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STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS

Annual Report

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

Progress during the reporting period included: i) mapping of antibody binding sites for the envelope (E) and non-structural-one (NS1) proteins specified by the Japanese encephalitis (JE) and dengue-one (DEN-1) viruses; ii) positive identification of JE virus coding segments that encode a previously undetected variant of the NS1 protein (larger), the previously undetected ns4 protein and one of the putative polymerase proteins, NS5; and iii) extension of the nucleotide sequence analysis of the JE and DEN-1 genetic material. The antigenic sites of the vaccine-candidate E and NS1 proteins were localized by analyzing binding activities of poly-and monoclonal antibodies to recombinant virus proteins produced in *E.coli* from deletion variants of the relevant cDNAs.

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FOREWARD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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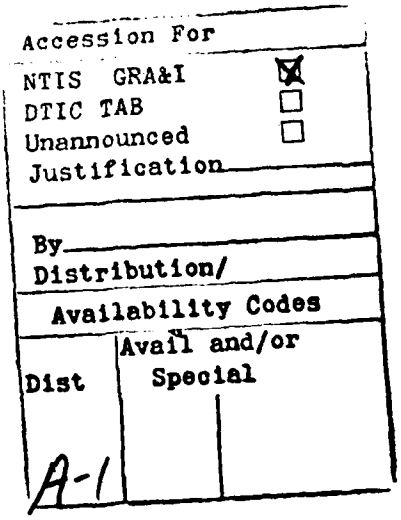

A. Other Contributing Personnel:

Name	Position
Mr. Jack McCown*	Professional Technician II
Dr. Peter W. Mason*	Former Postdoctoral Fellow (9/85-1/87) Currently Asst. Prof., YARU, Yale Univ.
Dr. Joe Liebeschuetz*	Postdoctoral Fellow (12/7/86-3/31/87)
Mr. Martin Zuegal	Graduate Student
Ms. Linda Niedzwicz*	Analytical Technician II (to Oct, 1986)
Mr. Anthony Semproni*	Research Assistant
Mr. Don Taylor*	Research Assistant
Dr. Deepak Gadkari	Guest Scholar[1]
Dr. Chong-Lek Koh	Guest Scholar[2]

* Designates contract employees

1 Institute of Virology, Pune, India
1/87 - 12/87
Salary support provided by Indian travel grant

2 University of Malaya, Kuala Lumpur, Malaysia
2/87 - 10/87
Salary support provided by Univ. of Malaya

B. Project Aims:

Stated objectives for the current project period include:

1. continue sequencing of the genome of the Japanese encephalitis and dengue viruses.

2. development of recombinant proteins from cloned JE and DEN-1 cDNA. The proteins of interest are the envelope (E) and non-structural-one (NS1) proteins.

3. analyze the immunological properties of the recombinant E and NS1 proteins. Characterization includes 2) evaluation of protective potential as candidate subunit vaccines and b) mapping of antibody binding domains on the individual proteins.

C. Program

1. Sequencing of the Dengue-Type One (DEN-1) Genome.

A manuscript reporting a partial sequence for the DEN-1 virus genome (Nauru Island isolate) under study was submitted and accepted for publications. The title of the paper is 'Sequence of the Dengue-1 virus genome in the region encoding the three structural proteins and the major non-structural protein N1.' The paper (copy attached) describes the sequence of 3745 nucleotides at the 5' end of the DEN-1 genome. The sequenced region contains the beginning of a continuous open reading frame which specifies the capsid (C), membrane (M), and envelope (E) structural proteins and the non-structural protein NS-1. The sequences are compared with corresponding proteins for seven other flaviviruses, including two of the three remaining dengue serotypes, DEN-2 and DEN-4.

