JOURNAL OF VIROLOGY, Dec. 1992, p. 7225–7231
0022-538X/92/127225-07$02.00/0

Vol. 66, No. 12

Mutational Analysis of the Octapeptide Sequence Motif at the NS1-NS2A Cleavage Junction of Dengue Type 4 Virus

MICHELE PETHEL, BARRY FALGOUT,† AND CHING-JUH LAI*

Molecular Viral Biology Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 5 June 1992/Accepted 9 September 1992

We have previously shown that proper processing of dengue type 4 virus NS1 from the NS1-NS2A region of the viral polyprotein requires a hydrophobic N-terminal signal and the downstream NS2A. Results from deletion analysis indicate that a minimum length of eight amino acids at the C terminus of NS1 is required for cleavage at the NS1-NS2A junction. Comparison of this eight-amino-acid sequence with the corresponding sequences of other flaviviruses suggests a consensus cleavage sequence of Met/Leu-Val-Xaa-Ser-Xaa-Val-Xaa-Ala. Site-directed mutagenesis was performed to construct mutants of NS1-NS2A that contained a single amino acid substitution at different positions of the consensus cleavage sequence or at the immediate downstream position. Three to eight different substitutions were made at each position. A total of 50 NS1-NS2A mutants were analyzed for their cleavage efficiency relative to that of the wild-type dengue type 4 virus sequence. As predicted, nearly all substitutions at positions P1, P3, P5, P7, and P8, occupied by conserved amino acids, yielded low levels of cleavage, with the exception that Pro or Ala substituting for Ser (P5) was tolerated. Substitutions of an amino acid at the remaining positions occupied by nonconserved amino acids generally yielded high levels of cleavage. However, some substitutions at nonconserved positions were not tolerated. For example, substitution of Gly or Glu for Gln (P4) and substitution of Val or Glu for Lys (P6) each yielded a low level of cleavage. Overall, these data support the proposed cleavage sequence motif deduced by comparison of sequences among the flaviviruses. This study also showed that in addition to the eight-amino-acid sequence, the amino acid immediately following the NS1-NS2A cleavage site plays a role in cleavage.

The four serotypes of dengue virus are members of *Flaviviridae*, a family of 60 to 70 viruses that are transmitted by mosquitos or ticks. Dengue viruses and many other flaviviruses, including yellow fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus, cause major epidemics associated with human illnesses of various degrees of severity (17, 28). Results from studies on the morphology, chemical, and antigenic structure indicate that members of the flavivirus family share a high degree of similarity (33). The complete or nearly complete sequences of several flavivirus genomes have been determined (9, 10, 16, 19, 23–25, 30, 32, 36, 39, 41, 44). Analysis of these sequences showed that 95% of the flavivirus genome encodes a long polyprotein in the order NH_2–C–pre-M/M–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–COOH. The polyprotein is co- and posttranslationally processed to produce the 10 or more individual structural proteins and nonstructural proteins. There is evidence that the three virus structural proteins, C, pre-M/M, and E, are cotranslationally cleaved by a signal peptidase (26, 37). The proteolytic processing of the nonstructural protein NS2A-NS2B-NS3-NS4A-NS4B-NS5 domain has been studied for a number of flaviviruses, including dengue type 4 (DEN 4) virus (4, 12–14), DEN 2 virus (34, 35), yellow fever virus (6–8), and West Nile virus (43). These studies supported the proposal that NS3 contains a catalytically active proteinase domain (1, 15). In addition, NS2B is also required for cleavage, presumably as a cofactor for functional activity (8, 14, 43). This two-component viral proteinase mediates cleavage at the NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5 junctions. These cleavage junctions share a common sequence motif in which the sequence of two basic amino acids, Lys-Arg or Arg-Arg, is followed by Ala, Ser, or Gly. Cleavage at the NS4A-NS4B junction of the polyprotein can occur in the absence of the viral NS2B-NS3 protease, and this cleavage is presumably mediated by a signalase because the cleavage site is preceded by a long hydrophobic sequence (4).

Sequence analysis indicates that the cleavage sequence at the NS1-NS2A junction does not contain a dibasic amino acid motif or a long hydrophobic amino acid stretch, suggesting that a novel cleavage mechanism may be involved. We have shown earlier that efficient cleavage at the DEN 4 virus NS1-NS2A junction requires the N-terminal hydrophobic signal of NS1 and downstream NS2A in *cis* (12). The requirement for a signal suggests that cleavage of the NS1-NS2A precursor occurs at some intracellular site along the exocytic pathway (13). Deletion analysis has shown that an octapeptide sequence at the C terminus of NS1 is also required for efficient cleavage at this junction (18). On the basis of comparison of this eight-amino-acid sequence with the corresponding sequences of 13 other flaviviruses, we have proposed that the flavivirus consensus NS1-NS2A cleavage sequence appears to be P8-Met/Leu-Val-Xaa-Ser-Xaa-Val-Xaa-Ala-P1, in which amino acids at positions P6, P4, and P2 vary (18). The experimental evidence to support the proposed sequence motif is still lacking. For this reason, we analyzed the cleavage phenotype of DEN 4 NS1-NS2A constructs that contained a single substitution for one of these eight amino acids or the amino acid immediately following the cleavage site. For the most part, the results of this analysis are in good agreement with the cleavage sequence motif deduced from the sequence comparison.

* Corresponding author.
† Present address: Laboratory of Vector-Borne Viral Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.

