Cloning and Expression of Genes for Dengue Virus Type-2 Encoded-Antigens for Rapid Diagnosis and Vaccine Development

ANNUAL PROGRESS REPORT

by

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October 31, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH DEVELOPMENT COMMAND
Fort Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-85-C-5273

University of Kansas Medical Center
Department of Biochemistry
Kansas City, KS 66103

SECURITY CLASSIFICATION OF THIS PAGE

AD-A202018

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a REPORT SECURITY CLASSIFICATION unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release distribution unlimited
2b DECLASSIFICATION / DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION University of Kansas Medical Center 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code) Department of Biochemistry & Molecular Biology University of Kansas Medical Center 39th and Rainbow Blvd. Kansas City, KS 66103	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research and Development Command 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5273
8c ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 63750A; PROJECT NO. 3M4-63750D808; TASK NO. AH; WORK UNIT ACCESSION NO 031

11. TITLE (Include Security Classification)
Cloning and Expression of Genes for Dengue Virus Type 2 Encoded Antigens for Rapid Diagnosis and Vaccine Development

12. PERSONAL AUTHOR(S)
Radha K. Padmanabhan, Ph.D.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM 09/15/87 TO 09/14/88	1988 October 31	35

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
cDNA clones; complete sequence analysis; DEN-2 (New Guinea-C strain) RAI

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

1. Using oligodeoxynucleotide primers, we cloned the region of DEN-2 RNA encoding the structural proteins C, prM (M), and E glycoprotein. The cDNA clones were completely sequenced.

2. Using a synthetic primer complementary to the 3'-end of DEN-2 RNA, additional cDNA clones, mapping 3' to the previously characterized clones, # A4 and B2 were isolated. Genome 'walking' using these cDNA clones as probes, pPM-F12, pPM-A10, pPM53 were isolated, all mapping toward the 3'-end of the genome. These cDNA clones were sequenced.

3. To complete the cDNA cloning of DEN-2 genome, a novel approach was used which involved the polymerase chain reaction. Using this approach, the sequences between nucleotide 9950 and the 3'-terminus were amplified in a Taq polymerase-catalyzed chain reaction. The amplified DNA was cloned and sequenced. Keywords: Dengue Vaccine, Diagnosis, Medicine

20 DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	unclassified
22a NAME OF RESPONSIBLE INDIVIDUAL Ms. Virginia Miller	22b TELEPHONE (Include Area Code) 301/663-7325
	22c OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

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2.0 FOREWORD

The investigators have abided by the National Insitutes of Health Guidelines for research involving Recombinant DNA molecules (April 82) and the Administrative Practices Suppliments, as indicated in the Memorandum of Understanding and Agreement, approved by the Institutional Biosafety Committee and N.I.H.

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3.0 INTRODUCTION

Dengue, a human disease of global significance, is caused by dengue virus, a member of the newly formed family flaviviridae, which comprises of about 70 closely related enveloped viruses (Westaway et al., 1985). This group of viruses contains a single-stranded RNA of about 11 kb as their genome with a positive-stranded polarity (Russell, et al., 1980). The RNA has a type I cap structure, and a poly(A) track toward the 3' end is absent. Dengue viruses are of four distinct serotypes (DEN-1 to 4) and are transmitted to humans principally by Aedes aegypti mosquitos. In endemic areas of tropical Asia, apart from dengue fever (DF), a more severe form of the disease, dengue hemorrhagic fever (DHF), occurs in children, which could lead to dengue shock syndrome (DSS). Recently the pathogenesis of dengue was the subject of an excellent review by Halstead (1988).

The wide geographical occurrence of dengue infections combined with increasing number of epidemics in Central and South Americas and the Caribbean is a cause of major concern . An effective vaccine is not available to protect individuals against all four serotypes of DF. The major problem associated with dengue vaccine is that individuals having protection against one serotype are fully susceptible to infection with other DF serotypes. More often, the secondary infection with another serotype results in a serious form of the disease, DHF. Moreover, there are geographical heterogeneities in multiple dengue serotypes, as well as the genotypic variants of the same serotype (Trent et al., 1983; Repik et al., 1983; Kerschner et al., 1986; Walker et al., 1988). Using the techniques of RNA oligonucleotide finger printing and hybridizations with synthetic DNA probes, 15 genotypic variants for DEN-2, 7 for DEN-1 and 5 for DEN-3, have been characterized (Trent et al., 1983; Repik et al., 1983; Kerschner et al., 1986). Because of the rapid occurrence of variations in dengue viruses, there is a need to obtain the complete nucleotide sequence data of all the DEN serotypes and also of the important strains of the same serotype. This would make it possible to relate protein structure to specified surface epitopes and facilitate the development of a recombinant vaccine.

The first complete nucleotide sequence of a flavivirus reported was that of YF (Rice et al., 1985). This study established that there is a single long ORF coding for a large polyprotein, which is then cleaved by cellular and/or viral proteases to form the mature structural proteins; capsid (C); membrane (M); envelope (E); and nonstructural proteins: NS1, ns2a, ns2b, NS3, ns4a, ns4b, and NS5, respectively. Recently the nucleotide sequence data for DEN-2S1 candidate vaccine strain derived from the PR-159 isolate and the DEN-2JAM have been reported (Hahn et al., 1988; Deubel et al., 1988). Between the same topotypes, variations of about 10% in nucleotide sequences were noted. In addition, there were deletions of 20 nt in DEN-2S1 compared with the DEN-2JAM. A partial sequence totalling 5472 nt of cDNA clones from DEN-2NGS-C has been reported (Yaegashi et al., 1986; Putnak et al., 1988). In this report, we present the complete nucleotide sequence of the genome of DEN-2NGS-C strain and compare it with those of two other DEN-2 strains. Our results indicate that the DEN-2NGS-C is more similar to the DEN-2JAM than to the DEN-2S1 candidate strain from the PR-159 isolate.

4.0 Body of the Report

Cloning of the region of DEN-2 RNA encoding the structural proteins.

(a). Rationale:

One of the overall objectives of the Contract proposal is to sequence the entire dengue 2 virus genome. During the last ANNUAL REPORT dated November 17, 1987 [for the work done during September 15, 1986- September 14, 1987], the DNA sequence analysis of the regions encoding the nonstructural protein NS1, ns2a, ns2b, NS3, ns4a, ns4b, and up to 528 amino acids in the NS5 coding region was reported totalling 7446 nucleotides, which was about 74% of the viral genome. This report contains the complete sequence with the exception of only seven nucleotides in the protein noncoding region. Therefore, the specific aim # 1 of the Contract is essentially fulfilled.

