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Expression of the NS1 gene of dengue virus type 2 using vaccinia virus

Dimerisation of the NS1 glycoprotein

Brief Report

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Summary. A part of the genome of dengue virus type 2 spanning the coding region from the carboxy terminus of the envelope protein E to the beginning of the NS3 protein was expressed using recombinant vaccinia virus. Additional constructs which contained open reading frames terminating within the NS1 or NS2A genes were also expressed. NS1 dimers were formed by extended NS1 molecules containing 61 amino acids of NS2A. No dimers were detected when NS1 was shortened by 79 amino acids at its carboxy terminus.

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Dengue virus type 2 (DEN-2) is a member of the family *Flaviviridae* genus *Flavivirus* [26]. The virus is widely distributed in tropical and subtropical areas, and is the cause of significant human disease [18]. The single-stranded RNA genome of DEN-2 is approximately 10,700 nucleotides (nt) in length, contains an open reading frame of 10,100 nt, and encodes a polyprotein which is rapidly processed to form three structural and seven nonstructural (NS) proteins [4, 11, 14, 25]. Although the importance of the flavivirus structural proteins in virion formation and infectivity is clear, functions have not been definitively assigned to all the nonstructural proteins. The amino terminal portion of NS3 contains the viral protease active in cleaving at basic sites located at the junctions between some of the nonstructural proteins [19], and it is likely that both NS3 and NS5 have a role in viral RNA replication [8, 21].

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The flavivirus NS1 glycoprotein is unique among nonstructural proteins encoded by RNA positive strand viruses as it is glycosylated. It was suggested that the protein may be involved in virion assembly and release [16, 21]. The monomeric form of DEN-2 NS1 is approximately M_r 46,000, and is comprised of a polypeptide backbone of M_r 40,000 and two N-linked glycans [25]. The amino and carboxy termini were identified by sequencing of radiolabelled protein [1, 29]. Intracellular and extracellular forms of the protein were described, with both forms existing as dimers that dissociated on heating to 100 °C [16, 27, 28]. In addition, there has been considerable interest in the potential use of NS1 in protecting against flavivirus infection in man. Studies in laboratory animals show that NS1 induces protective immunity and that anti-NS1 monoclonal antibodies mediate passive protection against infection [9, 12, 22–24]. Since the dimer appears to be the major form of DEN-2 NS1 in cell culture, studies were initiated to obtain more information on its structure. In particular, we wished to determine the regions of the protein required for dimer formation. Thus the NS1 gene of DEN-2 was expressed using recombinant vaccinia viruses, and dimer formation by extended and truncated NS1 polypeptides was investigated.

The open reading frames of the DEN-2 constructs expressed in recombinant vaccinia viruses (designated vDV) are shown diagrammatically in Fig. 1. A site for the initiation of translation was provided by cDNA containing sequences from the 5′ end of the Kunjin virus genome (stippled area) [3]. The cDNA for DEN-2 (New Guinea C strain) was prepared from two overlapping clones which were obtained by reverse transcription and sequenced by the methods used for the genes of the structural proteins [10]. Only one change was detected in the deduced amino acid sequence of NS1 of our isolate of New Guinea C when