MATERIALS AND METHODS

Cells and viruses. CV-1 cells and human thymidine kinase-negative 143 cells were grown in minimal essential medium (MEM) containing 10% fetal bovine serum as described previously (45). The WR strain of vaccinia virus was used for construction of recombinant vaccinia viruses. Recombinant vaccinia virus vSC8, which contains a bacterial β-galactosidase gene but no dengue virus cDNA insert, was used as a negative control (5). All recombinant vaccinia viruses described in this report were grown and titered in CV-1 cells. The recombinant virus vTF7-3, which contains the T7 RNA polymerase gene under the control of the vaccinia virus early/late promoter p7.5, was kindly supplied by B. Moss (11).

Site-directed mutagenesis and construction of vaccinia virus recombinants. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., or New England BioLabs, Inc. A GEMSEQ kit (Promega Corp.) or Sequenase 2.0 kit (United States Biochemical Corp.) was used for DNA sequencing. Oligonucleotides encoding amino acid changes were synthesized on a model 391 PCR-Mate Oligosynthesizer (Applied Biosystems Inc.). Recombinant DNA construction was performed according to standard procedures. Initially, the pSC11/NS1-NS2A plasmid construct obtained earlier (12) was digested with *Bam*HI and *Eco*RI to produce the 1.8-kb NS1-NS2A DNA fragment for insertion into the M13mp19 cloning vector. Site-directed mutagenesis for the construction of substitutions at positions P1, P2, P3, and P1′ was conducted by using a Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad Laboratories) as suggested by the manufacturer. Mutations contained in the various M13mp19 recombinant DNA constructs were verified by sequence analysis, and the 1.8-kb *Bam*HI-*Eco*RI NS1-NS2A DNA fragment was introduced into the pSC11/NS1-NS2A intermediate cloning vector. The wild-type vaccinia virus WR strain and the recombinant plasmid pSC11/NS1-NS2A were used for the construction of recombinant vaccinia viruses as described previously (12, 45).

pTM-1 transient expression system. Cleavage of mutant NS1-NS2A constructs encoding an amino acid substitution at position P4, P5, P6, P7, or P8 was studied by using the pTM-1/T7 transient expression system (11, 29). To facilitate mutant DNA construction, two silent mutations (G-3473 to T and C-3476 to A) that generated a new *Pst*I site at nucleotide position 3472 of the DEN 4 virus sequence were introduced into the wild-type NS1-NS2A DNA fragment by site-directed mutagenesis. The mutated DNA fragment was subsequently inserted into the pSC11 vector to yield plasmid pSC11-NS1-NS2A (*Pst*I).

The ATG codon within the *Nco*I site (CCATGG) of the pTM-1 plasmid vector is the codon of choice for initiation of protein synthesis (29). To prepare the NS1-NS2A DNA sequence for insertion into the pTM-1 vector, a polymerase chain reaction (PCR) was performed, using the pSC11-NS1-NS2A (*Pst*I) DNA template, oligonucleotide D179 (5′-CCCA GATTCATGAACACTTCAATGGCTATG-3′), which contains an ATG codon within the flanking *Bsp*HI sequence, and oligonucleotide 1852 (5′-TCTGTGAGCGTATCCCAA ACG-3′), which is located within the pSC11 DNA downstream of the DEN 4 virus NS1-NS2A insert. The PCR DNA product (1.8 kb) was cleaved by *Bsp*HI and *Sma*I prior to its insertion between the *Nco*I and *Stu*I sites of the pTM-1 vector, yielding plasmid pTM-1-NS1-NS2A (*Pst*I). To construct mutant plasmids, the DNA fragment between the unique *Pst*I site at position 3472 and the unique *Nco*I site at position 3220 of the DEN 4 virus sequence was replaced with the corresponding DNA fragment encoding an amino acid change. For this purpose, a series of mutant DNA fragments was generated by PCR, using the wild-type NS1-NS2A DNA template primed by a negative-strand oligonucleotide carrying the mutation to be introduced and the *Pst*I cleavage sequence. The positive-strand primer was oligonucleotide 2410, which contains the sequence 5′-AAGGCTATGCC ACGCAAA-3′ located upstream of the unique *Nco*I site. The PCR product was cleaved by *Nco*I and *Pst*I and was used to replace the wild-type DNA fragment in pTM-1-NS1-NS2A (*Pst*I). All plasmid constructs were sequenced across the mutation site for verification.

Preparation of ^{35}S-labeled cell lysates and immunoprecipitation. Confluent CV-1 cells in a six-well dish were infected with a recombinant vaccinia virus at a multiplicity of infection of 4.2 PFU per cell in MEM containing 2% fetal bovine serum (MEM-2). At 24 h postinfection, cells were starved for methionine by replacing the MEM-2 with methionine-free MEM-2. After 1 h, the medium was replaced with 0.6 ml of the same medium containing 60 μCi of L-[^{35}S]methionine (>800 Ci/mmol; Amersham Corp.). At the end of 2 h, the labeling medium was removed and cells were lysed by adding 0.5 ml of radioimmunoprecipitation assay buffer (0.1 M Tris hydrochloride [pH 7.5], 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40) and 0.1% sodium dodecyl sulfate (SDS). The cell lysate was collected, centrifuged for 15 min in a microcentrifuge, and stored at −20°C until use.

To 50 μl of the ^{35}S-labeled cell lysate was added 1 μl of normal mouse ascitic fluid. The lysate mixture was incubated on ice overnight. Excess Pansorbin (Calbiochem) was added, and the incubation continued for an additional 1.5 h. The mixture was then centrifuged, and the resulting supernatant was transferred to another tube and immunoprecipitated with 1 μl of DEN 4 virus hyperimmune mouse ascitic fluid. Following incubation for 2 h on ice, excess Pansorbin was added. After 1.5 h, the lysate mixture was again centrifuged and the pellet was washed twice with radioimmunoprecipitation assay buffer containing 2% SDS.