(b). Experimental:

1. Cell culture, DEN-2 virus, and RNA

DEN-2 (New Guinea strain), originally isolated in 1944 (Sabin and Schlesinger, 1945) is the prototype strain of DEN-2 viruses. The virus stock used in this study was passed 38 times in suckling mouse brain, which was used to infect *Aedes albopictus* C6/36 cells in 175 cm^2 tissue culture flasks at a moi of < 0.5. The virus particles released into the growth medium were harvested by ultracentrifugation (100,000 x g for 3 h) seven and 13 days postinfection. The virus was adsorbed by immunoaffinity chromatography using the monoclonal antibody 4G2 raised against the structural glycoprotein E, which was linked to Protein-A Sepharose (Sigma Chemical Co.). The virus particles adsorbed to the affinity column were directly disrupted by passing a buffer (10 mM Tris.HCl, pH 7.5, 0.1 M NaCl, and 1 mM EDTA) containing 0.1% SDS. The viral RNA was collected in polypropylene tubes (Eppendorf) containing chloroform-saturated phenol. Subsequent to two extractions with phenol, the RNA was precipitated by the addition of 2.5 vols. of ethanol and stored at -70° C until use. The integrity of the RNA was checked by electrophoresis on an agarose gel and was found to be predominantly (>90%) full length when isolated by this procedure.

2. Synthesis of the cDNA copy of DEN-2 RNA

The nucleotide sequence of DEN-2 cDNA clones totalling 5472 nt, encoding the nonstructural proteins NS1, ns2a, ns2b, ns4b, and portions of NS3, ns4a, NS5 of the polyprotein precursor was reported previously (Yaegashi et al., 1986; Putnak et al., 1988). In order to clone the structural region upstream to the NS1 region, a synthetic primer CGTGAATTCATTCCTATCCAT (complementary to nt 2328-2348, in Fig. 2) was used for the reverse-transcriptase catalyzed cDNA synthesis. The experimental conditions of the cDNA synthesis were as described (Yaegashi et al., 1986). Briefly, the DEN-2 RNA was denatured with methylmercuric hydroxide (Bailey and Davidson, 1976) in the presence of the primer. Subsequent to an annealing step, the cDNA synthesis was carried out as described (Okayama and Berg, 1983

Gubler and Hoffman, 1983; Maniatis et al., 1982). Following methylation of EcoRI sites, the double-stranded cDNA was ligated to an EcoRI linker, digested by EcoRI, and size-fractionated by electrophoresis on an agarose gel. The cDNA fragments were cloned at the EcoRI site of pUC18 (Vieira and Messing, 1982). Alternatively, the blunt-ended cDNA fragments were cloned at the PstI-cut and PolIk-treated pUC18 vector. The transformants were screened by restriction enzyme digestion. One cDNA clone of about 2.4 kb in length and several independent clones of various lengths were obtained from the region upstream to the primer site. cDNA clones were also obtained by using random primers for the synthesis of the first strand cDNA (Taylor et al., 1976; Rice et al., 1981). They were ordered on the DEN-2 genome by hybridization using other cDNA clones, which were sequenced previously (pVV1, pVV17, and pVV9; Yaegashi et al., 1986), as probes.

To clone the cDNA containing the 3'-end of the genome, the DEN-2 RNA was tailed with poly(A) using E. coli poly(A) polymerase (Sippel, 1973; Gething et al., 1980). The first strand cDNA was synthesized using a primer containing a stretch of T residues (CCCCCGGGTCTAGA(T)15T-OH) to initiate DNA synthesis from the 3' terminus of DEN-2 RNA. Duplex cDNA was synthesized as described previously (Okayama and Berg, 1982; Gubler and Hoffman, 1983). This cDNA library was used to amplify the region containing the 3'-terminal sequences of the DEN-2 genome. For amplification, the chain reaction catalyzed by Taq polymerase (Perkin-Elmer-Cetus Corp., CT, U.S.A.) was used (Saiki et al., 1988; Scharf et al., 1986). The oligodeoxynucleotides GGACAAGTTGGTACCTATGG (nt 9373-9392 in Fig. 2) and CCCCCTCTAGA(T)15T-OH were used as primers for amplification by Taq polymerase. The amplified DNA, after a total of 25 cycles of denaturation, annealing and DNA synthesis, was purified by electrophoresis on an agarose gel. The 1.4-kb DNA fragment was digested with KpnI + XbaI prior to cloning at the corresponding sites of pUC18.

3. Sequencing methods

For sequencing the cDNA clones, either the chemical method of Maxam and Gilbert (1980) or the dideoxy chain termination method of Sanger et al. (1977) was used. Subclones from pPM-

F12 cDNA were generated by sequential digestion with exonuclease III and S1 nuclease, followed by treatment with PolIk and T4 DNA ligase as described by Henikoff (1984).

(c) Results and Discussion

1. Analysis of cDNA encoding the structural proteins

Based on our unpublished nucleotide sequence data in the region upstream of the N-terminus of NS1 previously reported (Putnak et al., 1988), a synthetic primer complementary to nt 2328-2348 of DEN-2 RNA in the C-terminal region of E glycoprotein was used for the cDNA synthesis. Subsequent cloning step gave rise to several independent cDNA clones of various lengths in the structural region, possibly due to some heterogeneity in the population of cDNA molecules (clones 1-6, in Fig. 1). The longest cDNA clone was about 2.4 kb (clone 4), which appeared to contain nearly all of the sequences upstream to the primer site, based on the comparison with the nucleotide sequence of DEN-2JAM (Deubel et al., 1986). The sequence analysis of the structural region was carried out on both strands of clones 4-7 (Fig. 1). The sequence of this region is shown in Fig. 2 with the exception of about seven nucleotides at the 5'-end.

2. Sequence analysis in the region encoding the nonstructural proteins and in the 3'-terminal noncoding region

A partial sequence in the region encoding NS3 was previously reported (Yaegashi et al., 1986). To complete the sequence analysis in NS3 region and extend our analysis toward the 3'-terminus of the DEN-2 RNA, the cDNA library was screened by hybridization using the clones previously sequenced (Yaegashi et al., 1986) as probes. The new clones were ordered along the genome by sequencing at their termini and by using them in rescreening the library, which gave rise to the clones 11-16 (Fig. 1). To obtain the clone(s) containing the entire 3'- terminal

sequence of DEN-2 RNA, a different strategy was used. The poly(A)-tailed RNA was used for cDNA synthesis as described in MATERIALS AND METHODS, Section **b**. From the ds cDNA mixture, the sequences containing the 3'-terminal end including the poly(A) tail were amplified using the Taq polymerase-catalyzed chain reaction (Saiki et al., 1988; Scharf et al., 1986) (Fig. 3) using the primers #1, containing the oligo(dT)$_{16}$ track, and #2 (nt 9373-9392). This strategy allowed us to amplify and identify the 3'-terminal cDNA clones that contained the poly(A) tail. However, in addition to the 1.4-kb fragment expected from the distance between the primer #2 (nt 9373 in Fig. 2) and the 3'-terminus based on the data of Hahn et al. (1988), two additional major DNA fragments of about 0.4-kb, 0.8-kb in length (Fig. 3A, lane 2) were also obtained. The possibility that the generation of additional DNA fragments (major fragments of 0.4-kb, 0.8-kb, and other minor fragments) is unique to the use of primer #1 in the PCR reaction was verified as follows. A different primer AGAACCTGTTGATTCAACAGCACC complementary to the 3'-terminal sequence of DEN-2S1 genome (Hahn et al., 1988) (primer #3), was substituted for the oligo(T)-containing primer #1 in the PCR reaction, and a single band of 1.4-kb was obtained (Fig. 3B, lane 2). It was further supported by the fact that, when the purified 1.4-kb DNA fragment from the PCR reaction product was used for the second set of PCR reaction cycles using the same primers #1 and #2, an identical pattern of additional DNA fragments was produced (Fig 3A, lane 3), confirming that these DNA fragments were the products unique to the primer #1 in the PCR reaction, possibly arising from its annealing to other sites. The origin of these spurious DNA fragments were not further investigated. Subsequent cloning of the 1.4-kb DNA fragment gave rise to several transformants. Three clones (clones 18-20 in Fig. 1) containing inserts of about 1.4-kb in length were selected for further characterization. Sequence analysis from their termini revealed the presence of the poly(A) tail of 22-26 nt in length.

