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Cleavage of Dengue Virus NS1-NS2A Requires an Octapeptide Sequence at the C Terminus of NS1

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The length of amino acid sequence at the NS1-NS2A juncture of dengue virus that is required for specific cleavage effected by the *cis*-acting function of NS2A was identified by deletion analysis. Recombinant DNA sequences of NS1-NS2A, each containing a deletion in NS1 followed by a sequence of 3 to 20 amino acids at the C terminus of NS1 preceding the cleavage site, were constructed and expressed with vaccinia virus as a vector. The NS1 product of recombinant vaccinia virus-infected cells was immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The occurrence of cleavage between NS1 and NS2A was indicated by the appearance of shortened NS1. Failure to cleave this site yielded a large NS1-NS2A fusion protein. This analysis indicated that a minimum length of eight amino acids at the NS1 C terminus preceding the NS1-NS2A juncture is required for cleavage to take place. Comparison of this eight-amino-acid sequence of the NS1 C terminus of dengue type 4 virus with the analogous sequences of 12 other flaviviruses suggests that the consensus cleavage site sequence is as follows:

							Cleavage
P–8	P–7	P–6	P–5	P–4	P–3	P–2	P–1	↓
Leu/Met -	Val -	Xaa -	Ser -	Xaa -	Val -	Xaa -	Ala	

Proteolytic cleavage of the long single polyprotein encoded by the positive-strand RNA genome of a large group of diverse viruses, including the picornaviruses, the flaviviruses, and the distantly related plant viruses such as potyviruses, is a prerequisite for processing of viral proteins (13). Dengue viruses (serotypes 1 to 4) and other members of the flavivirus family have the same genome organization, and it is probable that these viruses employ the same strategy for translation and posttranslational processing. The genes for the three structural proteins, i.e., capsid protein and premembrane (membrane) and envelope glycoproteins, are located at the 5′ end of the viral RNA genome, and nonstructural proteins designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in that order are located at the 3′ end of the genome (4, 5, 7, 9, 12, 14, 17, 18, 22, 26, 29). The map positions of most dengue virus proteins have been assigned by direct sequence analysis of the N-terminal amino acids or by alignment based on homology with sequences of other flaviviruses determined by amino acid sequence analysis (1, 2, 7, 9, 12, 14, 17, 19, 21, 27, 28, 30).

Analysis of amino acid sequences near or at the cleavage sites indicates that dengue virus and other flaviviruses employ two different strategies for posttranslational cleavage of their polyprotein. The first type of cleavage occurs after a long hydrophobic sequence, generating structural proteins such as the premembrane and envelope proteins. Some evidence from studies employing in vitro protein synthesis suggests that this form of cleavage occurs cotranslationally and is catalyzed by host cell signalase (16, 20). The second type of cleavage, which is responsible for the processing of most nonstructural proteins, has been proposed to take place after a dibasic amino acid sequence such as Lys-Arg or Arg-Arg (4, 14, 19). In addition, a third type of cleavage appears to be involved in posttranslational processing at the NS1-NS2A junction because this region contains neither a hydrophobic amino acid domain nor a dibasic amino acid motif. The presence of Val-Xaa-Ala at the NS1-NS2A juncture suggested the possibility that cleavage was effected by cell signalase (21). However, there were no experimental data to support this hypothesis.

Using cloned DNA segments for expression of dengue virus proteins, we have shown that proper synthesis of the NS1 glycoprotein requires the N-terminal hydrophobic signal and the downstream nonstructural protein NS2A. NS2A apparently functions in *cis* for the cleavage of NS1-NS2A (10). Further studies employing deletion analysis revealed that the N-terminal 70% of NS2A appears to be sufficient to mediate effective processing (11). Although it is not clear whether NS2A is a *cis*-acting proteinase, i.e., whether it autocatalytically cleaves itself from NS1, it was possible to employ the vaccinia virus-eucaryotic cell expression system to define the site on NS1 that is catalyzed directly or indirectly by NS2A. In this paper we describe the amino acid sequence at the juncture of NS1 and NS2A required for cleavage mediated by NS2A.