Alternatively, confluent CV-1 cells in a six-well dish were infected with recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 8.3 PFU per cell in 0.5 ml of MEM per well without fetal bovine serum. After 30 min, the inoculum was removed and cells were transfected with 3 μg of wild-type plasmid pTM-1-NS1-NS2A (*Pst*I) or a recombinant mutant plasmid plus 15 μl of Lipofectin (Life Technologies, Inc.) in 1 ml of MEM. At 3.5 h postinfection, 1 ml of MEM containing 20% fetal bovine serum was added to each well. At 24 h after infection, cells were starved for methionine by replacing the medium with methionine-free MEM containing a final concentration of 124 mM NaCl. After 20 min, the medium was removed and replaced with 0.6 ml of the same medium containing 100 μCi of L-[^{35}S]methionine per ml. At the end of 1.5 h, the labeling medium was removed and cells were lysed as described above. Immunoprecipitation of the lysate was performed by using hyperimmune mouse ascitic fluid except that the preabsorption step with normal mouse ascitic fluid was omitted.

Polyacrylamide gel electrophoresis and fluorography. The immunoprecipitate pellets were resuspended in 1× protein loading buffer (50 mM Tris hydrochloride [pH 6.8], 5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) (45) and boiled for 5 min prior to loading on an SDS–12% polyacrylamide gel. Fluorography was done as previously described (3).

Quantitation of NS1-NS2A cleavage. The percent cleavage of the wild-type or mutant NS1-NS2A polyprotein was determined by measuring the radioactivity present in the

NS1 and NS1-NS2A bands excised from the dried gel. The wild-type NS1-NS2A polyprotein expressed by recombinant vaccinia virus was completely processed to yield authentic NS1, as no labeled precursor NS1-NS2A band was detected following the 2-h labeling (see Results). Under the same infection and labeling conditions, the total radioactivity measured for NS1-NS2A of several mutants that did not produce detectable NS1 averaged 10.2-fold less than that found for the wild-type NS1 band. The reduced detection of the NS1-NS2A polyprotein compared with processed NS1 was probably not due to the instability of the uncleaved polyprotein, since this precursor could stably accumulate for at least 30 min and only slowly degraded thereafter under conditions that inhibited the polyprotein processing (13). This finding suggests that the NS1-NS2A precursor exhibited a lower level of binding affinity to hyperimmune mouse ascitic fluid than did NS1. This differential immunoprecipitation was taken into consideration in the quantitation of the cleavage levels for NS1-NS2A mutants.

In studies using the pTM-1/T7 transient expression system, wild-type NS1-NS2A DNA produced predominantly cleaved NS1, but a minor band representing the uncleaved NS1-NS2A precursor was also detected. The cleavage efficiency of wild-type NS1-NS2A was found to be approximately 80%. The cleavage efficiency of the wild-type NS1-NS2A polyprotein measured in each transfection experiment was used to normalize the cleavage efficiency for each mutant. Three or more independent transfection experiments were conducted for each mutant. A Student Q test (38) was used to determine whether any values of percent cleavage could be discarded. In general, the percent cleavage values obtained for each mutant were relatively consistent.

RESULTS

Experimental approach to the analysis of the DEN 4 virus NS1-NS2A cleavage sequence. By sequence comparison of a number of flavivirus polyproteins, we previously proposed that the consensus sequence for cleavage of flavivirus NS1-NS2A is, from position P8 to position P1, Met/Leu-Val-Xaa-Ser-Xaa-Val-Xaa-Ala (18). In this cleavage sequence, Xaa at positions P2, P4, and P6 are nonconserved amino acids and amino acids at the other positions are conserved.

To test this cleavage model in vivo, mutant constructs of DEN 4 virus NS1-NS2A cDNA were engineered to encode a single amino acid substitution at one of these eight positions or at the P1′ position immediately following the NS1-NS2A junction. NS1-NS2A mutants containing amino acid substitutions at positions P1′, P1, P2, and P3 were expressed by using the recombinant vaccinia virus-based system. The cleavage phenotype of these NS1-NS2A mutants was analyzed by detection of the cleaved NS1 product and its precursor NS1-NS2A polyprotein expressed in recombinant virus-infected cells. A second procedure of gene expression was used to facilitate analysis of a large number of amino acid substitutions at positions P4 to P8. The second approach utilized the T7 promoter-driven transient expression system (11). Mutant NS1-NS2A DNA was directly cloned into plasmid pTM-1 under the control of a T7 promoter. Plasmid constructs were used for transfection of CV-1 cells previously infected with a recombinant vaccinia virus, vTF7-3, expressing T7 polymerase. Cleavage of the expressed mutant NS1-NS2A polyprotein was similarly analyzed.

Mutational analysis of the octapeptide sequence at the DEN 4 NS1-NS2A junction. (i) Effects of substitutions for Ala (P1) or Thr (P2). Six amino acids, Asp, Arg, Leu, Phe, Ser, and Tyr, were selected to separately substitute for Ala at position P1, which is conserved among the previously sequenced flaviviruses. Analysis of the cleavage phenotype of this series of mutant NS1-NS2A constructs is shown in Fig. 1, and cleavage efficiencies are summarized in Table 1. It can be seen that each of the six substitutions for Ala expressed predominately uncleaved NS1-NS2A. Interestingly, substitution of Leu for Ala almost completely abolished cleavage of the NS1-NS2A polyprotein, presumably because Ala contains a small aliphatic side chain whereas Leu contains a much larger side chain. Similarly, substitution of Ser for Ala also greatly diminished the cleavage efficiency despite the fact that both amino acids contain small side chains. Although only a few substitutions were tested, these six amino acids contain a variety of side chains. This finding indicates that Ala (P1) is optimal for cleavage, consistent with the proposed sequence motif.

