyzed by SDS-PAGE. The construct from which RNA was transcribed is indicated above each lane. Protein species which were identified as P2A2B3″, P2B3″, P3, P3′, P3″, and NS2B by immunoreactivity and molecular mass are indicated. The molecular masses (kilodaltons) of ^{14}C-labeled protein standards (Amersham) are indicated. (B) Immunoprecipitations of in vitro translations from proteolytically inactive templates. Unprocessed precursors P2A2B3″ and P2A2B′ are designated as are the positions of molecular mass markers.

required for proteolytic activity, we analyzed the in vitro translation patterns produced from two deletion constructs, pT15 and pT16, which together delete almost all the P2A and NS2B sequences. When pT15-programmed translations were fractionated by using αNS2B, only low levels of immunoreactive species were observed upon overexposure of autoradiographs (Fig. 6B). The molecular masses of these species were not consistent with correct processing at the 2A/2B or 2B/3 cleavage site. The low level of immunoreactivity suggests that the major antigenic epitopes recognized by αNS2B have been deleted in this construct. Upon precipitation with αNS3, a small amount of aberrantly processed P3′ was detected. When pT16-programmed translations were immunoprecipitated and analyzed by SDS-PAGE, both αNS2B- and αNS3-immunoreactive species of aberrant molecular mass could be detected. Since the 2A/2B cleavage site had been removed by this deletion, only cleavage at the 2B/3 boundary could be examined. Thus, it is unclear whether sequences in P2A or NS2B are required for proteolytic activity or whether the deletions induce misfolding of the molecule such that the correct cleavage sites are not recognized.

Sequencing of viral proteins. In order to confirm that the cleavage events observed in vitro occur at the same sites as those utilized in vivo and to confirm the identities of the proteins produced in vitro, we compared the amino-terminal amino acid sequences of NS2B and NS3 produced in vitro with those of proteins isolated from infected cell lysates and obtained the N-terminal sequence of P2B3′. NS2B from dengue virus type 2-infected cells labeled with [^{35}S]methionine or [^{3}H]leucine was isolated by preparative immunoaffinity chromatography. Approximately 85% of the label in the purified NS2B used for sequencing consisted of mature NS2B, as determined by electrophoresis. The results clearly showed that serine 1345 of the dengue virus polyprotein is the amino-terminal residue of NS2B produced in vivo (Fig. 7A). This assignment is in agreement with data for other sequenced NS2B species (9, 48, 54), on the basis of sequence homology.

In order to sequence NS2B produced in vitro, pT11-programmed translations were immunoprecipitated with αNS2B. Approximately 85% of the NS2B immunoprecipitated was fully cleaved, and approximately 10% of the label was in P2B3′, which is coterminal with mature NS2B and contributes to the observed NS2B signal (see below). The amino terminus of in vitro-produced NS2B was indistinguishable from that found in vivo (Fig. 7B).

Sequencing of P3′ labeled in vitro in pT11-programmed translations identified the amino-terminal residue of P3′ as Ala-1476 of the dengue virus type 2 polyprotein (Fig. 7C). Peaks of [^{3}H]leucine at cycles 4 and 18 and of [^{3}H]valine at cycles 3, 7, and 13 make this assignment unambiguous. This result agrees perfectly with the sequence of NS3 isolated from dengue virus type 2-infected cells (4). The P3′ results are also of interest because the cleavage which produces the amino terminus of dengue virus type 2 NS3 is unique in that it appears to occur after a single basic amino acid; the cleavages to produce NS3 in all other sequenced flaviviruses occur after dibasic residues. The fact that the amino terminus of P3′ produced in vitro is the same as that of NS3 isolated from infected cells suggests that the dengue virus 2B/3 cleavage site is in fact different and that the observed terminus of NS3 does not arise from cleavage at an upstream