Efforts to complete the sequencing of the genome continue, primarily through the work of two visiting scholars. Dr. Koh Chong-lek (Associate Professor from the University of Malaya in Kuala Lumpur) and Dr. Deepak Gadkari (Institute of Virology, Pune, India) mastered the requisite cloning and sequencing technology. As part of this training, Drs. Koh and Gadkari spent several days each at WRAIR and YARU learning virus culturing and RNA isolation procedures. The dengue RNA prepared included preparations from isolates of special interest to the W.H.O. Collaborating Center in Kuala Lumpur. Hosts for their visits were Col. Donald Burke and LTC Charles Hoke (WRAIR) and Drs. Peter Mason and Robert Shope (YARU). Dr. Koh, with the assistance of Dr. Gadkari, developed new sequence information for approximately 2.4 kb of cDNA extending from NS1 through nucleotide 6094 of the NS3 region. In addition, hybridization probes were used to identify clones that extend the mapped sequences to within 1 kb of the 3' end of the DEN-1 genome. Sequencing of these clones is in progress.

2. Sequence analysis of the Japanese encephalites virus genome (Nakayama strain).

Dr. Gadkari also initiated work to extend our sequence analysis of the JE genome. To this end, he has been learning the methodologies for direct RNA sequencing and for cDNA cloning. Dr. Gadkari isolated RNAs from two Indian isolates and the Nakayama strain of JE during visits to WRAIR and Yale.

3. Functional mapping of the JE genome

We have continued to use immunological procedures for the identification of viral-encoded polypeptides in JE-infected cells. JE cDNA fragments (Nakayama strain) have been expressed in *E.coli* as trpE-JE fusion proteins. Antibodies raised against these fusion proteins are useful probes for specific segments of the viral polyprotein.

In collaboration with Dr. Peter Mason - now an assistant professor at Yale University, we continued our analysis of the NS1 protein. Antibodies against the fusion protein specified by pATH#32 (see Table I) were used in Western blots and radioimmune precipitation (RIP) experiments to detect NS1 and its longer form, NS1', in insect (C6/36) and mammalian (Vero) cells infected with the Nakayama strain of JE. Two forms of NS1 were also detected in the extracellular fluid from cultures of infected Vero cells, the smaller form being much more abundant. Preliminary pulse-chase studies have failed to show a precursor-product relationship between the NS1 and NS1' proteins found in JE-infected Vero cell lysates and cell supernatants. The immunoreactive forms of NS1 were compared in cell lysates of Vero cells infected with six JE strains and Murray Valley encephalitis. The five JE strains that contained the NS1' protein were natural isolates, collected from several geographical locations, over a period of almost 50 years. Interestingly, the one JE strain that failed to yield detectable amounts of NS1 was an attenuated vaccine strain.

Antibodies against fusion proteins have also been used to identify proteins translated from the ns4 and NS5 regions of the JE genome. Immunoprecipitation and western blot analyses revealed the presence of a 29kDa protein that appears to be specified by the ns4a region of the JE genome. In addition, sequences related to this 29 kDa polypeptide appear to be present in a much larger polypeptide, possibly a higher molecular weight form of NS5.

Table I. Summary of reactivity of sera raised to trpE-JE fusion proteins

			SPECIFICITY			
	NUCLEOTIDES	PROTEIN CODING	C6/36		Vero	
CLONE*	EXPRESSED	REGIONS EXPRESSED	WESTERN BLOTS	RIPs	WESTERN BLOTS	RIPs
pATH#6	1821-2225	E	E	E	E	E
pATH#13	1275-2519	E, (NS1)	E	nd	E	nd
pATH#32	2658-3902	NS1, 'ns2a'	NS1, NS1' (p24, p30, p35)	NS1, NS1'	NS1, NS1' (p24)	NS1, NS1'
pATH#Aa	3522-3923	NS1, 'ns2a'	-	-	-	-
pATH#Ae	6660-7250	'ns4a'	p130,p150(p29)	-	(p38)	(p29)
pATH#Af	6660-8180	'ns4a,b,NS5	NS5,p130,p150 (p29)	NS5, p130	NS5, p130	NS5,p29
pATH#134	8230-9380	NS5	NS5,p130,p150	NS5.p130	NS5	NS5

4. Mapping of antigen binding domains of the JE envelope protein

Expression of JE cDNA in *E.coli* using the bacteriophage λgt11 vector (1,2) led to the identification of two separate regions of the E protein that were efficiently expressed as JE-β-galactosidase fusion proteins and reacted with antibodies present in polyclonal hyperimmune mouse ascites fluid (HMAF). The fusion protein derived from one of these regions, corresponding to amino acid residues 280 to 414 of the E protien, also reacted with 10 monoclonal antibodies (MAbs) generated against antigens expressed in JE-infected mice. These 10 MAbs appear to recognize different epitopes on the E protein based on 1) competitive binding analyses (D.S. Burke, personal communication), 2) cross-reactivity with other flaviviruses, and 3) neutralization titers.