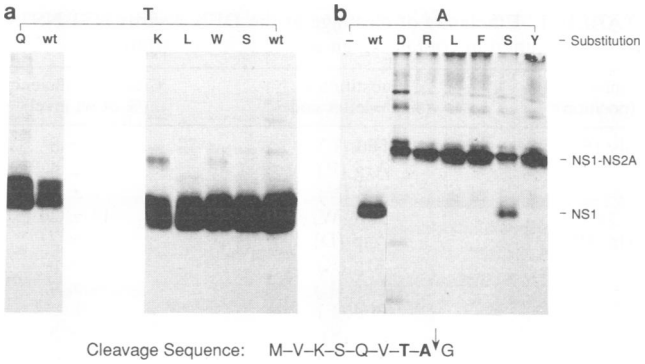

FIG. 1. Effects of substitutions for Thr (P2) (a) or Ala (P1) (b) on the cleavage of NS1-NS2A. Cleavage of DEN 4 virus NS1-NS2A was analyzed by immunoprecipitation of [^{35}S]methionine-labeled lysates prepared from recombinant vaccinia virus-infected cells. The precipitates were separated by polyacrylamide gel electrophoresis. The locations of the cleaved NS1 and uncleaved NS1-NS2A precursor are indicated. The vSC8-infected cell lysate is shown in lane –, and the cleaved NS1 product of wild-type NS1-NS2A is shown in lane wt. In all figures, the NS1-NS2A cleavage sequence is shown at the bottom; amino acids in bold letters indicate the positions for substitution. Amino acids are designated by the single-letter nomenclature system.

Six amino acids, Glu, Lys, Leu, Trp, Ser, and Gln, were also selected to replace Thr (P2), which is not conserved among flaviviruses. As shown in Fig. 1a and Table 1, four of the six substitutions produced the cleaved NS1 product in high or relatively high yield, ranging from approximately 67 to >100% of the wild-type level. This result indicates that these substitutions for Thr (P2) had little or no effect on NS1-NS2A cleavage. However, substitution of charged amino acid Glu or Lys yielded mutant NS1-NS2A polyproteins which were cleaved at a reduced level (18 or 34% of wild-type cleavage, respectively). Thus, a number of uncharged amino acids can substitute for Thr (P2) with little or no effect on cleavage.

(ii) Effects of substitutions for Val (P3) or Gln (P4). Four amino acids, Lys, Glu, Leu, and Gly, were substituted for Val (P3), which is invariant among flaviviruses with the exception of DEN 3 virus. As shown in Table 1 and Fig. 2, each of the four mutant recombinant vaccinia viruses produced predominantly the uncleaved NS1-NS2A polyprotein precursor. This result showed that cleavage of NS1-NS2A did not occur when Val (P3) was replaced with charged

TABLE 1. Efficiency of cleavage at the DEN 4 virus NS1-NS2A junction containing an amino acid substitution

Amino acid (position)[a]	Substitution (single-letter code)	Cleavage efficiency (% of wt level)[b]
Gly (P1')[c]	Glu (E)	19 ± 8
	Arg (R)	7 ± 4
	Val (V)	7 ± 4
	Trp (W)	48 ± 24
Ala (P1)	Asp (D)	3 ± 2
	Arg (R)	5 ± 5
	Leu (L)	4 ± 4
	Phe (F)	0
	Ser (S)	10 ± 3
	Tyr (Y)	0
Thr (P2)	Glu (E)	18 ± 5
	Lys (K)	34 ± 16
	Leu (L)	78 ± 10
	Trp (W)	67 ± 22
	Ser (S)	107 ± 15
	Gln (Q)	132 ± 19
Val (P3)	Glu (E)	4 ± 2
	Lys (K)	1 ± 2
	Leu (L)	4 ± 3
	Gly (G)	10 ± 4
Gln (P4)	Glu (E)	20 ± 8
	Leu (L)	85 ± 18
	Lys (K)	48 ± 18
	Gly (G)	13 ± 3
	Ala (A)	41 ± 13
	Arg (R)	65 ± 28
	Cys (C)	37 ± 25
	Trp (W)	66 ± 5
Ser (P5)	Arg (R)	8 ± 3
	Pro (P)	132 ± 4
	Thr (T)	20 ± 10
	Glu (D)	3 ± 3
	Ala (A)	100 ± 11
	Phe (F)	7 ± 1
Lys (P6)	Gln (Q)	84 ± 26
	Leu (L)	50 ± 15
	Glu (E)	28 ± 13
	Val (V)	43 ± 6
	Arg (R)	73 ± 23
	Ala (A)	102 ± 21
Val (P7)	Asp (D)	5 ± 4
	Gly (G)	1 ± 1
	Arg (R)	1 ± 1
Met (P8)	Trp (W)	6 ± 0
	Gly (G)	1 ± 1
	Thr (T)	4 ± 2
	Glu (E)	2 ± 1
	Lys (K)	2 ± 3
	Val (V)	20 ± 5
	Leu (L)	70 ± 11

[a] Cleavage at the NS1-NS2A junction of DEN 4 virus requires an eight-amino acid sequence at the NS1 C terminus which, from P8 to P1, is Met-Val-Lys-Ser-Gln-Val-Thr-Ala.

[b] Two or more independent experiments were performed to determine the cleavage efficiency of mutant NS1-NS2A relative to that of the wild-type (wt) NS1-NS2A control. The average cleavage efficiency with variations from the mean was calculated for each mutant polyprotein.

[c] Amino acid position P1' immediately following the cleavage site is occupied by Gly in the DEN 4 virus NS1-NS2A sequence.

amino acid Lys or Glu or with Leu, which is a large aliphatic nonpolar amino acid similar to Val.