Because the N-terminal region of NS2A is required for cleavage, internal deletions were introduced at the C terminus of NS1 in such a manner that various lengths of amino acid sequence upstream of the cleavage site were retained. These ΔNS1-NS2A constructs were examined for their ability to be cleaved in the presence of NS2A to generate the appropriate truncated NS1. Failure to be cleaved at the junction was recognized by the expression of a fusion ΔNS1-NS2A protein. The expression of both the truncated NS1 and the ΔNS1-NS2A fusion protein was assayed by radioimmunoprecipitation. The construction of recombinant DNA coding for this series of ΔNS1-NS2A is shown in Fig. 1. Intermediate recombinant pSC11-NS1-NS2A DNA constructed earlier for expression of authentic NS1 was used in this study (10). The dengue virus DNA contained the coding

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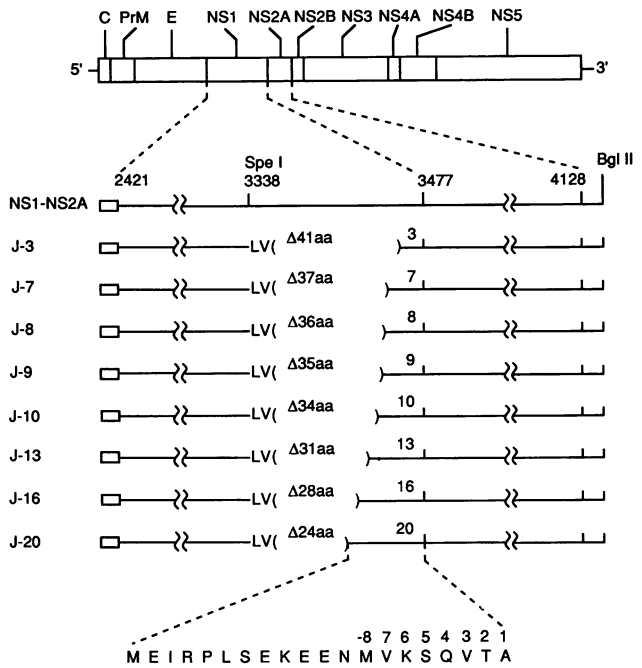

FIG. 1. Recombinant constructs of NS1-NS2A used for analysis of cleavage. A map of the dengue virus genome depicting the order of viral proteins is shown at the top. The DNA fragment coding for NS1-NS2A was cloned earlier in the pSC11 vector under the transcriptional control of the P7.5 promoter of vaccinia virus (6, 10). The unique *Spe*I site and the unique *Bgl*II site were used for insertion of recombinant DNA coding for NS1-NS2A generated by a PCR. Eight oligonucleotides, each containing the indicated sequence, were used to construct the recombinant shown on the left. The following oligonucleotides were used as the positive-strand primers for the indicated recombinants: v(J-3), oligo 2789 (5'-GATCACTAGTCGTGACGGCCGGACAGGGC-3'); v(J-7), oligo 2790 (5'-GATCACTAGTCGTCAAATCACAGGTGA CG-3'); v(J-10), oligo 2791 (5'-GATCACTAGTCGAGAACATGG TCAAATCA-3'); v(J-13), oligo 2792 (5'-GATCACTAGTCGAAAA AGAAGAGAACATG-3'); v(J-16), oligo 2793 (5'-GATCACTAGT CCCCTTGAGTGAAAAAGAA-3'); v(J-20), oligo 2794 (5'-GATC ACTAGTCATGGAGATTAGGCCCTTG-3'); v(J-8), oligo 2891 (5'-GATCACTAGTCATGGTCAAATCACAGGTG-3'); and v(J-9), oligo 2892 (5'-GATCACTAGTCAACATGGTCAAATCACAG-3'). The negative-strand primer was oligo 1852 (5'-CGTTTGCCATA CGCTCACAG-3'). The double-stranded DNA products from the PCR were cleaved with *Spe*I and *Bgl*II to allow replacement of the corresponding fragment in the expression vector pSC11-NS1-NS2A. The series of recombinant DNA constructs was sequenced to verify the deletion and the flanking regions of 50 to 150 nucleotides. The number of amino acids deleted in each recombinant is indicated (Δ41aa, etc.). Similarly, the number shown next to the NS1-NS2A juncture denotes the number of C-terminal NS1 amino acids retained. The sequence of the last 20 amino acids is shown beneath v(J-20). The small box at the left end of each line depicts the 24-amino-acid N-terminal hydrophobic sequence of NS1.