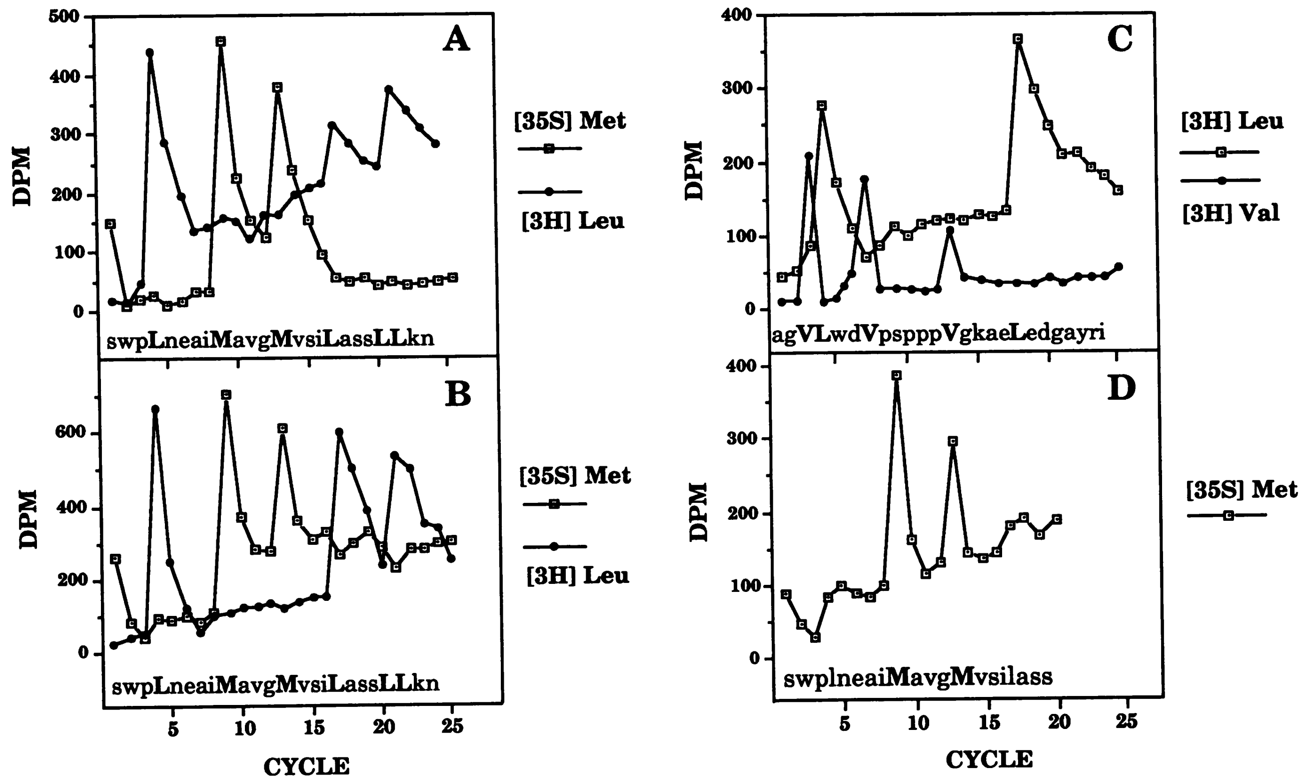

FIG. 7. Amino-terminal sequencing of viral proteins. Data in each panel are plotted as radioactivity per Edman degradation cycle versus cycle number. (A) In vivo-labeled NS2B. (B) In vitro-labeled NS2B. (C) In vitro-labeled P3'. (D) Precursor P2B3'. The amino-terminal sequences of NS2B, NS3, and NS2B3 deduced from the nucleotide sequence of the RNA genome are indicated below each sequence panel, with the leucine, valine, and methionine residues identified by Edman degradation highlighted in boldface capital letters. The burst of ^{35}S disintegrations per minute observed in the first sequencing cycle of immunoaffinity-purified NS2B (both in vivo and in vitro) is not consistent with the deduced sequence of NS2B and is presumably contributed by contaminants which coprecipitate with NS2B. The single-letter amino acid code used is as follows: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

site characterized by dibasic residues followed by amino-terminal nibbling (4).