It was reported that the products of 30 cycle-PCR amplifications contained a total of 17 misincorporations consisting of transitions and transversions distributed randomly throughout 28 separate clones of 239 bp DNA (Saiki et al., 1988). The overall error frequency of Taq polymerase in this case was 0.25% , although the actual rate of misincorporation per nucleotide per cycle is

estimated at 2×10^{-4} (Saiki et al., 1988). The sequence data derived from the conventional cDNA clones 16 and 17 extended up to nt 10,250. The sequence data for the region from nt 10,200 to the 3'-terminus was derived from the PCR clone 20. There are six nucleotide differences noted between DEN-2NGS-C and DEN-2JAM in this region. The possibility that some of these differences were due to the error frequency of Taq polymerase could not be ruled out.

3. Organization of DEN-2 genome

The complete sequence of the DEN-2NGS-C genome, with the exception of about seven nt from the 5'-noncoding region, based on the comparison with that of DEN-2JAM (Deubel et al., 1986), is shown in Fig. 2. It includes the previously published data (Yaegashi et al., 1986; Putnak et al., 1988) and is 10,723 nt in length, which is identical to that of DEN-2JAM. It is 20 nt longer than DEN-2S1 genome. The base composition is very similar to the other DEN-2 strains (Vezza et al., 1980; Hahn et al., 1988; Deubel et al., 1988) (data not shown). Comparison of the sequence of DEN-2NGS-C indicates that the genomic organization of the virus is similar to that of other flaviviruses, such as YF (Rice et al., 1985), WN (Castle et al., 1985; 1986), DEN-4 (Zhao et al., 1986; Mackow et al., 1987), PR-159 isolate of DEN-2S1 strain (Hahn et al., 1988), DEN-2 strain 1409 isolated in Jamaica in 1983 (DEN-2JAM (Duebel et al., 1988), JE (Sumiyoshi et al., 1987), and Kunjin (Coia et al., 1988). The length of the 5'- and 3'-nontranslated sequences are identical to that of DEN-2 JAM strain, being 96 and 454 nt, respectively. The sequences of the 5'-nontranslated segments of DEN-2NGS-C and DEN-2JAM are identical. Between DEN-2NGS-C and DEN-2S1, there are four ntucleotide differences in the 5'-noncoding region. In the region encoding NS3, there are nine additional nucleotides in DEN-2NGS-C, similar to the difference between DEN-2JAM and DEN-2S1 (Deubel et al., 1988). In addition, in the 3'-noncoding region of DEN-2NGS-C, there are 11 additional nucleotides, compared with that of DEN-2S1 strain; and it is more divergent in the 3'-distal half of the noncoding region than in the 3'-proximal half. The 3'-terminal 79 nt of a number of flavivirus genomes have been shown to have the potential to form a hairpin loop structure (Hahn et al., 1987). This is consistent with the notion that the 3'-proximal half of the genome, which is

conserved even among evolutionarily distant flaviviruses, may be involved in replication (Rice et al., 1985; Brinton et al., 1986; Wengler and Castle, 1986; Zhao et al., 1986; Takegami et al., 1986).

4. Deduced polyprotein sequence of DEN-2NGS-C genome and its cleavage sites

The translated sequence of the genome as shown in Fig. 2 indicates that one long ORF encodes 3391 aa residues. The codon usage is non-random, as noted by other investigators, and is very similar to the other DEN-2 strains (data not shown). The order of the gene products in the structural region is the capsid C, precursor of the membrane glycoprotein prM processed to M, and the envelope protein E, which is followed by the nonstructural proteins, NS1, NS2A, NS2B, NS3, ns4a, NS4B and NS5. This order was originally established for YF by Rice et al.(1985, and recently modified with respect to the location of NS2A and NS4B (Speight et al., 1988). The assignment of the cleavage sites indicated in Fig. 2 are based on the data from the direct N-terminal amino acid sequencing of these proteins isolated from DEN-2 virions for E (Bell et al., 1985), or from the DEN-2-infected cells for NS1, NS3 and NS5 (Biedrzycka et al., 1987), or by homology with the established cleavage sites of YF (Rice et al., 1985), WN (Castle et al., 1985; 1986; Wengler et al., 1985), and KUN-encoded proteins (Speight et al., 1988).

The C protein contains 16 R and 10 K residues (about 20% of the protein), which probably account for its affinity to the viral genome (Rice et al., 1985). The initiating M residue of C protein is probably removed by the cellular methionine peptidase, although this step is not well characterized. The C-terminal domains of C, M and E are hydrophobic, each of which probably serves as a signal sequence for the insertion of the respective protein that follows (prM, E, and NS1, respectively) across the membrane and into the lumen of endoplasmic reticulum, where it is cleaved by the host signal peptidase. The sequences V-M-A and V-Q-A conform to the consensus site proposed by von Heijne (1985; 1986) for cleavage by the cellular signal pepdidase, and might be involved in generating the N-terminus of prM and NS1, respectively. The cleavage at the prM-M junction occurs as a late step in virus maturation (Shapiro, et al., 1973). The prM

contains one putative N-glycosylation site which is not present in the mature M protein. The sequence of four residues preceding the cleavage site of E glycoprotein, P-A-Y-S is conserved in KUN, WN, MVE, SLE, JE, and YF (Trent et al., 1987). But in DEN-2 strains, it is P-S-M-T, which diverges to P-S-M-A in DEN-1 (Mason et al., 1987), or to P-S-Y-G in DEN-4 (Zhao et al., 1986).

The locations and identities in the polyprotein sequence of NS2A, NS2B, NS3, NS4B, and NS5 were recently established by partial N-terminal amino acid sequences of five KUN nonstructural proteins (Speight et al., 1988). The sites assigned for NS2A and NS4B of KUN are upstream to those originally proposed for the corresponding YF proteins (Rice et al., 1985), and are also present in the corresponding positions of WN (Castle et al., 1986), MVE (Dalgarno et al., 1986), and SLE (Trent et al., 1987) viruses. They conform to the consensus sequence proposed for cleavage by the host signal peptidase, V-X-A (von Heijne, 1985; 1986), rather than by the putative viral protease originally assigned for these cleavages (Rice et al., 1985). In the case of DEN viruses, the potential cleavage sites that would generate the N-termini of NS1 and NS2A, conforming to the consensus sequence of the type V-X-A, are conserved in all three DEN-2 strains, except that no stop transfer or translocation sequences occur upstream to this signal for NS2A (Coia et al., 1988). Moreover, for NS4B of DEN, this site (T-M-A) (see Fig. 4) does not strictly conform to the "-3 to -1" rule preceding the cleavage site, as identified for KUN virus (Speight et al., 1988), although the first four amino acids at the putative N-terminus of NS4B are identical in all DEN viruses so far examined, as well as in KUN and WN viruses. Interestingly, the sequence of three amino acids preceding T-M-A is V-A-A (Fig. 4), which conform to "-3 to -1" rule. So it is possible the requirements for cleavage by signalase are not absolute, and it remains to be seen whether this nearby sequence might be able to serve for signalase recognition. The cleavage sites preceding the N-termini of NS2B, NS3, ns4a, and NS5 are the same as originally assigned by Rice et al. (1985). In general, they contain a pair (or a cluster) of basic amino acids, followed by a short chain amino acid residue, which are probably recognized by a viral-encoded protease. The location of the N-terminus of the hypothetical ns4a and its sequence at

its putative cleavage site are tentative for any flavivirus polyprotein. It is based on the measured size of NS3 and the occurrence of the pair of basic amino acids, followed by a short chain amino acid.