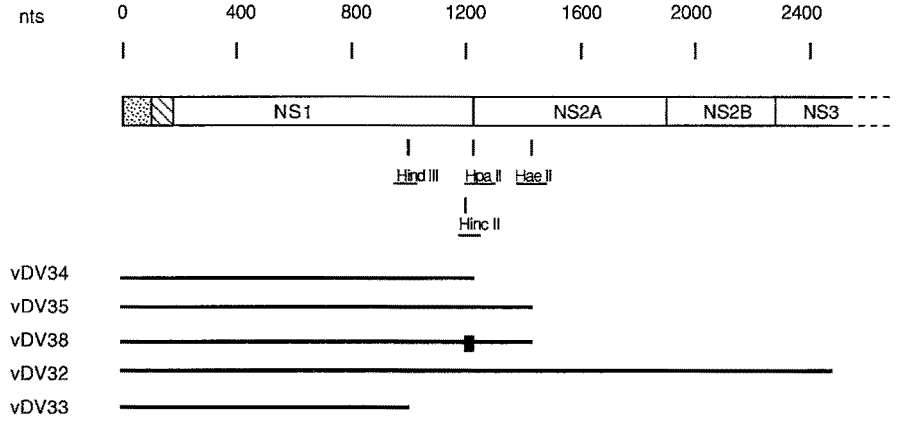
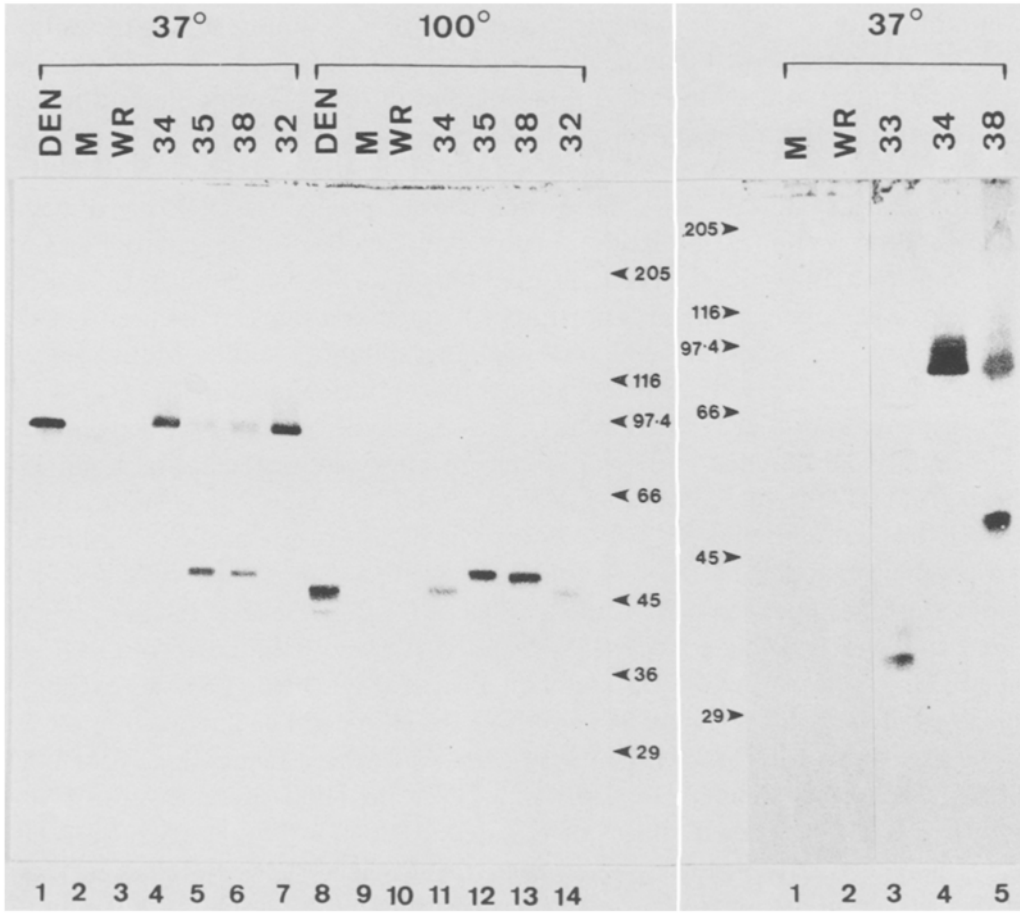


Fig. 1. Constructs of DEN-2 cDNA expressed in recombinant vaccinia virus. The open reading frames encoded by the various constructs (vDV) are indicated by solid lines. The stippled box represents 96 nt from the 5′ end of the Kunjin virus genome; the cross-hatched box represents nt encoding the carboxy terminal 27 aa of E. The solid box in vDV38 indicates nt encoding a substitution of six aa at the carboxy terminus of NS1