Eight amino acids, Glu, Leu, Lys, Gly, Ala, Arg, Cys, and Trp, were chosen to substitute for Gln (P4), which is not conserved among flaviviruses. Analysis of NS1-NS2A cleavage observed in mutant DNA-transfected cells (Table 1 and Fig. 2a) showed that with the exception of Glu or Gly

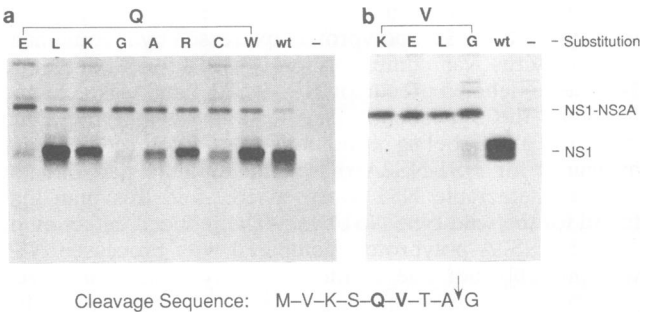

FIG. 2. Effects of substitutions for Gln (P4) or Val (P3) on the cleavage of NS1-NS2A. (a) Cleavage of NS1-NS2A polyproteins containing substitutions for Gln (P4) as produced by the vTF7-3/pTM-1 transient expression system; (b) cleavage of recombinant vaccinia virus-expressed NS1-NS2A mutants containing amino acid substitutions for Val (P3). Analysis of NS1-NS2A cleavage was performed as described for Fig. 1. Lane –, pTM-1-transfected cell lysate; lane wt, wild-type cleavage.

substitution, a moderate to high level of NS1-NS2A cleavage (37 to 85%) was observed for the amino acid substitutions. Both Glu and Gly substitutions reduced the cleavage efficiency to 20 and 13%, respectively. This result indicates that a number of amino acids can occupy position P4 and produce high levels of cleavage of NS1-NS2A.

(iii) Effects of substitutions for Ser (P5) or Lys (P6). Six substitutions (Glu, Thr, Pro, Arg, Ala, and Phe) were made for the conserved Ser at position P5 to test the effects of these mutations on the cleavage of the NS1-NS2A precursor. The results in Table 1 and Fig. 3 show that a charged amino acid in this position, such as Arg or Glu, reduced the level of cleavage of NS1-NS2A to 8 or 3%, respectively. Substitution of Thr for Ser, despite the fact that both amino acids have a hydroxyl group, also diminished the level of cleavage, as did the substitution of Phe (20 and 7%, respectively). Interestingly, Pro or Ala substituting for Ser had no effect on cleavage (132 or 100%, respectively, of the wild-type level). These substitutions were well tolerated.

Gln, Leu, Glu, Val, Arg, and Ala were selected to substitute for Lys at position P6, which is not conserved among

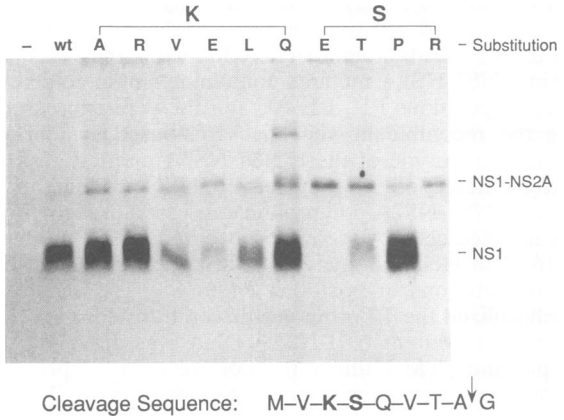

FIG. 3. Effects of substitutions of Lys (P6) or Ser (P5) on the cleavage of NS1-NS2A. DEN 4 virus NS1-NS2A polyproteins containing the indicated substitutions were produced by the vTF7-3/pTM-1 expression system. Analysis of NS1-NS2A cleavage was performed as described for Fig. 1. Lane –, infected cell lysate; lane wt, wild-type cleavage.

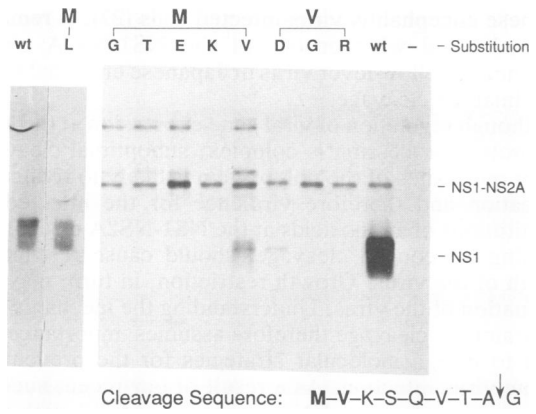

FIG. 4. Effects of substitutions for Met (P8) or Val (P7) on the cleavage of NS1-NS2A. The vTF7-3/pTM-1 transient expression system was used to analyze cleavage of NS1-NS2A polyproteins containing the indicated amino acid substitutions. Cleavage of NS1-NS2A was analyzed as described for Fig. 1. Lane wt, wild-type product; lane –, pTM-1-transfected cell lysate.