Previously published data (Yaegashi et al., 1986) on the comparison of the amino acid sequences between YF and DEN-2NGS-C in the region encoding the nonstructural proteins revealed that these amino acid sequences are much less conserved except for NS3 and NS5, consistent with their postulated role in viral replication. Three regions of NS5 were shown by Rice et al. (1986b) to share some similarities with regions of RNA-dependent RNA polymerases of ten positive-stranded RNA genomes. Although the primary amino acid sequences are less conserved among different serotypes of DEN viruses, and more so among members of different serological groups, the hydrophobicity plots of all these flavivirus genomes are strikingly similar (data not shown), suggesting a common function for the viral-coded proteins.

5. Comparison of DEN-2NGS-C genome with those of other DEN viruses

Nucleotide divergence between the three DEN-2 strains was determined. The results shown in Table I indicate that there are a total of 836 nt changes consisting of 749 transitions and 87 transversions between DEN-2NGS-C and DEN-2S1 (7.8%). However, there are only 82 aa (2.4%) changes, 20 in the structural, and 62 in the nonstructural proteins. On the otherhand, DEN-2NGS-C and DEN-2JAM strains are more closely related, as there are only a total of 489 nt changes (4.6%) comprising 394 transitions and 95 transversions, which resulted in 58 aa changes (1.7%). The nucleotide sequence identities between DEN-2 and DEN-4, or between DEN-2 and DEN-1 are considerably less, indicating that these serotypes of DEN viruses diverged from DEN-2 strains much earlier.

Fig. 4 shows the alignment of the deduced amino acid sequence of DEN-2NGS-C strain with those of DEN-2JAM (Deubel et al., 1988), DEN-2S1 (Hahn et al., 1988), DEN-4 (Zhao et al., 1987), and a partial amino acid sequence of DEN-1 (Mason et al., 1987). The differences in the amino acid sequences between DEN-2 and DEN-4 range from 30% in the NS2A to 80% in the

NS4B protein (Table II). The overall similarity between DEN-2 and DEN-4 is only 68%, and between DEN-2 and DEN-1 in the regions sequenced (C, prM [M], E, and NS1), it is 69%.

Recently, the nucleotide sequence of the region encoding the structural proteins of DEN-2NGS-C, which was originally derived from Queensland Institute of Medical Research (DEN-2NGS-C-QIMR) was published (Gruenberg et al., 1988). Comparison of these data with ours reveals that the two sequences are essentially identical except for a few differences, which are as follows. The sequence of the first 76 nt in the 5'-noncoding region was not reported by Gruenberg et al. (1988). In addition, within the structural region reported, there are three amino acid changes resulting from nucleotide changes at second-codon positions. The amino acid residues at positions 171, 327, and 734 of the polyprotein sequence are K, E, and I in our study (Fig. 2), whereas they are R, K, and T, respectively in the report published (Gruenberg et al., 1988). Two independent clones were sequenced on both strands in our study, and they were identical in having these three changes in the amino acid residues. Therefore, these differences between the sequences could be attributed to the viral RNA resulting from different passage history and/or changes due to error by the reverse transcriptase during cDNA synthesis. Similar to the report of Hahn et al. (1988), we have also observed a number of clonal variations in our sequence analysis. For example, the aa # 451 is T in one cDNA clone (clone 4) and I in an independent cDNA clone (clone 6). Similarly, the amino acid residues at #2391 and 2392 are D and G in clone 15, and G and R in clone 13 (Yaegashi et al., 1986). Since some of these variations are at sites which are highly conserved among the different DEN viruses, the conserved amino acid residues were chosen (for example, the amino acid residues at positions 451, 2391 and 2392).

6. Conservation of glycosylation sites and cysteine residues

The potential sites of glycosylation in E or in the nonstructural proteins other than NS1 are not relatively conserved among the various flaviviruses. The single glycosylation site of prM at Asn-69 is conserved in all DEN viruses (Fig. 4), but is not conserved in YF or WN-MLV-SLE subgroup (Hahn et al., 1988). The glycosylation sites of NS1 at Asn-130 and Asn-207 are

conserved in all DEN strains (Fig. 4), as well as in the NS1 of other flaviviruses (Putnak, et al., 1988; Hahn et al., 1988), suggesting that glycosylation might be important for its function. They are present at identical location in the protein, except in YF, one of them is shifted by one residue (aa #208) (Hahn et al., 1988). As noted by others (Rice et al., 1986b), the cysteine residues are highly conserved in the structural proteins and NS1 of all DEN strains and other flaviviruses so far sequenced, with the exception of a single C residue in the NS1 of DEN-4 (corresponding to C residue # 1087 of DEN-2NGS-C in Fig. 4, which is substituted by V in DEN-4).

(d). Conclusions

The sequence data for the prototype DEN-2NGS-C provides additional information regarding the evolution of the geographically distinct isolates of the same DEN serotype. These comparative data point out that the DEN-2NGS-C isolated in 1944 and the DEN-2JAM isolated in 1983 have undergone very little divergence (<2%), compared with an attenuated strain of DEN-2 isolated in 1969 in Puerto Rico. Many conserved amino acid substitutions are present in the structural and nonstructural protein domains of the three DEN-2 strains. Future studies directed toward examining the differences in these domains would be expected to provide valuable insight into the relationship between the structure and function of these viral proteins, once a suitable mammalian expression system is established.

5.0 Figure Legends

Fig. 1. Sequencing strategy of DEN-2NGS-C genome.

The various cDNA clones and their map positions with respect to the viral genome are shown. The numbers 1-20 refer to the clones, p74-A28 (1), p72-A13 (2), p72-C15 (3), pKT2.4 (4), pKT1.8 (5), pKT1.6 (6), pRP-2 (7), pVV9 (8), pVV18 (9), pVV1 (10), pYS505 (11), pPM-A10 (12), pVV17 (13), pYS-B2 (14), pKT-A4 (15), pPM53 (16), pPM-F12 (17), pPM-PCR1 (18), pPM-PCR2 (19), and pPM-PCR3 (20), respectively. The nucleotide sequence of the cDNA clones pRP2, pVV9, pVV18, pVV1, and pVV17 have already been published (Yaegashi et al., 1986; Putnak et al., 1988). cDNA clones (clones 18-20) are derived from the poly(A)-tailed RNA and subsequent amplification by the PCR reaction. Sequencing was carried out by either the dideoxy chain termination method (Sanger et al., 1977) (dotted arrows), or by the chemical method of Maxam and Gilbert (1980) (solid arrows). The solid arrows refer to sequencing in the 3'-5' direction, and those preceded by dots represent sequencing in the 5'-3' direction. Subclones for pPM-F12 were generated by the method of Henikoff (1984).