In order to define further the specificity of cleavage events and definitively identify processing intermediates, the putative P2B3' was isolated from pT11-programmed translations by preparative SDS-PAGE and sequenced. Peaks of ^{35}S were observed at positions 9 and 13, aligning this species perfectly with the amino terminus of NS2B and confirming the previous identification based on gel mobility and immunoreactivity (Fig. 7D).

DISCUSSION

Cleavage of the dengue virus polyprotein in vitro. The kinetic experiments indicated that in vitro cleavage at the 2A/2B junction precedes that at the 2B/3 junction. These junctions were inactive as substrates in a *trans* cleavage assay and cleavage was insensitive to dilution, implying that cleavage at these two junctions occurs by an intramolecular mechanism. It is formally possible that NS3 is extremely active in *trans* such that sensitivity to dilution could not be demonstrated and that the negative results of the *trans*-processing experiments resulted from the perturbed tertiary structure of substrates, although we have used similar methods to demonstrate *trans* cleavage of the Sindbis virus nonstructural polyprotein (29). Differences in the rate of translation and in the association of membranes with processing intermediates could also influence the observed rate of cleavage in vivo.

Cleavage at the 2A/2B and 2B/3 junctions in vitro proceeds with the same specificity as that found in vivo. Translation products are cleaved at the correct sites of cleavage, and nonspecific cleavage is rarely observed. While the kinetics of cleavage in vitro are much slower than those observed in vivo, it is highly probable that the fidelity and order of in vitro cleavage are a true reflection of in vivo processing. The use of improved expression systems and in vitro mutagenesis of cleavage sites will be useful in establishing whether the order of cleavage observed is obligatory or whether it simply represents the kinetically favored order.

Testing the flavivirus protease model. Positive identification of NS3 as a flavivirus proteinase will stimulate research on the nature of the catalytic and substrate recognition residues involved in proteolysis. The specific predictions as to which histidine, aspartate, and serine residues form the catalytic triad are testable through site-specific mutagenesis, and mutants which are catalytically active in vitro can be tested for biological activity in vivo through the use of infectious cDNA clones (40). NS3 shares the greatest amount of sequence similarity with the Sindbis virus capsid protein protease domain, and site-directed mutagenesis of

the NS3 protease domain will be greatly assisted by molecular modeling studies once the coordinates of the Sindbis capsid protein become available (5).

Four specific predictions of the protease model were addressed by experiments presented here (3, 21). Our experimental results are consistent with the predictions that the protease domain consists of approximately 180 amino acids at the N terminus of NS3 and could retain function when severed from the remainder of NS3, which forms a putative helicase domain. However, our results are not consistent with the prediction that an internal conserved cleavage site in NS3 is utilized to separate the helicase and protease domains or the prediction that cleavage of the flavivirus polyprotein by NS3 is an ATP-dependent process. The antiserum used in our experiments was directed specifically against the protease domain, and therefore any internal cleavage of NS3 in vitro or in vivo should have been detected. Proteolysis occurred even in those cases in which the helicase domain had been deleted, making it unlikely that hydrolysis of ATP is absolutely required for proteolysis, although the possibility that it has an effect in vivo has not been eliminated.

The presence of protease and helicase domains within a single viral protein is a common structural motif found in alphavirus, pestivirus, potyvirus, and coronavirus proteins as well as flaviviruses (22). The primary function of a viral proteinase is to posttranslationally regulate the production of individual gene products from a polyprotein precursor. The viral proteinase can also produce processing intermediates which may themselves be functional components of the viral life cycle. The choice between emphasizing proteinase function and emphasizing helicase function would depend on the specific needs of the virus at that point in the life cycle. It appears likely that the main function of mature NS3 is as a helicase, since the initial cleavages releasing NS2B and NS3 most likely occur in *cis* and mature NS3 does not appear to work efficiently in *trans*.

