

Internal proteolysis of the NS3 protein specified by dengue virus 2

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The NS3 protein of flaviviruses is a multifunctional polypeptide required for virus replication. Enzymic activities that have been demonstrated or predicted from the presence of sequence motifs include protease, NTPase, helicase and RNA triphosphatase. Both full-length and truncated forms of NS3 have been identified in infected cells. To examine internal cleavage of the NS3 protein of dengue virus 2 (DEN-2), infected cells or COS cells transfected with cDNA encoding NS2B/3 were radiolabelled and immunoprecipitated with anti-serum against NS3 or hyperimmune mouse ascitic fluid. The polypeptides detected were NS2B/3 (M_r 83 000), NS3 (M_r 69 000), NS3' (M_r 50 000) and NS3'' (M_r 19 000). The latter polypeptide has not been previously identified. For DEN-2, it has been proposed that NS3' results from cleavage at the site ... R₄₅₇R | GR₄₆₀ ... within an RNA helicase sequence motif of NS3. Our results demonstrated that cleavage occurred at this site, and that prior cleavage between NS2B/NS3 was not necessary.

The flavivirus genome is translated as a polyprotein of C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 which is cleaved into individual proteins by host and viral proteases (Chambers *et al.*, 1990a). NS3 is the second largest nonstructural polypeptide. It contains approximately 580 amino acids, representing 17% of the genomic coding capacity, and has motifs characteristic of protease, NTPase and helicase (Koonin & Dolja, 1993). The protease has been most widely studied; its active form is a complex of NS2B and NS3, with the amino-terminal 185 amino acids of NS3 sufficient for proteolysis (Preugschat *et al.*, 1990; Chambers *et al.*, 1990b; Wengler *et al.*, 1991; Falgout *et al.*, 1991). It cleaves following basic amino acids at the junctions NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5, and within C (Nowak *et al.*, 1989; Speight & Westaway, 1989 a) and NS4A (Lin *et al.*, 1993). NTPase activity (motifs in the central region of the protein) was shown

for West Nile, yellow fever and Japanese encephalitis viruses (Wengler & Wengler, 1991; Warrenner *et al.*, 1993; Takegami *et al.*, 1994). RNA triphosphatase activity was demonstrated for West Nile virus (Wengler & Wengler, 1993).

A truncated form of NS3 (M_r 50 000), designated NS3', was detected in tick-borne encephalitis virus (TBE)- and dengue virus 2 (DEN-2)-infected cells. It was proposed that NS3' is produced by cleavage within NS3 at the site ... RR | GR ... (residues 457–460 in DEN-2 NS3), which is highly conserved in known flavivirus sequences (Falgout *et al.*, 1991; Pugachev *et al.*, 1992; Arias *et al.*, 1993). However, the literature contains conflicting reports regarding this cleavage. The results obtained from *in vitro* translation for TBE suggested that it was effected by a cellular protease (Pugachev *et al.*, 1992, 1993), whereas expression experiments using DEN-2 cDNA indicated that the viral NS2B/3 protease was responsible (Arias *et al.*, 1993). The other product of cleavage remained to be identified. We now provide experimental evidence that proteolysis occurs at the proposed internal site of DEN-2 NS3 in the presence of NS2B/NS2B3, and identify the second product of the cleavage. We have designated this protein NS3''.

The vector pSV.SPORT 1 (Gibco BRL) was used to express cDNA encoding NS3 or NS2B/3 in COS cells. Viral cDNA (DEN-2, New Guinea C strain) was inserted into the *Sma*I site of the vector. The complete and abbreviated names for the constructs are listed in Fig. 1. The cDNA for constructs S1 and S2 was prepared by RT-PCR and sequenced completely. The 5' ends of the NS3 (S1) or NS2B (S2, S3, S4) genes in the respective constructs were preceded with nucleotides 22–93 of the 5' untranslated region of Kunjin virus (Khromykh & Westaway, 1994) and the sequence CATG. These start codons, and the stop codon at the 3' end of the NS3 gene, were generated by PCR using appropriately designed primers. Mutations were introduced into the pSV.NS2B/3 (S2) construct by overlap PCR (Ho *et al.*, 1989) using S2 DNA as template. These changed either the encoded sequence at the proposed NS3 internal cleavage site from ... R₄₅₇R | GR₄₆₀ ... to ... A₄₅₇A | GR₄₆₀ ... (pSV.NS2B/3_{457,458} i.e. S3), or the sequence at the NS2B/NS3 junction from ... K₁₂₇KQR | AG₂ ... to ... A₁₂₇IQG | AG₂ ... (pSV.NS2B₁₂₇₋₁₃₀/3 i.e. S4). The sequence of constructs S3 and S4 was confirmed at the sites of mutagenesis and at the 5' and 3' ends of the inserts. The constructs were introduced into COS cells by electroporation