Fig. 2. Composite nucleotide sequence of DEN-2NGS-C derived from cDNA clones.

The nucleotide sequences of the cDNA clones shown in Fig. 1 overlapped with the previously reported sequences for NS1, ns2a, ns2b, ns4b, and portions of NS3, ns4a, and NS5 (Putnak et al., 1988; Yaegashi et al., 1986). The complete sequence with the exception of about seven nucleotides at the 5'-noncoding region, based on the comparison with DEN-2JAM (Deubel et al., 1986), is shown along with the deduced amino acid sequence of the polyprotein precursor. The confirmed N-linked glycosylation sites are boxed and the potential ones are circled. The nomenclature of the viral proteins originally proposed by Rice et al. (1985) and recently modified by Speight et al. (1988) for NS2A, NS2B, and NS4B is followed. The horizontal arrows indicate the start points of these viral proteins. The cleavage sites for the generation of the N-terminus of the various proteins are based on the partial amino acid sequencing of E (Bell et al., 1985), and NS1, NS3, and NS5 (Biedrzycka et al., 1987), or on the homology with other flaviviruses (Rice

et al., 1985; Castle et al., 1985; 1986; Speight et al., 1988) (see section d of RESULTS AND DISCUSSION).

Fig. 3. Amplification of the 3'-terminal cDNA clone by polymerase chain reaction.

Panel A: DEN-2 RNA was tailed with poly(A) using E. coli poly (A) polymerase (Sippel, 1973; Gething et al., 1980). The first strand cDNA was synthesized using a primer containing a stretch of T residues and potential to form SmaI and XbaI sites (Primer #1; CCCCCGGGTCTAGA$(T)_{15}$T-OH), and the second strand of cDNA as described (Okayama et al., 1982; Gubler and Hoffman, 1983). For amplification, chain reaction catalyzed by Taq polymerase (Perkin-Elmer-Cetus Corp., CT, USA) was used. Primer #1 (see above) and the oligodeoxynucleotide GGACAAGTTGGTACCTATGG as primer #2 (nt 9373-9392 in Fig. 2) were used in a reaction mixture (100 µl) containing 10 mM Tris.HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin (w/v), dNTPs (200µM), 1µM each of the primers and 2.5 U of Taq polymerase. The sample was overlaid with mineral oil to prevent evaporation. The sample was incubated sucessively for one min at 94° C, 2 min at 37° C and 3 min at 72° C, and this cycle was repeated 25 times. Subsequent to the reaction, the sample was loaded on to an agarose gel (1%) and electrophoresed. The gel was stained with ethidium bromide and photographed. A. Lane 1, λ DNA digested with HindIII and used as size markers; the bands from top to bottom have sizes of 23-kb, 9.7-kb, 6.6-kb, 4.3-kb , 2.3-kb, 2,1-kb, and 0.56-kb; lane 2, PCR reaction product after 25 cycles; the sizes of the three major bands from top to bottom are 1.4-kb, 0.8-kb, and 0.4-kb, respectively; lane 3, second PCR reaction performed using the primers # 1 and # 2 and the 1.4-kb fragment purified from lane 2 as the template.

Panel B: PCR reaction carried out using the primer # 2 (see above) and the primer # 3, AGAACCTGTTGATTCAACAGCACC, which is complementary to the 3'-terminal sequence of DEN-2 RNA (Hahn et al., 1988) and the cDNA mixture that was used in the experiment in lane 2. The size of the single band is about 1.4-kb.

Fig. 4. Alignment of the complete amino acid sequences of DEN viruses.

The amino acid sequences of DEN-2NGS-C, DEN-2JAM (Deubel et al., 1988), DEN-2S1 from the PR-159 isolate (Hahn et al., 1988), DEN-4 (Zhao et al., 1986; Mackow et al., 1987), and DEN-1 (Mason et al., 1987) are compared. The dots indicate identical amino acid residues. The horizontal arrows represent the start points of the various viral proteins as shown in Fig. 2.

Legend to Tables

TABLE I. Divergence in nucleotide sequences among Dengue 2 strains

[a]Using the nucleotide sequence of DEN-2NGS-C as the reference, DEN-2JAM (JAM; Deubel et al., 1988) and DEN-2S1 (PR/S1; Hahn et al., 1988) are compared to calculate the number of transitions (purine--purine, or pyrimidine--pyrimidine) and transversions (purine -- pyrimidine, and vice versa) in the regions encoding the structural and nonstructural proteins.

TABLE II. Divergence in amino acid sequences among Dengue viruses

[a]The amino acid sequences of DEN-2NGS-C is used as the reference, and compared with DEN-2JAM (Deubel et al., 1988), DEN-2S1 (Hahn et al., 1988), DEN-4 (Mackow et al., 1987) and DEN-1 (Mason et al., 1987). The total The total number of amino acid residues in each of the structural and nonstructural proteins of DEN-2NGS-C are shown. [b] Proteins encoded by the virus refer to: C, Capsid; prM, precursor of membrane protein, M; E, envelope glycoprotein; NS1, NS2A, NS2B, NS3, ns4a, NS4B, and NS5 are the nonstructural proteins. [c] length of each protein as number of amino acids is given. [d] The number of dissimilar amino acid residues and the % identities are also shown. The values were calculated from the alignment of the amino acid residues of the various DEN viruses from Fig. 4.

Table I

Divergence in nucleotide sequences among DEN-2 strains[a]

	Structural Proteins		Nonstructural Proteins	
Transitions	JAM	PR/S1	JAM	PR/S1
G > A	20	35	71	114
A > G	18	36	68	113
C > U	22	37	110	187
U > C	25	55	97	172
TOTAL	85	163	346	586
Transversions				
G > C	2	2	5	1
G > T	2	3	5	5
A > C	0	1	3	16
A > T	4	3	11	19
Pu > Py	8	9	24	41
C > G	0	1	3	6
T > G	0	3	2	3
C > A	1	1	13	9
T > A	1	2	8	12
Py > Pu	2	7	24	30

Table II

Divergence in amino acid sequences among DEN-2 strains[a,b]

	DEN-2NGS-C	DEN-2JAM		DEN-2S1		DEN-4		DEN-1	
Protein	Length	Dissimilar[c]	%Similar	Dissimilar[c]	%Similar	Dissimilar[c]	%Similar	Dissimilar[c]	%Similar
C	114	3	97.4	1	99.1	36	68.4	36	68.4
prM	91	5	94.5	2	97.8	26	71.4	19	79.1
M	75	1	98.7	2	97.3	26	65.3	23	69.3
E	495	10	98.0	14	97.2	190	61.6	157	68.3
NS1	352	2	99.4	12	96.6	95	73.0	93	73.6
NS2A	218	7	96.8	4	98.2	152	30.3		
NS2B	130	1	99.2	3	97.7	57	56.2		
NS3	618	9	98.5	13	97.9	155	74.9		
NS4a	150	1	99.3	2	98.7	57	62.0		
NS4B	248	6	97.6	6	97.6	49	80.2		
NS5	900	13	98.6	22	97.6	242	73.1		