sequence for the 24-amino-acid N-terminal signal and the entire polypeptide sequences of NS1 and NS2A. This plasmid contained a unique *Spe*I site at dengue virus nucleotide 3338 within the NS1-coding sequence and a unique *Bgl*II site inserted at the *Sma*I site of pSC11 DNA immediately following the NS2A-coding sequence. The NS1-NS2A cleavage juncture is located at nucleotide 3477. In order to introduce deletions into the region between the *Spe*I site and the cleavage juncture and at the same time retain a variable length of C-terminal amino acid sequence of NS1 at the cleavage junction, a series of oligonucleotides was synthesized for use as primers in a polymerase chain reaction (PCR). Each of the positive-strand primer series contained the *Spe*I cleavage sequence ACTAGT and an in-phase deletion followed by the coding sequence for the length of C-terminal NS1 amino acids to be retained. In this manner, nonviral sequence was not introduced into the constructs. The negative-strand primer was oligo 1852 (see legend to Fig. 1 for sequence), which is located downstream of the *Bgl*II site within the pSC11 vector. For construction of intermediate recombinant DNA containing the deletions, the *Spe*I-*Bgl*II DNA fragment of the full-length pSC11-NS1-NS2A DNA was replaced with the series of PCR DNA products that were cleaved with *Spe*I and *Bgl*II.

Initially, oligonucleotide (oligo) primers (oligo 2789 to oligo 2794; for sequence, see legend to Fig. 1) that each specified a deletion of 41, 37, 34, 31, 28, or 24 amino acids within NS1 while retaining 3, 7, 10, 13, 16, or 20 amino acids at the cleavage junction were used for construction of the first series of recombinant DNA. All recombinant constructs contains the same Leu-Val sequence upstream of the deletion. This upstream sequence did not substitute for amino acids that were removed in all the deletion constructs. Recombinant vaccinia viruses expressing these mutant sequences were made by previously described procedures (10, 30). CV-1 cells were infected with these recombinants at 5 PFU per cell, and [^{35}S]methionine-labeled cell lysates were prepared in RIPA buffer (10, 30). The labeled lysates were immunoprecipitated with dengue virus hyperimmune mouse ascitic fluid and then electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. The result of this analysis is shown in Fig. 2A. Recombinant vaccinia virus v(NS1-NS2A) produced apparently authentic NS1 (40 to 46 kilodaltons) of the monomeric form and a smaller fraction of dimeric NS1 (86 kilodaltons), as seen in the unboiled sample. Boiling of the immunoprecipitate converted most dimeric NS1 to monomeric NS1. Each of the recombinant vaccinia viruses v(J-20), v(J-16), v(J-13), and v(J-10), which retained, respectively, 20, 16, 13, and 10 NS1 C-terminal amino acids at the NS1-NS2A junction, produced a labeled band smaller than the full-length NS1. These protein products were further reduced in size after endoglycosidase F digestion, and the size estimates were consistent with the values predicted for the monomeric form of shortened NS1 (see Fig. 3).