compared with that of the published sequence [14]. At amino acid (aa) position 141, the Ala residue of the published sequence was replaced by Pro. The DEN-2 numbering system of Irie et al. [14] is used in the following description of the constructs. Each contained ninety-six base pairs corresponding to nt 1–96 of the published sequence of Kunjin virus [3] ligated to DEN-2 cDNA at a filled in *Eco*R I site (nt 2340). Thus each construct began with 75 nt from the 5′ noncoding region of the Kunjin genome and encoded a protein containing the first seven aa of the Kunjin C protein joined to the last 27 aa of the DEN-2 envelope glycoprotein E. This portion of E acted as a signal sequence to insert NS1 into the endoplasmic reticulum; the corresponding coding sequences are cross-hatched in Fig. 1. The open reading frame of the longest construct (vDV32) was truncated in other recombinant viruses by modification of the sequence at conveniently located restriction endonuclease sites as indicated in Fig. 1, namely at an *Hin*d III site (nt 3235) for vDV33, at *Hpa* II (nt 3476) for vDV34, and at *Hae* II (nt 3658) for vDV35 and vDV38. The details of the carboxy termini of the proteins encoded by the five constructs were as follows: (*i*) vDV34 ended with -Ala-Gly-Leu, where Ala is the final aa of NS1, Gly is the first aa of NS2A and Leu is vector-encoded, (*ii*) vDV35 and vDV38 both ended with -Gly-Leu, where Gly is aa 61 of NS2A and Leu is vector-encoded, (*iii*) the carboxy-terminus of vDV32 contained 64 aa of NS3 fused to eight aa encoded by vector sequences, and (*iv*) vDV33 ended with -Leu-Ala, where Leu is aa 273 of NS1 and Ala is vector-encoded. In addition, vDV38 encoded a six aa substitution precisely at the carboxy terminus of NS1 (solid block in Fig. 1); -Asn-Ser-Leu-Val-Thr-Ala- was replaced by -Asp-Ser-Arg-Gly-Ser-Pro- encoded by a fragment of the pUC19 polylinker inserted between the *Hinc* II and *Hpa* II sites at nt 3457 and nt 3476 respectively.

To prepare recombinant viruses, the DEN-2 constructs were inserted into the plasmid pBCB06* at a site immediately downstream of the vaccinia virus P-7.5 promoter and flanked by vaccinia thymidine kinase (TK) gene sequences [2]. The plasmids were used to transfect mouse L929 cells which had been infected with vaccinia virus (WR strain) using methods described by Mackett et al. [17]. TK^- viruses were selected in TK^- human 143 B cells maintained in the presence of 25 μg/ml bromodeoxyuridine. Plaques formed by recombinant viruses were identified by DNA hybridisation and the viruses were plaque purified three times. To confirm the presence of the desired DEN-2 sequence in each virus, viral DNA was extracted from infected cells, digested with appropriate restriction endonucleases, electrophoresed and analysed by Southern blotting using the methods of Rice et al. [20].

The NS1 polypeptides synthesised in infected cells were detected by Western blot analysis. For controls, cells (143 B) were infected with DEN-2 (New Guinea C strain) at 10 pfu/cell, or with vaccinia virus (WR or recombinant) at 25 pfu/cell, or were mock infected. Cells were lysed in 2% (w/v) SDS three days after infection for DEN-2, or 24 h after infection for vaccinia. The lysates were heated for 5 min in the presence of 14 mM 2-mercaptoethanol at either 37 °C (unboiled)



A **B**

Fig. 2 A, B. Analysis by gel electrophoresis and Western blotting of NS1 proteins synthesised in cells infected by DEN-2 (*DEN*), or parental vaccinia virus (*WR*), or recombinant vaccinia viruses (*vDV32, 33, 34, 35, 38*). Cells were also mock infected (*M*). The positions of molecular weight markers are indicated. Samples were heated for 5 min either at 37 °C (unboiled) or 100 °C (boiled) before electrophoresis

or 100 °C (boiled) before electrophoresis in 7.5–15% acrylamide gels [15], and were then transferred to 0.45 micron nitrocellulose membranes overnight at 300 mA per gel. The membranes were incubated in 5% (w/v) non-fat dried milk in Tris-buffered saline (50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 0.05% Tween 20), and were then incubated with rabbit anti-NS1 antiserum [25]. After washing and incubation with anti-rabbit IgG antiserum conjugated with horse radish peroxidase, the membranes were washed and incubated with the substrate 4-chloronaphthol in 16% (v/v) methanol and 0.02% (v/v) H_2O_2.

The results are shown in Fig. 2 A. NS1 in DEN-2 virus-infected cells was detected in the form of dimers (lane 1), which were converted to monomers by heating at 100 °C (lane 8). NS1 was cleaved from the polyprotein encoded by

vDV32. This was apparent by comparison of the mobilities of the NS1 monomers obtained using vDV32 (lane 14) with those detected in vDV34 (lane 11) and DEN-2 virus-infected cells (lane 8). The dimers produced by the two recombinant viruses were stable at 37 °C (lanes 4 and 7).