Fig. 1

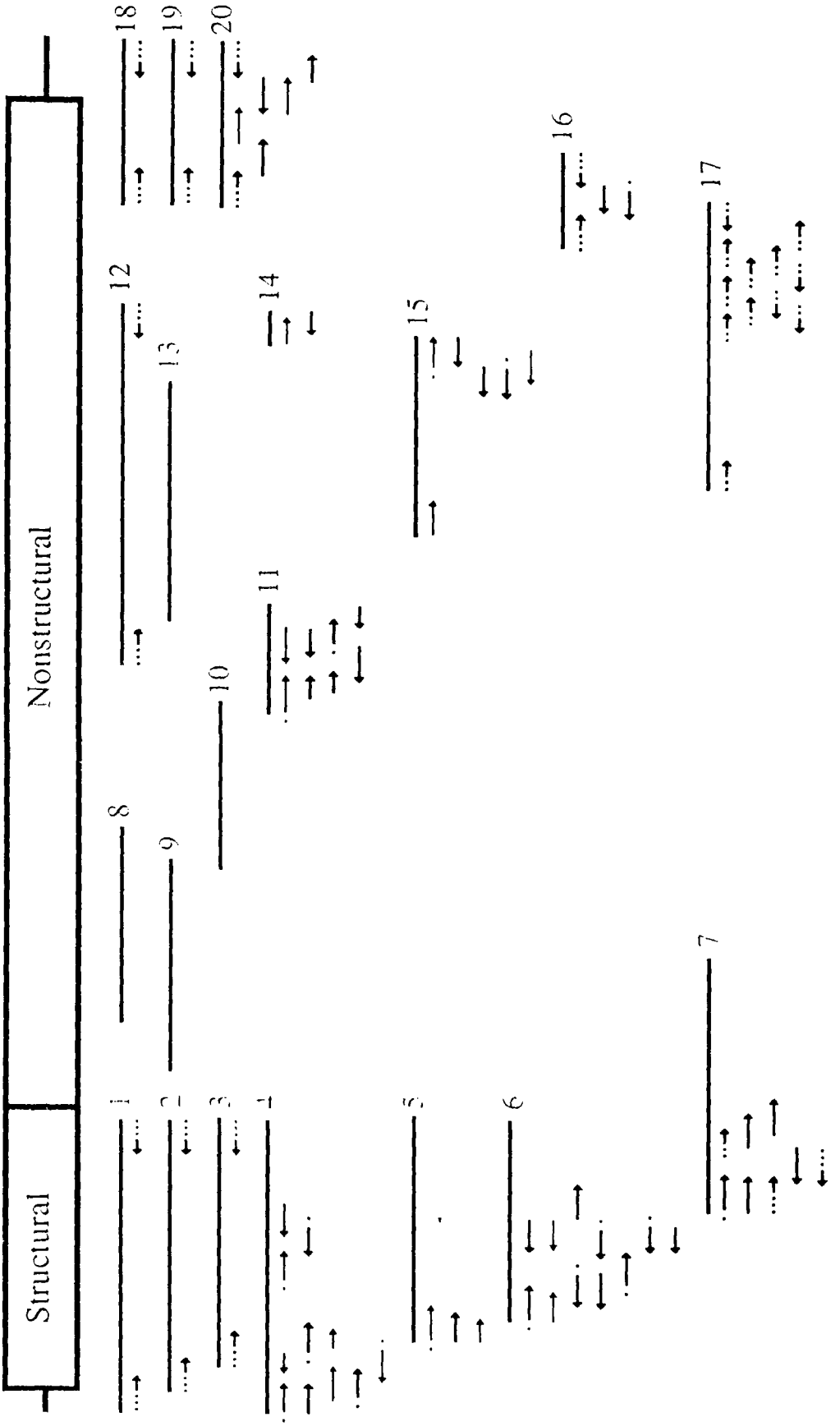
Structural
Noustructural
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Fig. 2

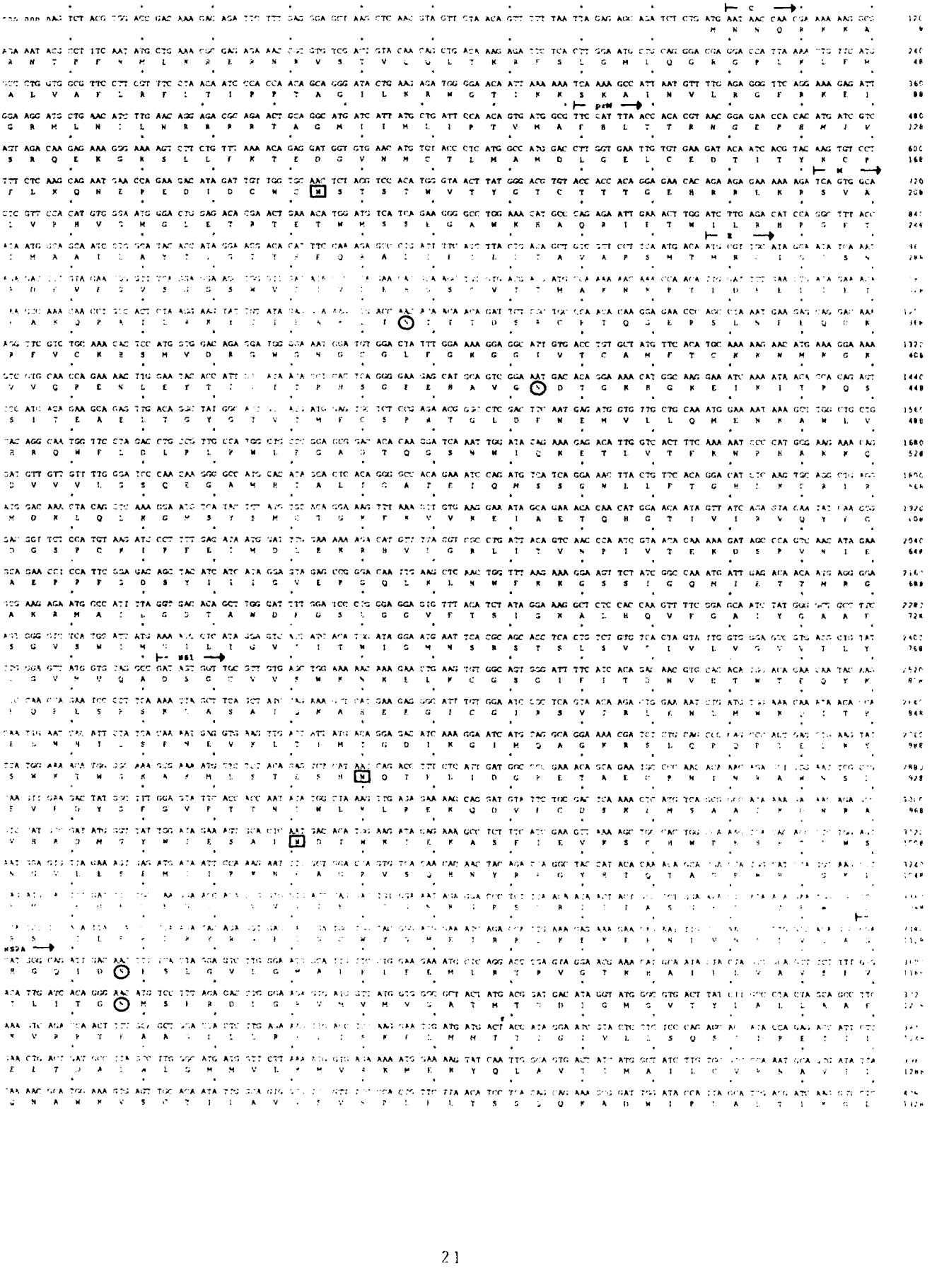

Fig. 2 cont.