It should be noted that all the shortened NS1 species were detected at a lower level than the authentic NS1. This may be the result of decreased binding affinity to hyperimmune mouse ascitic fluid, since immunoprecipitation with an antiserum prepared in rabbits against a 14-amino-acid peptide of NS1 (peptide 9, amino acids 132 to 145 of NS1) detected a 5- to 10-fold increase in the amounts of shortened NS1 species (data not shown). Little or no uncleaved NS1-NS2A accumulated during infection with these deletion recombinants or with v(NS1-NS2A). Also, dimeric shortened NS1 was not detected in the unboiled samples, suggesting that the deleted amino acid sequences directly or indirectly played a role in the intermolecular interaction during dimerization. NS1 dimerization did not appear to be required for the cleavage to take place, and cleavage between NS1 and NS2A apparently occurred normally for the mutant NS1-NS2A sequences expressed by these recombinants. On the other hand, recombinants v(J-7) and v(J-3) did not express shortened NS1. Instead a band larger than NS1 of the size predicted for the NS1-NS2A fusion protein was detected, indicating that both recombinants failed to cleave NS2A from ΔNS1. It was possible that the observed defective cleavage was due to the

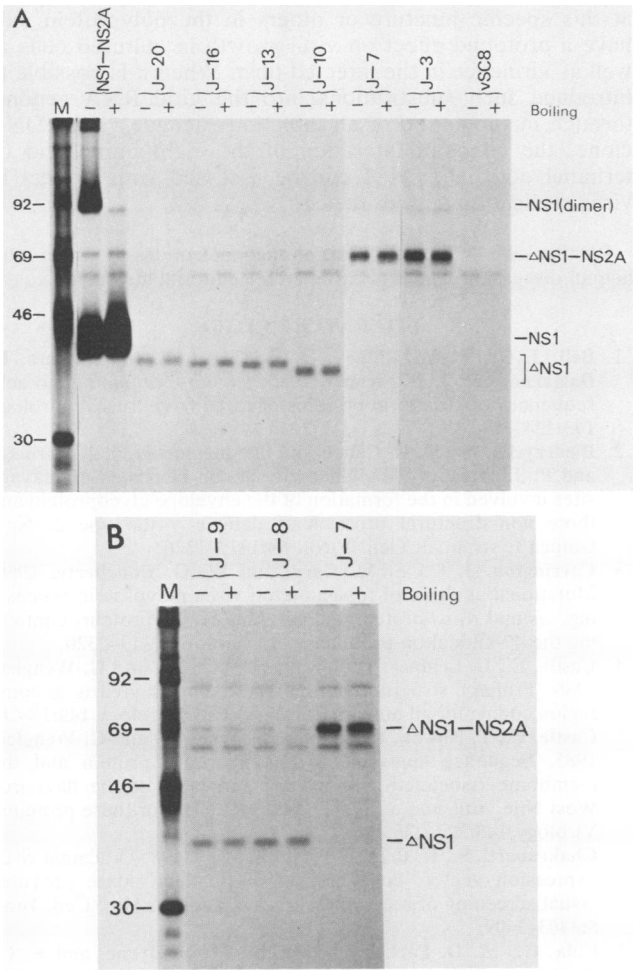

FIG. 2. Detection of cleaved or uncleaved dengue virus NS1-NS2A expressed by recombinant vaccinia viruses. Recombinant v(NNS1-NS2A) expressing authentic dengue virus NS1 was described earlier (10). Deletion recombinants that retained 20, 16, 13, 10, 9, 8, 7, or 3 NS1 C-terminal amino acids at the cleavage juncture, as respectively designated v(J-20), v(J-16), v(J-13), v(J-10), v(J-9), v(J-8), v(J-7), and v(J-3). vSC8 is the control vaccinia virus which expresses no dengue virus-specific proteins. In the experiments illustrated in panels A and B, CV-1 cells were infected with these recombinant viruses at 5 PFU per cell and labeled with [^{35}S] methionine 15 h after infection for a 2-h period. Labeled lysates were prepared in RIPA buffer and immunoprecipitated for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10). Cleaved NS1 expressed by these recombinants migrates to the position indicated by ΔNS1, whereas uncleaved NS1-NS2A is the larger band designated ΔNS1-NS2A. One-half of each test sample was boiled before being loaded on the gel. The molecular size markers (lanes M) in kilodaltons are shown on the left.