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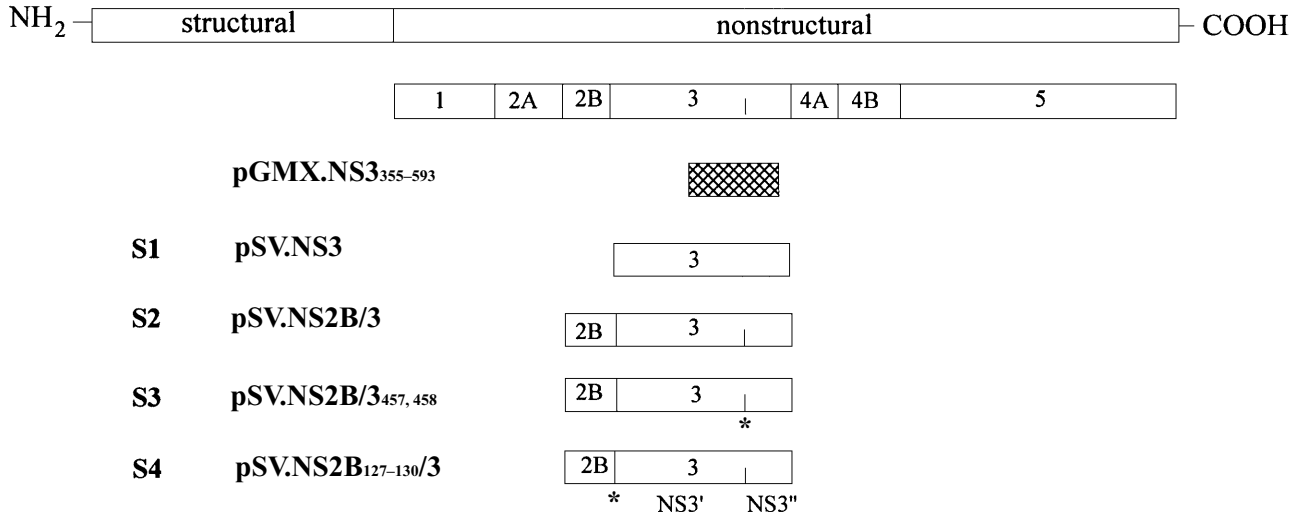


Fig. 1. DEN-2 polypeptides synthesized using pGEMEX (in *E. coli*) or pSV.SPORT 1 (in COS cells). The abbreviated and complete designations of the constructs are shown. Subscripts show the positions of residues in the individual proteins and either define the limits of viral polypeptide synthesized (pGMX), or the locations of mutagenesis (pSV). Asterisks mark the mutagenized sites; amino acid changes are given in the text.