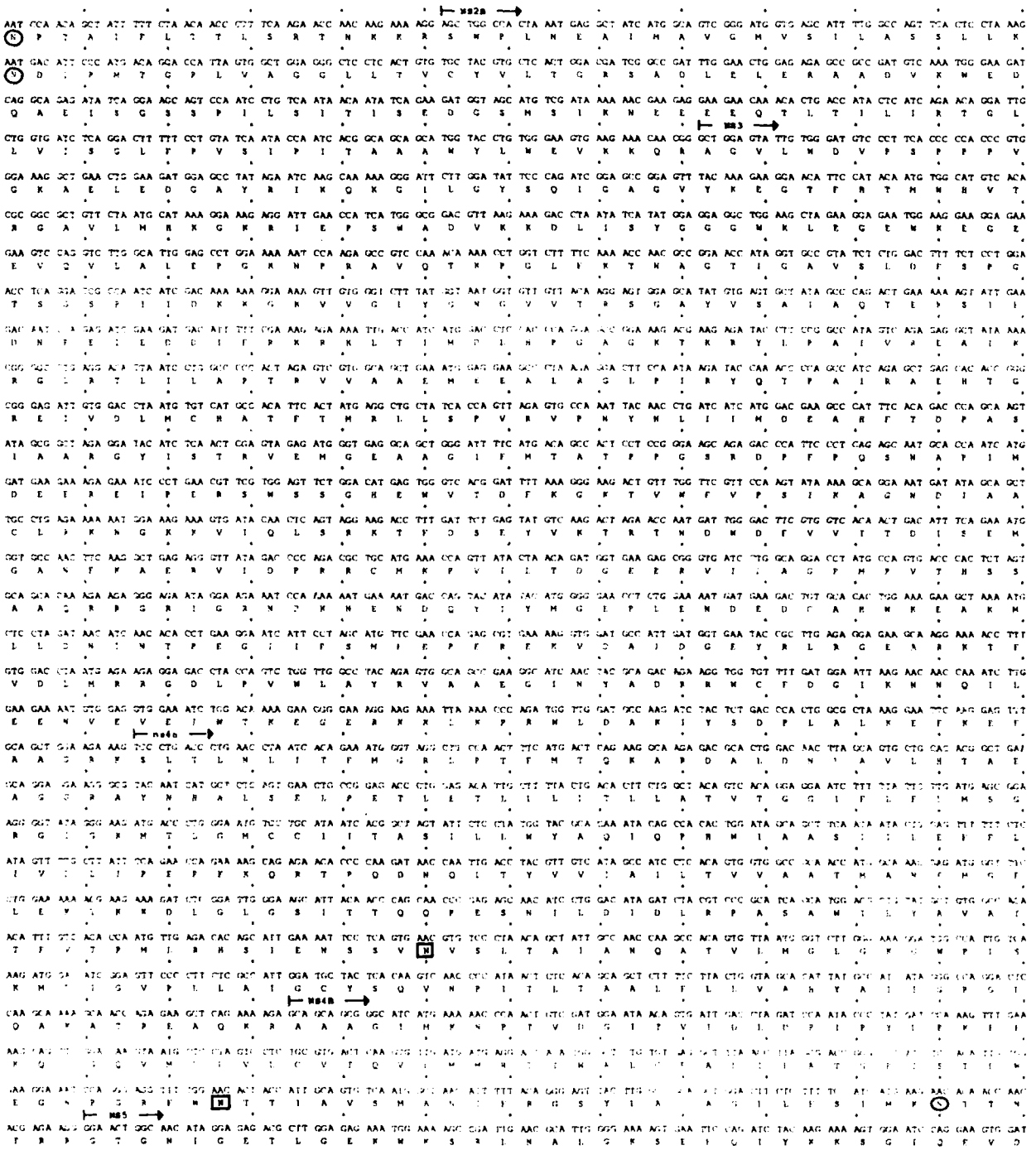

Fig. 2 cont.

Fig. 3

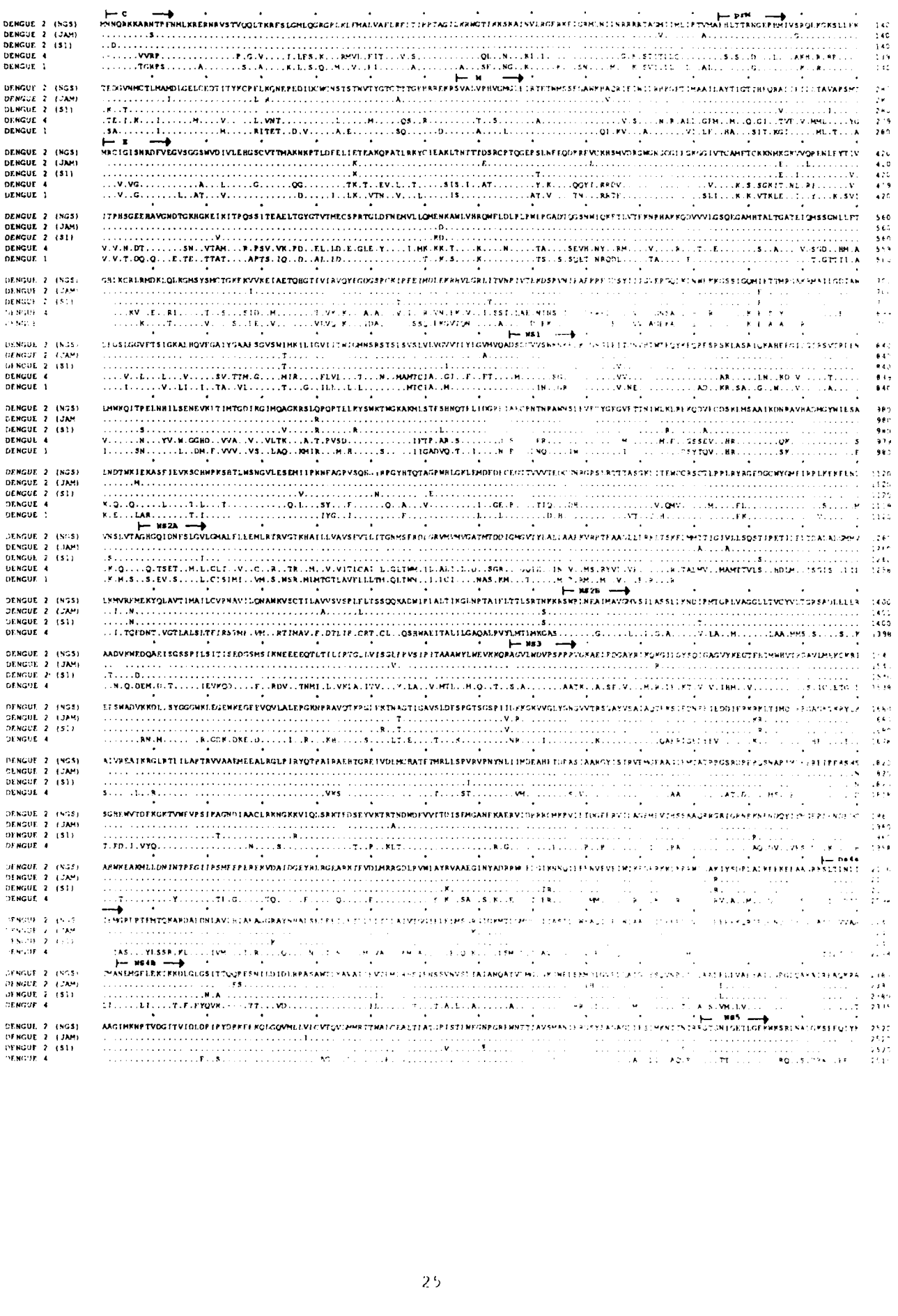
C
prM
M
E
NS1
NS2A
NS2B
NS3
NS4A
NS4B
NS5
DENGUE 2 (NGS)
DENGUE 2 (JAM)
DENGUE 2 (S1)
DENGUE 4
DENGUE 1