failure of targeting the ΔNS1-NS2A polyproteins to a specific intracellular site, such as the endoplasmic reticulum or the Golgi apparatus, at which the responsible protease for cleavage might be located. This does not appear to be the case, as indicated by the experiment shown in Fig. 3 involving endoglycosidase F digestion. Both ΔNS1-NS2A fusion proteins of v(J-7) and v(J-3) were sensitive to endoglycosidase F digestion and therefore were glycosylated. Similarly, cleaved NS1 expressed by other recombinants was also glycosylated. This indicates that translocation of NS1-NS2A containing the deletions across the endoplasmic reticulum membrane was apparently normal. The most plausible explanation for this finding is that three or seven amino acids preceding the cleavage junction are not sufficient to permit cleavage to occur. It appears that a sequence of at least 8 to 10 C-terminal NS1 amino acids is required to effect such cleavage mediated by the *cis* function of NS2A.

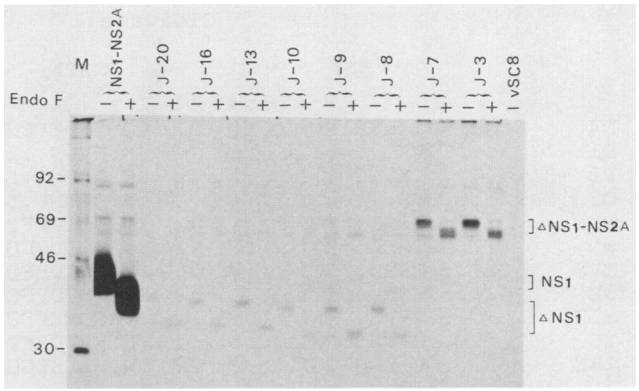

FIG. 3. Endoglycosidase F (Endo F) digestion of cleaved and uncleaved dengue virus NS1-NS2A. The cleaved and uncleaved dengue virus NS1-NS2A proteins expressed by recombinant vaccinia viruses were prepared from the same lysates used for the experiments described in Fig. 2. Immunoprecipitates were digested with endoglycosidase F (+) or mock digested (−). vSC8 is the control vaccinia virus which contains no dengue DNA sequences. The molecular mass markers (lane M) in kilodaltons are shown on the left.

In order to further delineate the length of this cleavage site sequence, two additional deletion recombinants, v(J-9) and v(J-8), which retained the Leu-Val sequence followed by the last nine and eight amino acids, respectively, preceding the cleavage site were similarly constructed. For this purpose, the positive-strand primers oligo 2891 and oligo 2892 (see legend to Fig. 1) were synthesized, and the respective DNA product from a PCR using the same negative-strand primer as described earlier was prepared for replacement of the sequences between *Spe*I and *Bgl*II in intermediate pSC11-NS1-NS2A DNA. Recombinant vaccinia viruses constructed from the intermediate pSC11 plasmid were used for infection of CV-1 cells, and the NS1 products were analyzed similarly as described above. The expressed NS1 products (Fig. 2B and Fig. 3) were similar to those previously observed with recombinants v(J-20), v(J-16), v(J-13), and v(J-10). Recombinants v(J-9) and v(J-8) each produced a shortened NS1 band that is indicative of cleavage between NS1 and NS2A. On the other hand, recombinant v(J-7) again expressed an uncleaved ΔNS1-NS2A fusion protein, as in the previous test shown in Fig. 2A. These observations suggest that the NS1-NS2A cleavage site sequence contains the eight NS1 amino acids preceding this site. This 8-amino-acid domain is considerably shorter than the hydrophobic sequences (usually a stretch of 15 to 25 amino acids) that are functionally recognized by host cell signalase for cotranslational cleavage (25). The NS1-NS2A cleavage site sequence also differs from the dibasic amino acid sequences that are utilized for the processing of most flavivirus nonstructural proteins. Also, the cleavage sites recognized by poliomyelitis virus 3C proteinase contain a short sequence of Gln-Gly, Tyr-Gly, or Asp-Ser (23). Recently, however, an extended cleavage sequence consisting of seven amino acids has been defined for the 49-kilodalton proteinase of tobacco etch virus, which belongs to the plant potyvirus family (3).