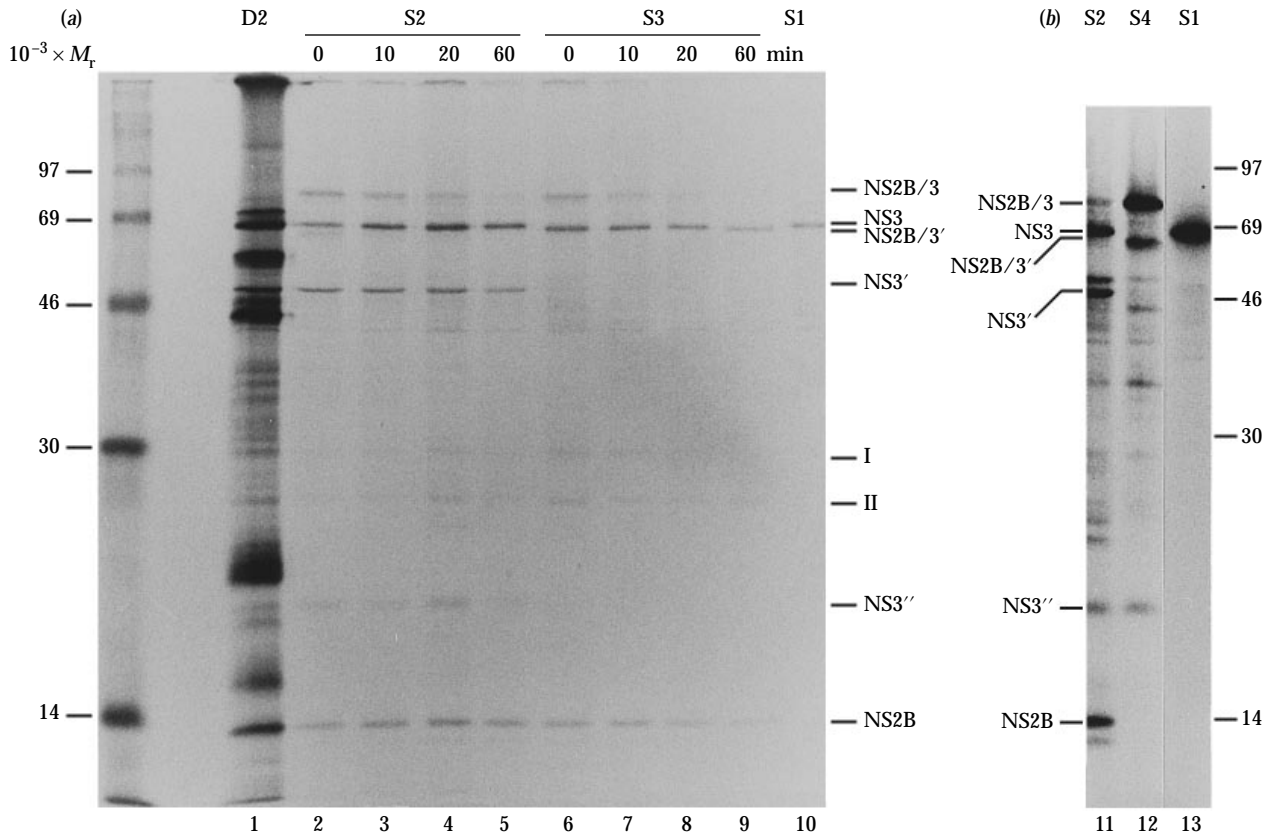


Fig. 2. Analysis by radioimmunoprecipitation with HMAF and gel electrophoresis of polypeptides synthesized in transfected COS cells. (a) Cells were transfected with S2 (lanes 2–5) or S3 (lanes 6–9), radiolabelled for 10 min and chased with excess methionine for the times shown. Vero cells infected with DEN-2 (D2, lane 1), and COS cells transfected with S1 (lane 10) were radiolabelled for 1 h. (b) Cells were transfected with S2, S4 or S1 and radiolabelled for 1 h. Size markers are shown.