Implications for dengue virus polyprotein processing. A number of viral proteinases function both in *cis* and in *trans* to cleave polyproteins in vivo (32). Like other proteinases of positive-stranded RNA viruses, NS3 may also function both in *cis* and in *trans*. The amino terminus of NS4B is believed to be generated by a signalase cleavage event which presumably occurs cotranslationally upon insertion of the hydrophobic tail of NS4A into the lumen of the endoplasmic reticulum (9, 48). Once this event occurs, the dengue virus polyprotein backbone is severed, and by definition, any processing events that occur downstream of this scission must occur in *trans*, and if NS3 is the proteinase responsible for cleavage at the 4B/5 boundary then it must cleave in *trans*.

Processing events consistent with scission at the 3/4A junction are observable in vitro (F. Preugschat, unpublished data), and putative NS34A intermediates can be detected in vivo (Fig. 2B). This implies that the kinetics of 3/4A cleavage are slower than the kinetics of 2A/2B and 2B/3 cleavage. It is possible that NS34A is a precursor that is restricted to the plane of the endoplasmic reticulum by a carboxy-terminal membrane-spanning segment and that this is the form of proteinase that is responsible for *trans* cleavage at the 4B/5 junction. By restricting the proteinase to the plane of the endoplasmic reticulum, the concentration dependence of the *trans* cleavage step would be reduced and result in rapid kinetics of cleavage in infected cells.

The flavivirus capsid protein undergoes a complex two-step maturation process in infected cells. As the carboxy terminus of the capsid protein is translated, it acts as a membrane insertion sequence for a cotranslational signalase-mediated cleavage that generates the amino terminus of the prM protein. Nascent intracellular capsid protein possesses a C-terminal membrane-spanning segment that is cleaved prior to assembly of the virion (37). This cleavage event occurs on the carboxy-terminal side of several basic residues and could be potentially mediated in *trans* by NS3. The initial signalase-mediated cleavage can be observed by using an in vitro translation system, but the secondary maturation cleavage that removes the membrane insertion sequence has not been observed in vitro (37, 44). By using an in vitro expression system designed to analyze cleavages at the 3/4A, 4B/5, and C/prM junctions, it should be possible to reconstruct the flaviviral protein-processing pathway by using the experimental approaches described in this paper.

ACKNOWLEDGMENTS

We thank Richard Kuhn and Ellen Strauss for stimulating discussions and editorial prowess; David Teplow and Tammy Bauer for expertise and assistance with protein sequencing; and Joel Dalrymple for furnishing cells, virus, and hyperimmune antiserum. We also thank A. E. Gorbalenya for communicating results prior to publication.

Frank Preugschat gratefully acknowledges the financial support of the Canadian Natural Sciences and Engineering Research Council (NSERC) and Gordon Ross. This work was supported by Public Health Service grant AI20612 from the National Institutes of Health and by a grant from the World Health Organization.