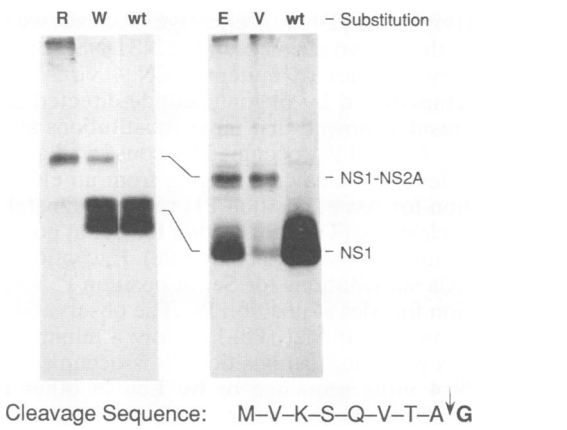

FIG. 5. Effects of substitutions for Gly (P1′) downstream of the NS1-NS2A cleavage site. Wild-type and mutant DEN 4 virus NS1-NS2A polyproteins were expressed by recombinant vaccinia viruses. Cleavage of NS1-NS2A was analyzed as described for Fig. 1. The cleavage sequence shown below indicates substitutions for Gly at position P1′. Lane wt, cleavage of the wild-type NS1-NS2A; lane –, pTM-1-transfected cell lysates.

flaviviruses. Analysis of the expressed DEN 4 virus products showed that most amino acid substitutions at this position had little or no effect on cleavage of the NS1-NS2A precursor (Fig. 3). On the other hand, substitution of negatively charged Glu for positively charged Lys (P6) reduced the cleavage efficiency to 28% (Table 1).

(iv) Effects of substitutions for Val (P7) or Met/Leu (P8). Val (P7) in the NS1-NS2A cleavage sequence is conserved among flaviviruses. Three amino acids, Asp, Gly, and Arg, were selected to replace Val at this position for analysis of the effect on cleavage of the mutant NS1-NS2A polyproteins. As shown in Fig. 4 and Table 1, Asp, Gly, or Arg substitution produced cleavage-defective NS1-NS2A. This analysis indicates that Val (P7) is required for NS1-NS2A cleavage.

Seven amino acid substitutions, Trp, Gly, Thr, Glu, Lys, Val, and Leu, were tested for the requirement of Met (P8) in the cleavage sequence of the DEN 4 virus NS1-NS2A polyprotein. Analysis of the cleavage phenotype of these seven NS1-NS2A mutants is shown in Fig. 4. With the exception of the Val substitution, which yielded a low level of NS1-NS2A cleavage (20%; Table 1), NS1-NS2A containing one of five substitutions for Met (P8) was cleaved at a very low level (1 to 6%). However, substitution of Leu for Met had a minimal effect on NS1-NS2A cleavage (approximately 70% of the wild-type level), indicating that Leu at this position of the cleavage sequence can be tolerated. Similar to other positions occupied by amino acids that are conserved among flaviviruses, position P8 appeared to be relatively invariant and restricted to Met or Leu for DEN 4 virus NS1-NS2A cleavage.

Effects of substitutions for Gly (P1′) on cleavage of NS1-NS2A. Comparison of flavivirus sequences indicates that Gly (P1′) immediately following the DEN 4 virus NS1-NS2A cleavage site is not a conserved amino acid. Three other amino acids, Tyr, Phe, and Asn, occupy this position in the sequences of other flaviviruses (18). The cleavage phenotype of NS1-NS2A containing amino acid substitutions for Gly (P1′) was also studied. Four substitutions, Glu, Arg, Val, and Trp, were made at the P1′ position. The results in Table 1 and Fig. 5 revealed that substitution of Trp for Gly (P1′) resulted in a moderate reduction of NS1-NS2A cleavage to 48% of the wild-type level. Substitution of Glu, Arg, or Val for Gly resulted in a significant reduction of cleavage (19, 7, or 7%, respectively). These results indicated that the amino acid at position P1′ also plays a role in cleavage.

DISCUSSION

Processing of viral polyproteins plays a central role in the gene expression of positive-strand RNA viruses (40). These viruses encode proteinases that cleave the viral polyproteins at certain sites with a stringent specificity (20). Analysis of the sequence near the cleavage sites has shown that viral proteinases generally recognize a sequence motif composed of a small number of amino acid residues. For example, picornavirus 3C or 2A proteinase has been shown to cleave the sequence Q-G or T-Y/F-G, respectively (20). The Nla proteinase of tobacco etch virus, a member of the potyvirus group, cleaves a heptapeptide sequence containing the Glu-Xaa-Xaa-Tyr-Xaa-Gln-Ser/Gly motif, with cleavage occurring between Gln and Ser or Gly (31). The proteolytic processing of the DEN 4 virus polyprotein at the junction between nonstructural proteins NS1 and NS2A requires an eight-amino-acid sequence at the C terminus of NS1 (18). Cleavage also requires the downstream NS2A protein apparently acting in *cis* (12). Evidence demonstrating that a fusion protein containing a portion of pre-M, the C-terminal eight amino acids of NS1, and all of NS2A was cleaved at the predicted NS1-NS2A junction has been obtained (13). This finding indicates that the NS1 sequence is not required for cleavage except for the C-terminal eight amino acids. Computer searches have not identified any sequences in the flavivirus NS2A protein with homology to the catalytic site of any proteinase family. In vitro translation-processing studies have not revealed evidence that NS2A autocatalyzes its cleavage from NS1 (13). Thus, it seems likely that cleavage at the NS1-NS2A junction may be mediated by a novel host cell protease.