To map the antigenic determinants for these MAbs on the E protein, we isolated the cDNA fragment from the shortest immunoreactive λgt11 clone and ligated it into the polylinker region of the appropriate pATH expression plasmid. Large quantities of E antigen were expressed from the resulting plasmid in the form of a trpE fusion protein. Deletions were generated by Bal31 exonuclease digestion. The truncated fusion proteins expressed by the deleted plasmids were then used in immunological tests to define the borders of the minimal sequence required for reactivity with the MAbs and HMAF. The epitopes recognized by HMAF and all 10 MAbs map to a region within amino acid residues 303 and 396 of the E protein. This sequence includes two cysteine residues that are known to form a structurally important disufide bridge in the E protein of flaviviruses (cf. ref. 4), and one of these residues (Cys-304) lies at the N-terminal border of the shortest immunoreactive sequence. Biochemical studies with the authentic viral protein confirmed the importance of disulfide bridges in the formation of this antigenic structure. These results show that epitopes for at least some strongly neutralizing MAbs lie within a 94 amino acid stretch of the E protien sequence. Furthermore, the presentation of these epitopes apparently requires the formation of a disulfide bridge between Cys-304 and Cys-335.

5. Mapping Epitopes on Recombinant DEN-1 Antigens.

DEN-1 E and NS1 protein coding sequences were expressed in *E.coli* by subcloning two large fragments of cloned cDNA into the pATH expression vector. A 1236 bp fragment coding for the first 412 amino acid residues of the 496 residues E protein and a 1184 bp fragment coding for the 355 C-terminal residues of NS1 plus 40 amino acids of ns2a were fused in-frame with the *E.coli trpE* gene in pATH-11 and pATH-1, respectively. The fusion proteins expressed by these plasmids cross-reacted with antisera and MAbs against DEN-1. Of 60 MAbs tested, five reacted with the E-*trpE* fusion and five others reacted with the NS1-*trpE* fusion. The known properties of these MAbs are given in Table 2.

We used deletion analysis to define the minimal sequences necessary to express the antigenic determinants for these antibodies. For mapping the C-terminal ends of the antigenic regions, we linearized the vector through random cutting with DNase I and inserted a *Bam*HI linker. After identifying plasmids with linker insertions within the viral cDNA inserts, the new restriction site was used to delete downstream coding sequences. This method produced a series of 19 nested deletions for the E-*trpE* protein with an average size increment between clones of approximately 70 bp. Similarly, 12 incremental deletions of about 100 bp were obtained in the NS1 sequence. For creating N-terminal deletions, the sequences downstream from the linker insertion were subcloned back into pATH vectors to establish new in-frame fusions to *trpE*. Precise mapping was accomplished by Bal31 exonulease digestion of selected plasmids.

The immunoreactivity of the truncated fusion proteins was examined by ELISA and western blotting. Two antigenic regions have been mapped in the E protein sequence. Domain I contains the epitopes for two MAbs and lies between amino acid residues 76 and 93. Domain II contains epitopes for three MAbs, at least one of which (4E5-6) confers passive protection in mice (7). This domain lies between residues 293 and 401 (109 aa) and contains an essential disulfide bridge. Although the N-terminal border of this region has not been precisely determined by Bal31 digestion, an analogous determinant on the E protein of JE has an N-terminal border at residue 303 (see above). Two overlapping domains have been mapped in the NS1 protein; the first is between residues 57 and 104 (48 aa), the second is between residues 81 and 125 (45 aa). These results are summarized in Figs. 2 and 3.

Table II. Properties of monoclonal antibodies that are reactive to recombinant DEN-1 antigens expressed in *E.coli*.