LITERATURE CITED

1. **Bancroft, W. H., R. M. Scott, K. H. Eckels, C. H. Hoke, T. E. Simms, K. D. T. Jesrani, P. L. Summers, D. R. Dubois, D. Tsoulos, and P. K. Russell.** 1984. Dengue virus type 2 vaccine: reactogenicity and immunogenicity in soldiers. J. Infect. Dis. **149:**1005–1010.
2. **Bancroft, W. H., F. H. Top, Jr., K. H. Eckels, J. H. Anderson, Jr., J. M. McCown, and P. K. Russell.** 1981. Dengue-2 vaccine: virological, immunological, and clinical responses of six yellow fever-immune recipients. Infect. Immun. **31:**698–703.
3. **Bazan, J. F., and R. J. Fletterick.** 1989. Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology **171:**637–639.
4. **Biedrzycka, A., M. R. Cauchi, A. Bartholomeusz, J. J. Gorman, and P. J. Wright.** 1987. Characterization of protease cleavage sites involved in the formation of envelope glycoprotein and three nonstructural proteins of dengue virus type 2, New Guinea C strain. J. Gen. Virol. **68:**1317–1326.
5. **Boege, U., M. Cygler, G. Wengler, P. Dumas, and J. Tsao.** 1989. Sindbis virus core protein crystals. J. Mol. Biol. **208:**79–82.
6. **Calisher, C. H., N. Karabatsos, J. M. Dalrymple, R. E. Shope, J. S. Porterfield, E. G. Westaway, and W. E. Brandt.** 1989. Antigenic relationships between flaviviruses as determined by cross neutralization tests with polyclonal antisera. J. Gen. Virol. **70:**37–43.
7. **Calisher, C. H., R. E. Shope, W. Brandt, J. Casals, N. Karabatsos, F. A. Murphy, R. B. Tesh, and M. E. Wiebe.** 1980. Proposed antigenic classification of registered arboviruses. I. Togaviridae, *Alphavirus*. Intervirology **14:**229–232.
8. **Chamberlin, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98:**132–135.
9. **Chambers, T. J., D. W. McCourt, and C. M. Rice.** 1989. Yellow fever virus proteins NS2a, NS2b and NS4b: identification and partial N-terminal amino acid sequence analysis. Virology **169:** 100–109.
10. **Cleaves, G. R.** 1985. Identification of dengue type 2 virus-specific high molecular weight proteins in virus-infected BHK

cells. J. Gen. Virol. **66**:2767–2771.