Chemically synthesized peptides of various sequences have been used for analysis of cleavage sequence motifs in a number of in vitro cleavage systems (2, 42). Because the protease responsible for cleavage at the NS1-NS2A junction has not been identified and no in vitro processing system is available, this approach has not been possible for the anal-

ysis of flavivirus NS1-NS2A cleavage. Instead, we turned to a study of the in vivo cleavage of the NS1-NS2A polyprotein encoded by a series of mutant DEN 4 virus NS1-NS2A cDNAs constructed by oligonucleotide-directed mutagenesis. Our results showed that most substitutions at positions P1, P3, P5, P7, and P8, occupied by conserved amino acids, yield low levels of cleavage ranging from no cleavage (Phe substitution for Ala at position P1) to approximately 20% of wild-type cleavage (Thr substitution for Ser at position P5 or Val substitution for Met at position P8). Exceptions were the Pro and Ala substitutions for Ser at position P5 and the Leu substitution for Met at position P8. The observation that the Leu substitution for Met (P8) has only a minimal effect on cleavage is predicted, as position P8 is occupied by Met in the DEN 4 virus sequence or by Leu in other flavivirus sequences (18). On the other hand, most substitutions of amino acids at the nonconserved positions generally yielded moderate to high levels of cleavage. These data are in agreement with the proposed consensus cleavage sequence for the NS1-NS2A junction; the conserved amino acids are essential for cleavage, while substitutions at the nonconserved positions are tolerated.

Variation from the consensus sequence may offer additional insight into the specificity of the sequence requirements for NS1-NS2A cleavage. As mentioned above, a noticeable exception has been found for the substitution of Pro or Ala for Ser (P5), which is conserved among flaviviruses. Ala and Pro differ in polarity from Ser, but like Ser, they have small side chains and appear to be recognized equally efficiently at position P5 by the responsible protease. In the DEN 3 virus NS1-NS2A cleavage sequence, Ala replaces the conserved Val at the P3 position (30). This represents the only natural variant of the cleavage sequence motif at position P3. This substitution is tolerated presumably because both Val and Ala are aliphatic amino acids. Our study revealed that replacement of Ser for Ala (P1), Val for Met (P8), or Thr for Ser (P5) permits partial cleavage. For the most part, substitutions for conserved amino acids, even with amino acids having similar side chains, yielded cleavage-deficient NS1-NS2A polyproteins. This finding indicates that the responsible enzyme recognizes a well-defined sequence at the cleavage site with a highly stringent specificity.

Although amino acids at positions P2, P4, and P6 of the cleavage sequence are not conserved among flaviviruses, not all substitutions at these positions produce a cleavage-competent NS1-NS2A polyprotein, and several sequence constraints exist. In the case of DEN 4 virus NS1-NS2A cleavage, the substitutions of Gly or Glu for Gln (P4) and Glu for Lys (P6) permitted only a low level of cleavage. These findings indicate that these amino acids are not completely unimportant for cleavage. Nonetheless, it is clear that amino acids at positions P2, P4, and P6 are less important determinants for NS1-NS2A cleavage than are those at the other five positions in the consensus cleavage sequence. In addition, it appears that the amino acid at position P1′ plays a role in cleavage, since the substitution of Glu, Arg, or Val for Gly reduced the level of cleavage of NS1-NS2A.

The wild-type DEN 4 virus NS1-NS2A sequence appears to be optimal for polyprotein processing, since some substitutions for the nonconserved amino acids also caused a reduction in cleavage efficiency. This finding suggests that the DEN 4 virus cleavage sequence may have been evolutionarily selected for optimal protein processing at this junction, presumably conferring a growth advantage on the virus. A cleavage product, identified as NS1-NS2A′, has been detected in yellow fever virus-infected cells (7) and Japanese encephalitis virus-infected cells (27). It remains to be established whether or not the NS1-NS2A cleavage sequence of yellow fever virus or Japanese encephalitis virus is optimal for cleavage.

Although regulation of viral gene expression at the level of polyprotein processing is complex, suboptimal cleavage at one or more sites of the polyprotein is likely to reduce virus replication and therefore virulence for the infected host. Substitutions of amino acids at the NS1-NS2A cleavage site, resulting in reduced cleavage, should cause restriction of growth of the virus. Growth restriction, in turn, may cause attenuation of the virus. Understanding the mechanisms and constraints of cleavage therefore assumes importance in our effort to design molecular strategies for the prevention of dengue virus infection. As a result of our recent success in obtaining an infectious RNA transcript of full-length DEN 4 virus cDNA (22), it is now possible to construct DEN 4 virus mutants containing defined amino acid substitutions at the NS1-NS2A cleavage site. Analysis of these mutants by plaque assay has shown that several such single amino acid substitution mutants exhibited a small-plaque morphology (21). These mutants should be further evaluated for attenuation in animals and eventually in humans. Furthermore, understanding of the substrate specificity of the responsible enzyme may allow us to design uncleavable peptide mimics that might serve as specific inhibitors that interrupt the viral proteolytic processing. Interruption of the polyprotein processing is an especially attractive approach to the prevention or treatment of flavivirus infections. While effective vaccines directed against all four dengue virus serotypes as well as other members of the large flavivirus family will have to be developed, a therapeutic agent specific to the novel protease, if it can be successfully designed, might have the general applicability to all flaviviruses.

ACKNOWLEDGMENTS

We thank R. Chanock for continuing encouragement of this research project, M. Bray for helpful discussions, H. Kawano for cleavage analysis of the Leu substitution of Met at position P8, T. Heishman for expert typing of the manuscript, and Terry Popkin for photographic assistance.

REFERENCES

1. **Bazan, J. F., and R. J. Fletterick.** 1989. Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology **171**:637–639.
2. **Blundell, T. L., R. Lapatto, A. F. Wilderspin, A. M. Hemmings, P. M. Hobart, D. E. Danley, and P. J. Whittle.** 1990. The 3-D structure of HIV-1 proteinase and the design of antiviral agents for the treatment of AIDS. Trends Biochem. Sci. **15**:425–430.
3. **Bonner, W. H.** 1983. Use of fluorography for sensitive isotope detection in polyacrylamide gel electrophoresis and related techniques. Methods Enzymol. **96**:215–222.
4. **Cahour, A., B. Falgout, and C.-J. Lai.** 1992. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junction is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J. Virol. **66**:1535–1542.
5. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
6. **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.