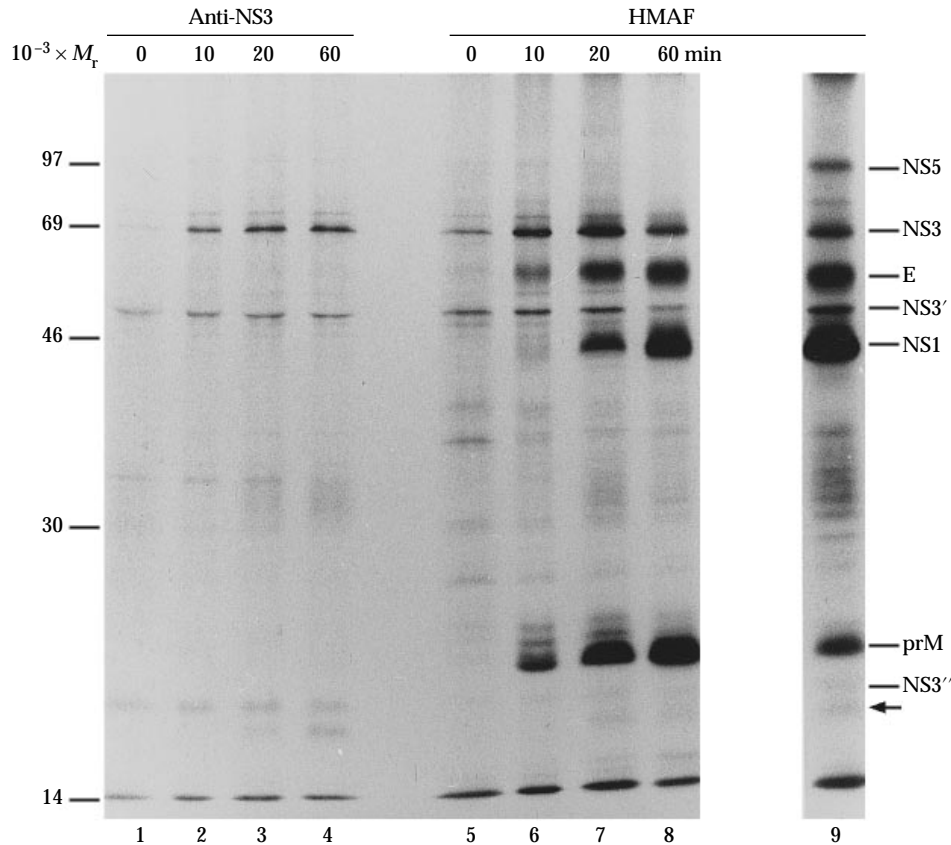


Fig. 3. Analysis by radioimmunoprecipitation and gel electrophoresis of polypeptides synthesized in DEN-2-infected Vero (lanes 1–8) and C6/36 cells (lane 9). Cells were radiolabelled for 10 min and chased for the times indicated. Rabbit anti-NS3 serum (lanes 1–4) or HMAF (lanes 5–9) were used to prepare the immunoprecipitates. The M_r 14 000 molecular marker migrated in the gel front. An arrow indicates the position of the protein with an M_r 18 000 migrating immediately ahead of NS3''.

and, 48 h later, polypeptides were labelled with [35 S]methionine as described previously (Pryor & Wright, 1993). DEN-2-infected Vero or *Aedes albopictus* C6/36 cells were radiolabelled at 60 h after infection. Proteins were immunoprecipitated with DEN-2 hyperimmune mouse ascitic fluid (HMAF) or antiserum prepared in rabbits against a bacterial fusion protein. The latter was synthesized using the bacterial expression vector pGEMEX (Promega) and contained a segment of NS3 (residues 355–593) as shown in Fig. 1. The fusion protein was designed to induce antibodies against NS3' and NS3''; in experiments described by others, most immunoprecipitates were prepared with antibodies restricted to recognition of the amino-terminal regions of NS3 (Pugachev *et al.*, 1992, 1993; Arias *et al.*, 1993).

Cells were transfected with pSV.NS2B/3 (S2), radiolabelled for 10 min, chased with excess cold methionine, and immunoprecipitated with HMAF (Fig. 2*a*, lanes 2–5). In addition to NS2B/3, NS3 and NS2B, polypeptides NS3' (M_r 50 000) and NS3'' (M_r 19 000) were detected. The sizes of NS3' and NS3'' calculated from their migration through the gel were identical to those predicted from their deduced amino acid sequences

following cleavage at ...R₄₅₇R|GR₄₆₀... Polypeptides NS3' and NS3'' were readily detected using HMAF or anti-NS3 serum (not shown). Mutagenesis of the proposed internal proteolysis site abolished cleavage; neither NS3' nor NS3'' were detected in cells transfected with pSV.NS2B/3_{457, 458} (S3) (Fig. 2*a*, lanes 6–9). Neither NS3' nor NS3'' were detected in cells electroporated with pSV.NS3 (S1) (Fig. 2*a, b*, lanes 10 and 13, respectively), supporting the conclusion that the viral NS2B/3 protease (Arias *et al.*, 1993) rather than a cellular protease (Pugachev *et al.*, 1993) was responsible for the cleavage.

Pugachev *et al.* (1993) suggested that release of NS3 from NS2B/3 may be required for internal cleavage of NS3. We obtained two results showing that prior cleavage was not necessary. In cells transfected with pSV.NS2B/3 (S2) and radiolabelled for 10 min (no chase), a small amount of protein corresponding in size to NS2B/3' (M_r 64 000) was detected (Fig. 2*a*, lane 2). It migrated immediately ahead of NS3 (M_r 69 000). Stronger evidence came from the examination of proteins in cells transfected with pSV.NS2B_{127–130}/3 (S4) and radiolabelled for 60 min (Fig. 2*b*, lane 12). This construct

encoded changes at the NS2B/NS3 site which prevented proteolysis at this location. NS3 and NS2B were not formed. However, NS3'' and a protein corresponding to NS2B/3' (M_r 64 000) were detected.