The monomers encoded by vDV35 and vDV38 (Fig. 2 A, lanes 12 and 13) were larger and migrated more slowly than genuine NS1, suggesting that they remained uncleaved at the NS1/NS2A junction. Without cleavage, the calculated extra M_r of polypeptide at the carboxy terminus of NS1 was 6,000 (28% of NS2A). Dimeric forms of NS1 were detected for both these recombinant viruses (lanes 5 and 6). However, either dimer formation was incomplete, or the dimers were unstable at 37 °C, since monomeric forms were also detected in unboiled samples. Furthermore, the dimers in the unboiled samples did not show the expected retardation in mobility. This was probably due to anomalous migration of the larger dimers, rather than to the formation of some dimers containing correctly cleaved NS1, since no correctly cleaved NS1 was revealed after boiling of the same samples (Fig. 2 A, lanes 12 and 13).

The construct vDV33 encoded a truncated form of NS1 lacking the 79 carboxy terminal aa (22%) of the protein (Fig. 2 B, lane 3). No dimers were observed in the unboiled sample, and the migration of the truncated NS1 was consistent with a M_r of 37,000 calculated from the deduced aa sequence, and allowing for two N-linked glycans.

From these results it was apparent that the Kunjin sequences functioned satisfactorily in initiating translation, and that the carboxy terminus of E acted as a signal sequence to insert NS1 into the endoplasmic reticulum. The electrophoretic mobility of NS1 (Fig. 2) and the susceptibility of NS1 to digestion by endoglycosidase F (not shown) both indicated that the protein was fully glycosylated. The NS1 genes of dengue virus type 4 (DEN-4) and Japanese encephalitis virus (JE) have been expressed using recombinant vaccinia viruses, also with the aim of examining cleavage at the NS1/NS2A site and NS1 dimerisation [5–7, 13]. The data presented in Fig. 2 supports and extends observations made with the NS1 proteins of these two viruses. Cleavage at the NS1/NS2A junction of DEN-4 was blocked if 68 or more aa were removed from the carboxy terminus of NS2A [6], and thus predictably the protein encoded by DEN-2 vDV35, which lacked 157 aa, was not cleaved. Fan and Mason [7] expressed a JE construct similar to vDV32 (encoding NS1/NS2A/NS2B/part NS3) using a transient expression system, but detected additional larger monomers and dimers of NS1, possibly arising from alternative cleavages within NS2A. Our results with vDV32 showed that correct cleavage occurred at the DEN-2 NS1/NS2A junction. There was no apparent ambiguity of cleavage generating additional products as occurred for the expressed JE constructs.

The dimers produced in cells infected by DEN-2, vDV34, and vDV32 were stable at 37 °C. The lack of any monomers in unboiled samples demonstrated that the dimers were the predominant form of NS1. The results with vDV34 showed that unlike cleavage at the NS1/NS2A site [5, 6], dimer formation did

not require a substantial part of NS2A. Thus dimers are probably formed after cleavage of NS1 from NS2A in DEN-2 virus-infected cells. However, the presence of additional uncleaved NS2A sequences (vDV35 and vDV38) did not prevent dimer formation, although the dimers were apparently less stable than those containing correctly cleaved NS1. Dimers were also formed by extended molecules of NS1 of JE [7]. Recently, the importance of the last eight aa of NS1 of DEN-4 in determining cleavage at the NS1/NS2A junction was demonstrated [13]. Amino acids in NS1 at positions − 8, − 7, − 5, − 3, and − 1 relative to the cleavage site are highly conserved among flaviviruses. Our results with vDV38 showed that substitution of five of the final six (Ser at − 5 was unchanged) did not prevent dimer formation, at least in an extended NS1 molecule. Finally, no dimers were detected when the last 79 aa of NS1 were deleted (vDV33). Either certain amino acids within this region are directly involved in dimer formation, or the 79 aa deletion changes the native state of NS1 such that the conformation of one or more distant sites required for dimerisation is modified. The NS1 protein is being analysed further by deletion and substitution of aa residues to locate more precisely the regions required for the formation of stable dimers.

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