MAb [a]	Group [b]	Protein specifity [c]	PRNT [d]	Cross-reactivity [e]
8C2	I	E	nd	type
8B9	I	E	nd	subcomplex (D1, D3)
9D12	II	E	>10,000	type
4E5-6	II	E	>10,000	flavivirus subgroup
13D-4	II	E	nd	flavivirus subgroup
13B-6	I	NS1	nd	type
13A1	I	NS1	nd	flavivirus subgroup
15F3	II	NS1	nd	subcomplex (D1, D4)
5C11	II	NS1	nd	type
7E11	II	NS1	nd	subcomplex (D1, D4)

[a] The monoclonal antibodies are described in refs.(5-7) Two of the MAbs 9D12 and 4E5-6, bind to topographically related sites (6).
[b] The antibody groups were defined by the 3' deletion series.
[c] Protein specificity was determined by western blot analysis with the native viral antigens.
[d] PRNT determined as described by Henchal *et al.*, (6).
[e] The cross-reactivity determined by either western blot or ELISA.

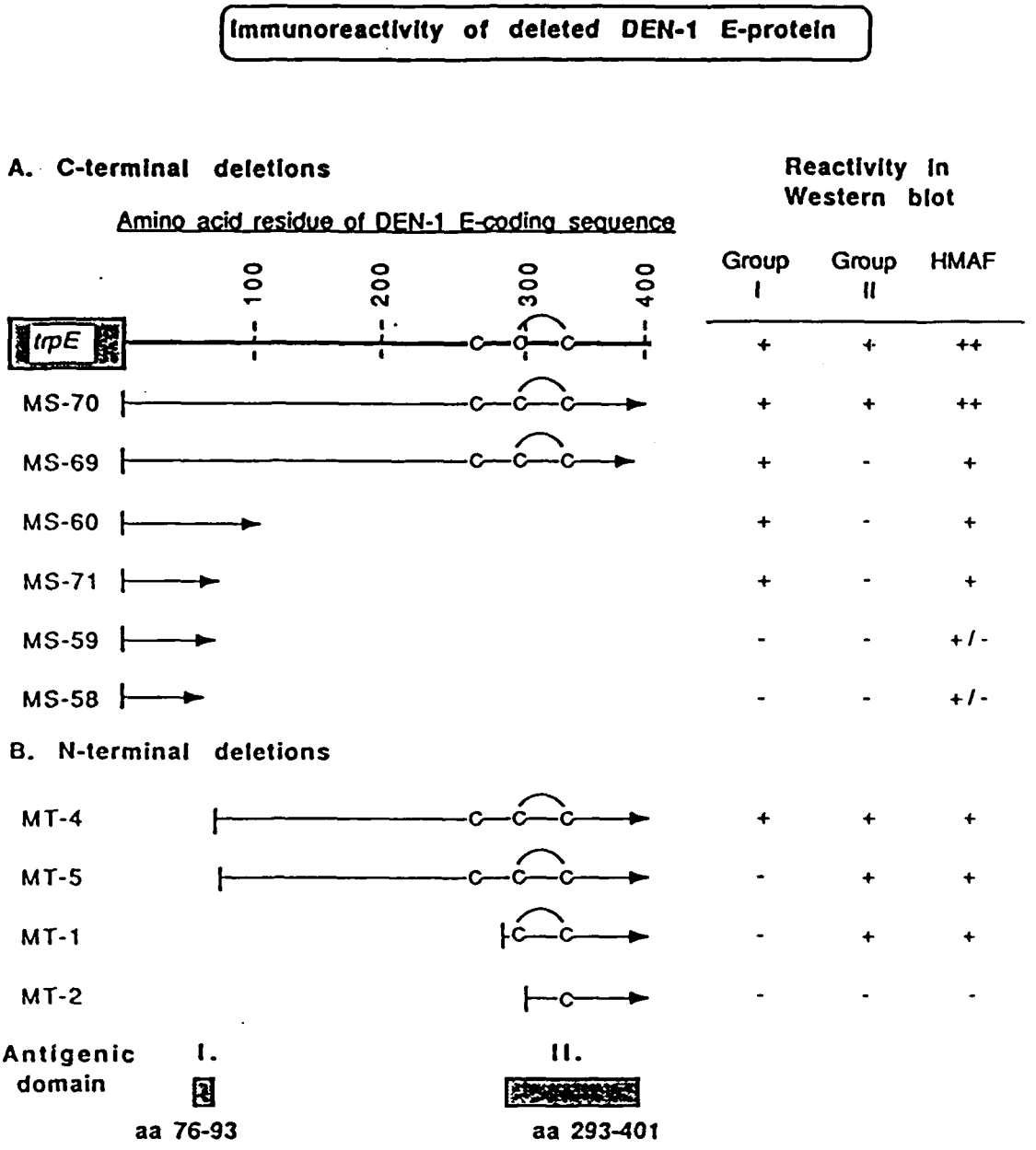

Fig.1. Summary of the deletion analysis used for mapping epitopes in the recombinant DEN-1 E protein. The clone designations for deletions extending from the carboxy terminus and the amino terminus, Figs 1A and 1B, respectively, are given on the left. The immunoreactivities of the derived recombinant proteins as determined by ELISA and western blot analyses are shown on the right. Group I and Group II antibodies are defined in Table II.