Some additional proteins visible as bands in Fig. 2 are worthy of comment. First, NS2B was readily detected following the use of HMAF, possibly due to the presence of antibodies recognizing NS2B alone, but more likely due to co-precipitation with NS3-related proteins. Co-precipitation of NS2B and NS3 has been reported for DEN-2 (Arias *et al.*, 1993) and Japanese encephalitis virus (Jan *et al.*, 1995). Second, in some cells expressing pSV constructs and producing NS2B, but not in DEN-2-infected Vero cells, a protein slightly smaller than NS2B was detected (Fig. 2*b*, lane 11). The ninth and thirteenth codons in the NS2B gene encode methionine with the flanking sequences ... ATCATGG ... and ... GGGATGG ..., thus matching the initiating consensus of ... (A/G)XXATGG ... (Kozak, 1987). Initiation at either of these codons rather than at the introduced ATG at the 5' terminus of the NS2B gene (... TC_{KUN93}CATGAGC ...) may have generated the smaller protein. The corresponding difference in molecular mass of protein NS2B/3 would be too small for resolution in the gels used. Third, bands I and II were frequently observed in cells expressing cDNA of NS2B/3, but not NS3 cDNA alone (Fig. 2*a, b*, lanes 2–9, and 11 and 12, respectively). Their origin is unknown.

Infected Vero and *Aedes albopictus* C6/36 cells were also examined for the presence of NS3''. Cells were infected with DEN-2 (New Guinea C strain), radiolabelled and immunoprecipitated with rabbit anti-NS3 serum or HMAF. The complete results for Vero cells are shown in Fig. 3; the results for C6/36 cells were similar and are not shown in full (lane 9 only). NS3' and NS3'' were detected within 10 min of the addition of label (Fig. 3, lanes 1 and 5), as was the case for cells transfected with cDNA constructs (Fig. 2*a*, lanes 2 and 6). NS3'' was not detectably degraded during the 60 min chase in infected cells (Fig. 3, lanes 4 and 8), whereas it was unstable in transfected cells (Fig. 2*a*, lane 5).

The anti-NS3 serum and HMAF immunoprecipitated a protein with an apparent M_r of 18 000, which appeared following a 20 min chase in DEN-2-infected cells. It migrated slightly ahead of NS3'' (Fig. 3). It was also detected in infected cells pulse-labelled for 60 min (Fig. 2*a*, lane 1), but not in transfected cells (Fig. 2*a, b*, lanes 2–13). The protein may represent an NS3-related polypeptide unique to infected cells. Alternatively, it may represent a protein co-precipitating with NS3 or an NS3-related polypeptide. One candidate is NS4A, since it follows NS3'' in the polyprotein and from its deduced amino acid sequence has an M_r of 16 000. However, NS4A is a difficult protein to identify; it is hydrophobic, its migration through gels is anomalous (Speight & Westaway, 1989*b*) and no anti-NS4A serum is available.

The significance of cleavage at the NS3'/NS3'' junction in virus replication is presently unknown. It was proposed that

cleavage of NS3 may contribute to the regulation of virus replication (Arias *et al.*, 1993). For this to occur, it would be necessary for cleavage to significantly alter one or more of the enzymic activities associated with NS3. For example, the NS3'/NS3'' junction lies in the final RNA helicase motif (VI) (Koonin & Dolja, 1993), which is proposed to have a role in ATP hydrolysis and RNA binding (Pause *et al.*, 1993; Fuller-Pace, 1994). Presumably NS3' would lack these activities. However, the NS2B/3' complex may be an active protease, since proteolysis has been detected in a number of expression systems containing truncated forms of NS3 (Preugschat *et al.*, 1990; Chambers *et al.*, 1990*b*; Wengler *et al.*, 1991; Falgout *et al.*, 1991; Arias *et al.*, 1993; Jan *et al.*, 1995); it is possible that such a complex may possess either modified specificity for cleavage sites, or altered *cis/trans* activity, or both of these. The relative contributions of NS3 and NS3'' to RNA triphosphatase activity, and thus to RNA capping, are unknown. It is proposed that the sequence elements for triphosphatase lie in the carboxy-terminal region of NS3 (Wengler & Wengler, 1993), corresponding approximately to NS3''. Therefore, to understand the importance of internal cleavage within NS3 for flavivirus replication, further analyses of the protein and its fragments in assays for NS3-associated activities are required. Useful information may be obtained by examining NS3 cleavage and replication rates of other flaviviruses.

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