Fig. 4 cont.

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DENGUE 2 (NGS)   FSGIGEVDRTLANEGIKRGETDHHAVSRGSAKLRWFVERNMVTPEGKVVDLGCGRGGWSYYCGGLKNVREVKGLTKGGPGHEEP IPMSTYGWNLVRLQSGVDVFFTPPEKCDTLLCDIGESSPNPTVEAGRTLRVLNLVE   2660
DENGUE 2 (JAM)   ...................................................................................................................................   2660
DENGUE 2 (S1)    ....................................L............................................................V......................................   2660
DENGUE 4         P...L.....E..SAL.D.SKHK.......S.I..I...GHEK.K.E..............HAT..H.T....Y.............A........K.H...KL.YK.T.QV............S...I.E.........YM..   2655

DENGUE 2 (NGS)   NWLNNNTGFCIKVLNPYMPSVIEKMEALQRKYGGALVRNPLSRNSTHEMYWVSNASGNIVSSVNMISRMLINRFTMRHKKATYEPDVDLGSGTRNIGIESEIPNLDIIGKRIEKIKQEHETSWHYDQDHPYKTWAYHGSY   2800
DENGUE 2 (JAM)   ........................T.............................................K.......T..............................................................   2800
DENGUE 2 (S1)    ..........V.............R..T................................................................T.................................................   2800
DENGUE 4         P..SSKPE.M.........T...EL.K....H..N...C..............G..........TP.K..L....T..R.P....K.....A...SVST.TKK.DMT.S.R.LQRIQE..KFT.....FN..R.........   2795

DENGUE 2 (NGS)   ETRQTGSASSMVNGVVRLLTKPWDVVPMVTQMAMTDTTPFGQQRVFKEKVDTRTQEPKEGTKKLMKITAEWLWKELGKKKTPRMCTREEFTRKVRSNAALGAIFTDENKWKSAREAVEDSRFWELVDKERNLHLEGKCET   2940
DENGUE 2 (JAM)   ...............................................................................................................R.........   2940
DENGUE 2 (S1)    ...............................................................................F......................................................   294[illegible]
DENGUE 4         .PPS...P.........K.........I.....L.V...AIH..............PQ..P...PMV.T...N.  AI  ....N..I   ..S.........V.QF.Q[illegible]....S...N..   ...A..Q   S   29[illegible]

DENGUE 2 (NGS)   [illegible]   [illegible]
DENGUE 2 (JAM)   [illegible]   [illegible]
DENGUE 2 (S1)    ..........E..........I...I......A......   [illegible]
DENGUE 4         ..............R.........................M.........R.....EEID..D.DI.........LD..Q..DLT.EQ.APH...I..K.......F.L.......A.   [illegible]

DENGUE 2 (NGS)   [illegible]   32[illegible]
DENGUE 2 (JAM)   ..................................I.......................................................V.............   32[illegible]
DENGUE 2 (S1)    ..................................I........AS......D...........................R.................................T.......   32[illegible]
DENGUE 4         ......KE.................M...V...S....A...ITQDDNQNPKGLKER.EK...KEC.VD...K..................E..VTS.LF..........F...P......K..RN.QE.....A...KTF.......SI....   3215

DENGUE 2 (NGS)   [illegible]GPARISQGAGWSLRETACLGKSYAQMWSLMYFHRRDLRLAANAICSAVPSHWVPTSRTTWSIHAKHEWMTTEDMLTVWNRVWIQENPWMEDKTPVESWEE[illegible]SATWAKNIQTAINQ   3360
DENGUE 2 (JAM)   .........................K..............T..............................................A......................................   3360
DENGUE 2 (S1)    .....................................................................S.................K....L.................................   3360
DENGUE 4         ...............................A...........................SH.SE....TE.F...........H.Q..........K.......FD...M.T.....H..............S.........S....T.   3355

DENGUE 2 (NGS)   VRSLIGNEEYTDYMPSMKRFRKEEEEAGVLW   3391
DENGUE 2 (JAM)   ......................R.........   3391
DENGUE 2 (S1)    ......................R.........   3391
DENGUE 4         .N...K...V..N.V....YSAFS.SF....   3386
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Personnel Supported from September 15, 198[illegible] September 14, 1988

Personnel	% Effort in the reporting period	Months	Period
Research Associates			
Dr. Akihiko Nakashima	29	3.5	March 11, 1988-June 26, 1988
Dr. Gunwar Sripad	12.5	1.5	Nov. 1, 1986-Oct. 31, 1987
Dr. Marutlin Mohan	62.5	7.5	Nov. 1, 1987-June 12, 1988
Dr. Koji Irie	83.3	10	Sept. 15, 1987-Junr 12, 1988
	Subtotal Months	22.5	
Research Assistants			
Dianne Vassmer	41.7	5	Sept. 15, 1987-Feb. 20, 1988
	23.3	7	Feb. 21, 1988-present
Karin Page	8.3	1	Sept. 15, 1987-Oct. 7, 1987
Subtotal			
Kevin Graham	50	6	March 21, 1988-Sept. 18 1988
Melissa Larson	25	3	June 26, 1988-Sept 18, 1988
Graduate Student			
Thaweesak Trirawatanapong	50	6	Sept. 15, 1987-present
Laboratory Aide			
Cindy Smith (25%)	33.3	8	Sept. 15, 1987-May 28, 1988
Anthony Martinez	21	5	April 29, 1988-Sept. 9, 1988
Principal Investigator			
Radha K. Padmanabhan	35	4.2	Sept. 15, 1987-present
	Total	45.2	