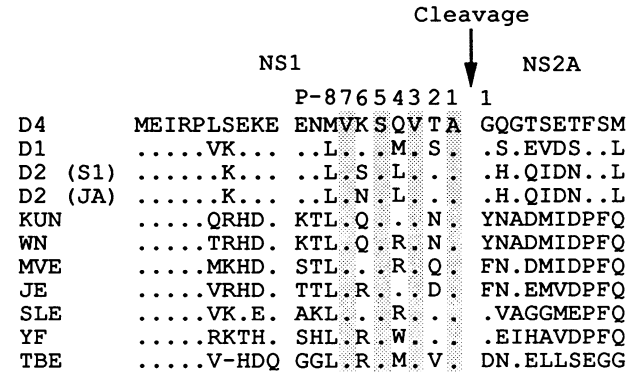

FIG. 4. Comparison of NS1-NS2A cleavage site sequences among flaviviruses. Dengue type 4 virus (D4) sequence at and near the NS1-NS2A junction including the eight-amino-acid cleavage site (positions P−1 to P−8) (14) was used for alignment and comparison with the analogous sequences of dengue type 1 virus (D1) (17); dengue type 2 virus, strain S1 [D2 (S1)] (12); dengue type 2 virus, strain Jamaica [D2 (JA)] (9); Kunjin virus (KUN) (7); West Nile flavirirus (WN) (4); Murray Valley encephalitis virus (MVE) (8); Japanese encephalitis virus (JE) (22); St. Louis encephalitis virus (SLE) (24); yellow fever virus (YF) (19); and tick-borne encephalitis virus (TBE) (15, 18). Dengue type 2 virus (New Guinea-C) and dengue type 2 virus (Jamaica) have identical sequences in this region, and so do the two strains of tick-borne encephalitis virus. An amino acid identical to the corresponding one in dengue type 4 virus is indicated by a period, and a deletion is indicated by a dash.

At least 13 complete or nearly complete amino acid sequences encompassing several major flavivirus subgroups have been determined (Fig. 4). A comparison of the eight amino acid residues at the NS1-NS2A juncture and the flanking sequences of dengue type 4 virus with the analogous sequences from 12 other flaviviruses suggests a shared specificity for recognition by the responsible proteinase. In the eight-amino-acid region, valine at position P–7, serine at P–5, valine at P–3, and alanine at P–1 appear to be strictly conserved among these viruses. The amino acid at position P–8 appears to be relatively conserved; methionine is found in dengue type 4 virus, and leucine is found in all 12 other flaviviruses. Five different amino acids, including two charged amino acids in the majority of these flaviviruses (54%), are found at P–6. There are also five different amino acids with a considerable degree of side chain diversity at P–4. Six different amino acids also occur at P–2. Four different amino acids are found at the N terminus of NS2A, i.e., P+1, and likewise amino acid variations occur in other downstream positions, as the homology of NS2A sequences among these viruses is low. Interestingly, in the eight-amino-acid region, conserved amino acids at P–7, P–5, P–3, and P–1 appear to alternate with variable amino acids at P–6, P–4, and P–2. Because a consensus sequence of Leu/Met-Val-Xaa-Ser-Xaa-Val-Xaa-Ala appears to be emerging for the NS1-NS2A cleavage site among this family of related flaviviruses, it will be of interest to introduce amino acid substitutions at each of these eight positions. This will allow us to test rigorously whether the observed consensus sequence is optimal for the polyprotein NS1-NS2A processing step during expression of this region of the dengue virus genome by a vaccinia virus recombinant. Conceivably, dengue virus gene expression during virus replication involves a more complex and highly regulated processing mechanism. In any case, it is likely that amino acid substitutions in the cleavage site sequence that result in increased or suboptimal cleavage at this specific juncture or others in the polyprotein may have a profound effect on viral growth in cultured cells as well as virulence in the infected host. When it is possible to introduce such substitutions into the viral RNA genome through manipulation of an infectious dengue virus cDNA clone, the effect of alteration of the eight-amino-acid C-terminal domain of NS1 can be assessed with respect to virus replication and virulence.

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