7. **Chambers, T. J., O. 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.
8. **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.
9. **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.
10. **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.
11. **Elroy-Stein, O., T. R. Fuerst, and B. Moss.** 1989. Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. Proc. Natl. Acad. Sci. USA **86**:6126–6130.
12. **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.
13. **Falgout, B., L. Markoff, and C.-J. Lai.** Unpublished data.
14. **Falgout, B., M. Pethel, Y.-M. Zhang, and C.-J. Lai.** 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J. Virol. **65**:2467–2475.
15. **Gorbalenya, A. E., A. P. Donchenko, E. V. Koonin, and V. M. Blinov.** 1989. N-terminal domains of putative helicase of flavi- and pestiviruses may be serine proteases. Nucleic Acids Res. **17**:3889–3897.
16. **Hahn, Y. S., R. Galler, T. Hunkapiller, J. M. 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.
17. **Henchal, E., and J. R. Putnak.** 1990. The dengue viruses. Clin. Microbiol. Rev. **3**:376–396.
18. **Hori, H., and C.-J. Lai.** 1990. Cleavage of dengue virus NS1-NS2A requires an octapeptide sequence at the C terminus of NS1. J. Virol. **64**:4573–4577.
19. **Irie, K., P. M. Mohan, Y. Sasaguri, R. Putnak, and R. Padmanahban.** 1989. Sequence analysis of cloned dengue virus type 2 genome (New Guinea C strain). Gene **75**:197–211.
20. **Krausslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
21. **Lai, C.-J., R. Men, M. Pethel, and M. Bray.** 1992. Infectious RNA transcribed from stably cloned dengue virus cDNA: construction of growth-restricted dengue virus mutants, p. 265–270. *In* F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Vaccines 92: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Lai, C.-J., B. Zhao, H. Hori, and M. Bray.** 1991. Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus. Proc. Natl. Acad. Sci. USA **88**:5139–5143.
23. **Mackow, E., Y. Makino, B. Zhao, Y.-M. Zhang, L. Markoff, A. Buckler-White, M. Guiler, R. Chanock, and C.-J. Lai.** 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. Virology **159**:217–228.
24. **Mandl, C. W., F. X. Heinz, C. Kunz.** 1988. Sequence of the structural proteins of tick-borne encephalitis virus (Western subtype) and comparative analysis with other flaviviruses. Virology **166**:197–205.
25. **Mandl, C. W., F. X. Heinz, E. Stoekl, and C. Kunz.** 1989. Genome sequence of tick-borne encephalitis (Western subtype) and comparative analysis of nonstructural proteins with other flaviviruses. Virology **173**:291–301.
26. **Markoff, L.** 1989. In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein. J. Virol. **63**: 3345–3352.
27. **Mason, P. W.** 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology **169**:354–364.
28. **Monath, T. P.** 1990. Flaviviruses, p. 763–814. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
29. **Moss, B., O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst.** 1990. New mammalian expression vectors. Nature (London) **348**:91–92.
30. **Osatomi, K., and H. Sumiyoshi.** 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176**: 643–647.
31. **Parks, T. D., and W. G. Dougherty.** 1991. Substrate recognition by the NIa proteinase of two potyviruses involves multiple domains: characterization using genetically engineered hybrid proteinase molecules. Virology **182**:17–27.
32. **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.
33. **Porterfield, J. S.** 1980. Antigenic characteristics and classification of Togaviridae, p. 13–46. *In* R. W. Schlesinger (ed.), Togaviruses: biology, structure, replication. Academic Press, New York.
34. **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.
35. **Preugschat, F., C.-W. Yao, and J. H. Strauss.** 1990. In vitro processing of dengue 2 nonstructural proteins NS2A, NS2B, and NS3. J. Virol. **64**:4364–4374.
36. **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.
37. **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.
38. **Shoemaker, D. P., C. W. Garland, and J. I. Steinfeld.** 1974. Experiments in physical chemistry. McGraw-Hill Co., New York.
39. **Speight, G., G. Coia, M. D. Parker, and E. G. Westaway.** 1988. Gene mapping and positive identification of non-structural proteins NS2a, NS2b, NS3, NS4b, and NS5 of the flavivirus Kunjin and their cleavage sites. J. Gen. Virol. **69**:23–34.
40. **Strauss, J. H., and E. G. Strauss.** 1988. Evolution of RNA viruses. Annu. Rev. Microbiol. **42**:657–683.
41. **Sumiyoshi, H., C. Mori, I. Fuke, K. Morita, S. Kuhara, J. Kondou, Y. Kikuchi, H. Nagamatsu, and A. Igarashi.** 1987. Complete nucleotide sequences of the Japanese encephalitis virus genome RNA. Virology **161**:497–510.
42. **Tritch, R. J., Y.-S. E. Cheng, F. H. Yin, and S. Erickson-Viitanen.** 1991. Mutagenesis of protease cleavage sites in the human immunodeficiency virus type 1 *gag* polyprotein. J. Virol. **65**:922–930.
43. **Wengler, G., G. Czaya, P. M. Fäeber, 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 center of the protease which cleaves the viral polyprotein after multiple basic amino acids. J. Gen. Virol. **72**:851–858.
44. **Zhao, B., E. Mackow, A. Buckler-White, L. Markoff, R. M. Chanock, C.-J. Lai, and Y. Makino.** 1986. Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for the structural proteins. Virology **155**:77–88.
45. **Zhao, B., G. Prince, R. Horswood, K. Eckels, P. Summer, R. Chanock, and C.-J. Lai.** 1987. Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. J. Virol. **61**:4019–4022.