11. **Cleaves, G. R., and D. T. Dubin.** 1979. Methylation status of intracellular dengue type 2 40S RNA. Virology **96**:158–165.
12. **Coia, G., M. D. Parker, G. Speight, M. E. Byrne, and E. G. Westaway.** 1988. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. J. Gen. Virol. **69**:1–21.
13. **Condreay, L. D., R. H. Adams, J. Edwards, and D. T. Brown.** 1988. Effect of actinomycin D and cycloheximide on replication of Sindbis virus in *Aedes albopictus* (mosquito) cells. J. Virol. **62**:2629–2635.
14. **Dasso, M. C., and R. J. Jackson.** 1989. On the fidelity of mRNA translation in the nuclease treated rabbit reticulocyte lysate system. Nucleic Acids Res. **17**:3129–3144.
15. **Deubel, V., R. M. Kinney, and D. W. Trent.** 1988. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology **165**:234–244.
16. **Dunn, S. D.** 1986. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal. Biochem. **157**:144–153.
17. **Eckels, K. H., V. R. Harrison, P. L. Summers, and P. K. Russell.** 1980. Dengue-2 vaccine: preparation from a small-plaque virus clone. Infect. Immun. **27**:175–180.
18. **Ehrenkranz, N., A. Ventura, R. Cuadrado, W. Pond, and J. Porter.** 1971. Pandemic dengue in Caribbean countries and the southern United States—past, present, and potential problems. New Engl. J. Med. **285**:1460–1469.
19. **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.
20. **Gorbalenya, A. E., A. P. Donchenko, V. M. Blinov, and E. V. Koonin.** 1989. Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases: a distinct protein superfamily with a common structural fold. FEBS Lett. **243**: 103–114.
21. **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.
22. **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1988. A conserved NTP-binding motif in putative helicases. Nature (London) **333**:22.
23. **Gruenberg, A., W. S. Woo, A. Biedrzycka, and P. J. Wright.** 1988. Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, New Guinea C and PUO-218 strains. J. Gen. Virol. **69**:1391–1398.
24. **Gubler, D. J.** 1989. *Aedes aegypti* and *Aedes aegypti*-borne disease control in the 1990s: top down or bottom up. Am. J. Trop. Med. Hyg. **40**:571–578.
25. **Hahn, C. S., E. G. Strauss, and J. H. Strauss.** 1985. Sequence analysis of three Sindbis virus mutants temperature-sensitive in the capsid autoprotease. Proc. Natl. Acad. Sci. USA **82**:4648–4652.
26. **Hahn, C. S., and J. H. Strauss.** 1990. Site-directed mutagenesis of the proposed catalytic amino acids of the Sindbis virus capsid protein autoprotease. J. Virol. **64**:3069–3073.
27. **Hahn, Y. S., R. Galler, T. Hunkapiller, J. Dalrymple, J. H. Strauss, and E. G. Strauss.** 1988. Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology **162**:167–180.
28. **Hardy, W. R., and J. H. Strauss.** 1988. Processing the nonstructural polyproteins of Sindbis virus: study of the kinetics in vivo by using monospecific antibodies. J. Virol. **62**:998–1007.
29. **Hardy, W. R., and J. H. Strauss.** 1989. Processing the nonstructural polyproteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in *cis* and in *trans*. J. Virol. **63**:4653–4664.
30. **Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid-solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
31. **Kelley, P. M., and M. J. Schlesinger.** 1982. Antibodies to two major chicken heat shock proteins cross-react with similar proteins in widely divergent species. Mol. Cell. Biol. **2**:267–274.
32. **Kraeusslich, H. G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
33. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Markoff, L.** 1989. In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein. J. Virol. **63**: 3345–3352.
36. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035–10038.
37. **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.
38. **Ozden, S., and B. Poirier.** 1985. Dengue virus induced polypeptide synthesis. Arch. Virol. **85**:129–137.
39. **Rice, C. M., R. Aebersold, D. B. Teplow, J. Pata, J. R. Bell, A. V. Vorndam, D. W. Trent, M. W. Brandriss, J. J. Schlesinger, and J. H. Strauss.** 1986. Partial N-terminal amino acid sequences of three nonstructural proteins of two flaviviruses. Virology **151**:1–9.
40. **Rice, C. M., A. Grakoui, R. Galler, and T. J. Chambers.** 1989. Transcription of infectious yellow fever RNA from full-length cDNA templates produced by *in vitro* ligation. New Biol. **1**:285–296.
41. **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.
42. **Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang.** 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J. Virol. **61**:3809–3819.
43. **Ruiz-Linares, A., M. Bouloy, M. Girard, and A. Cahour.** 1989. Modulations of the *in vitro* translational efficiencies of yellow fever virus mRNAs: interactions between coding and noncoding regions. Nucleic Acids Res. **17**:2463–2474.
44. **Ruiz-Linares, A., A. Cahour, P. Desprès, 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.
45. **Schatzmayer, H. G., R. M. R. Nogueira, and A. P. A. Travassos da Rosa.** 1986. An outbreak of dengue virus at Rio de Janeiro (1986). Mem. Inst. Oswaldo Cruz Rio J. **81**:245–246.
46. **Scott, R. M., K. H. Eckels, W. H. Bancroft, P. L. Summers, J. M. McCown, J. H. Anderson, and P. K. Russell.** 1983. Dengue 2 vaccine: dose response in volunteers in relation to yellow fever status. J. Infect. Dis. **148**:1055–1060.
47. **Smith, G. W., and P. W. Wright.** 1985. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and *Aedes albopictus* cells. J. Gen. Virol. **66**:559–571.
48. **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.
49. **Spindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**:132–141.
50. **Svitkin, Y. V., V. N. Lyapustin, V. A. Lashkevich, and V. I. Agol.** 1984. Differences between translation products of tick-borne encephalitis virus RNA in cell-free systems from Krebs-2 cells and rabbit reticulocytes: involvement of membranes in the processing of nascent precursors of flavivirus structural pro-

teins. Virology **135**:536–541.

51. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
52. **Wengler, G., M. Beato, and G. Wengler.** 1979. *In vitro* translation of 42S virus-specific RNA from cells infected with the flavivirus West Nile virus. Virology **96**:516–529.
53. **Wengler, G., G. Wengler, and J. Gross.** 1978. Studies on virus specific nucleic acids synthesized in vertebrate and mosquito cells infected with flaviviruses. Virology **89**:423–437.
54. **Wright, P. S., M. R. Cauchi, and M. L. Ng.** 1989. Definition of the carboxy termini of the three glycoproteins specified by dengue virus type 2. Virology **171**:61–67.