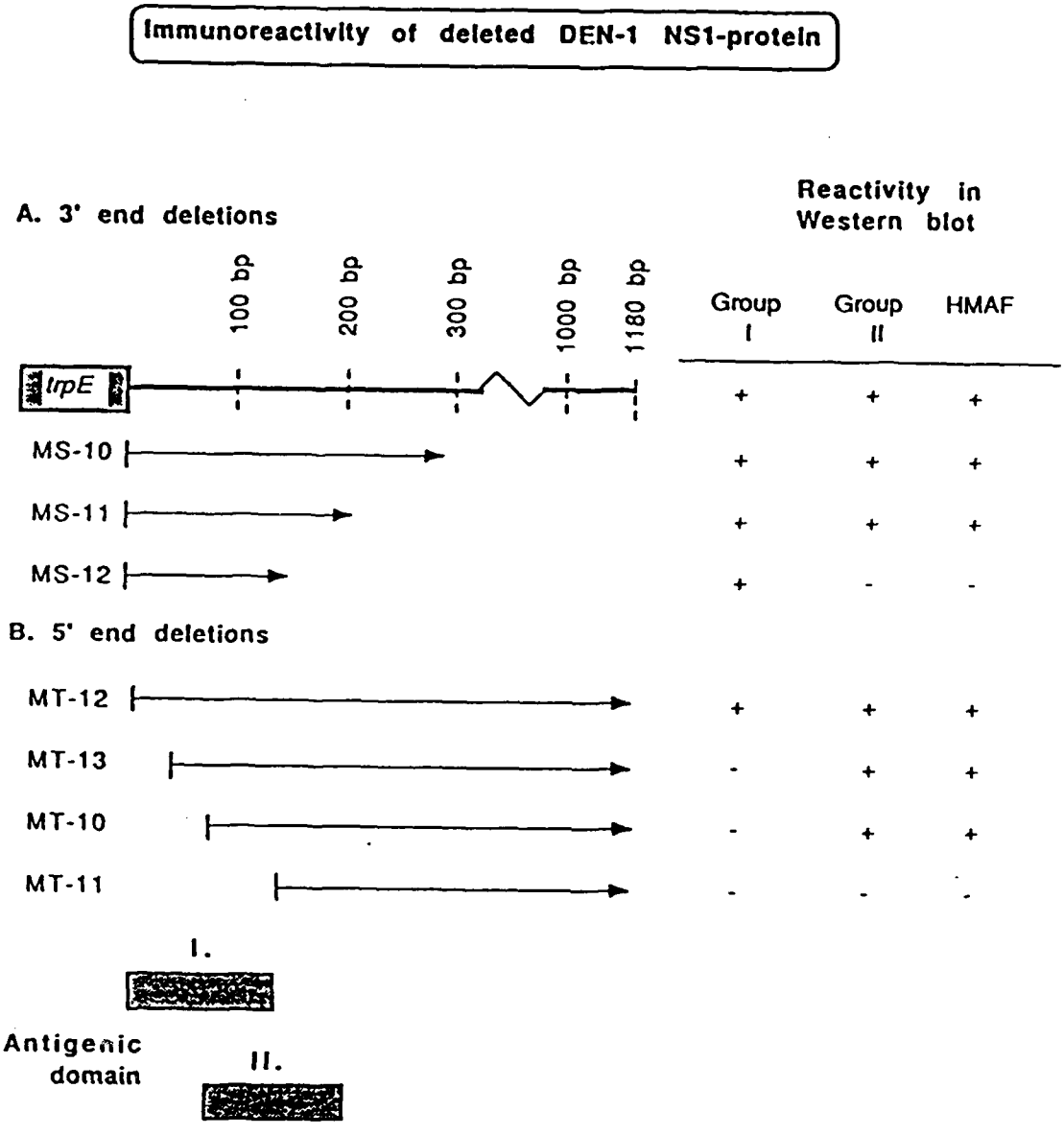

Fig.2. Summary of the deletion analysis used for mapping epitopes in the recombinant DEN-1 NS1 protein. The clone designations for deletions extending from the carboxy terminus and the amino terminus, Figs 2A and 2B, respectively, are given on the left. The immunoreactivities of the derived recombinant proteins as determined by ELISA and western blot analyses are shown on the right. Group I and Group II antibodies are defined in Table II.

6. Evaluation of recombinant JE E and NS-1 proteins as potential vaccines.

Earlier results had demonstrated that both the recombinant E and NS-1 proteins produced in *E.coli* reacted with antibodies present in the sera of humans who were seropositive for JE and were not reactive with control sera. The recombinant proteins were strongly immunogenic in mice as determined by western blot analysis using authentic viral proteins as the binding targets. The antisera elicited to the recombinant proteins had titers equivalent to those of sera obtained from virus-infected animals. Furthermore, the antisera displayed specific reactivities to either the E or NS1 proteins, depending on which recombinant protein was used as the immunogen. Based on these encouraging immunological properties, three different recombinant proteins (Fig.3) were selected for testing as protective immunogens in mice. Each protein was purified in milligram quantities and this material was forwarded to WRAIR for use in animal protection experiments directed by Dr. Charles Hoke. Results from these experiments will be available in the next quarter.

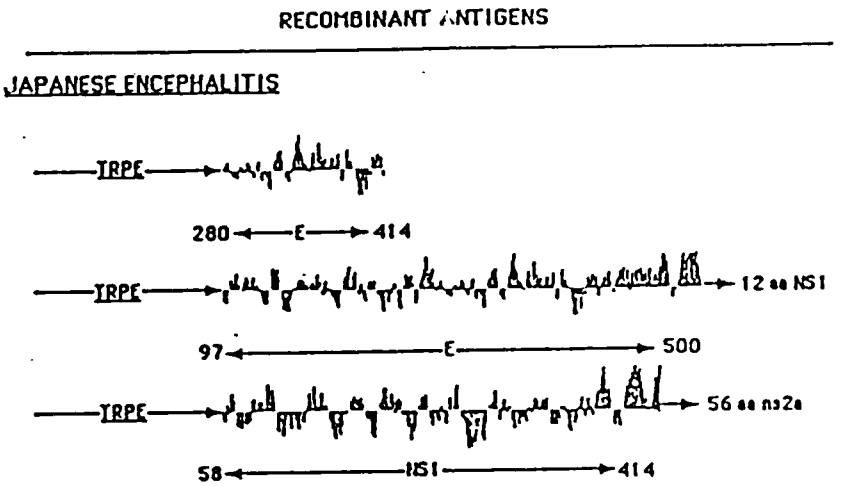

Fig. 3. Structure of JE fusion proteins showing the hydrophobicity profile of the respective JE protein sequences. Hydrophobic regions are indicated by areas above the line. The numbers below each construction indicate the amino acid residues of each JE protein expressed in the fusion.

D. Future work

Efforts for the future will include:

1. Continued sequencing of the DEN-1 genome
2. Extension of sequence analysis of the JE genome
3. Refinement of epitope mapping
4. Initiation of virus protein expression studies in insect and animal cell systems.
5. Assessment of the immunogenic potential of trpE-fusion proteins produced in *E.coli*.
6. Construct vectors for the expression of viral antigens in *E.coli* as non-fusion proteins.

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