SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

AD-A229 651

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 Massachusetts Department of Biochemistry 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Amherst, MA 01003	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-86-C-6156
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21702-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102A; PROJECT NO. 3M1 61102BS13; TASK NO. AA; WORK UNIT ACCESSION NO. 052

11. TITLE (Include Security Classification)
Structure and Expression of Genes for Flavivirus Immunogens

12. PERSONAL AUTHOR(S)
Maurille J. Fournier and Thomas L. Mason

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 6/14/87 TO 6/14/88	June 28, 1990	10

16. SUPPLEMENTARY NOTATION

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	~~RA 1~~; Flaviviruses; Subunit vaccines; Diagnosis; Mice; Gene mapping; Biotechnology; BW; CDNA; Cloning, Dengue Fever; DNA Probes; Recombinant DNA
06	01		
06	13		

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

Progress during the report period included: 1) Extension of the nucleotide sequence analysis of the Japanese encephalitis (JE) and dengue serotype 1 (DEN-1) genetic material; 2) expressed in E. coli both fusion and non-fusion derivatives of the envelope structural glycoprotein (E) and the nonstructural glycoprotein NS1; 3) used deletion analysis to define the minimal amino acid sequences necessary for the binding of anti-JE and anti-DEN-1 monoclonal antibodies; 4) determined that fusion derivatives of JE E and NS1 proteins expressed in E. coli do not provide protective immunity in mice; 5) developed nonradioactive nucleic hybridization probes for sensitive detection of DEN RNAs in virus-infected cells; 6) initiated collaborative efforts to express JE and DEN-1 antigens in baculovirus and vaccinia virus expression systems; 7) established a culture facility for the growth and analysis of DEN-1.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M. 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

November 21, 1990

STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS

Annual Summary Report

Maurille J. Fournier
Thomas L. Mason

June 28, 1990

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-86-C-6156

Department of Biochemistry
University of Massachusetts
Amherst, *Massachusetts* 01003

FOREWORD

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.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

A. Other Contributing Personnel:

Name	Position
Mr. Jack McCown*	Professional Techician II (6/87 to pres.)
Dr. Michael Beach*	Postdoctoral Research Associate (7/87 to pres.)
Mr. Anthony Semproni*	Research Assistant (6/86 to pres.)
Mr. Don Taylor*	Research Assistant (10/86 to pres.)
Dr. Deepak Gadkari	Guest Scholar[1]
Dr. Chong-Lek Koh	Guest Scholar[2]
Dr. Zhang Yong-he	Guest Scholar[3]
Dr. Xiao Ze-shuai	Guest Scholar[3]
Dr. Watcharee Attatippahohkun	Guest Scholar

* Designates contract employees

1 Institute of Virology, Pune, India
1/87 - 10/87
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

3 Institute of Virology and National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China
10/87 - pres.
Supported by WHO grant to M.Fournier and T.Mason

4 Mahidol University, Bangkok, Thailand
2/88 - present

B. Project Aims

Objectives for the project period include:

1) continue sequencing genomes of the Japanese encephalitis (JE) and dengue (DEN) viruses.

2) develop recombinant viral proteins - for potential use as vaccines against the JE and DEN-1 viruses. The proteins of interest are the envelope (E) and non-structural-one (NS1) proteins. Primary emphasis is on E. coli expression systems, but other host-vector systems are also under study.

3) characterize the vaccine potential of the recombinant viral proteins. Immunogenicity in mice is assessed by evaluation of: a) titer and specificity of antibodies reacted in vitro with authentic E and NS1 proteins and b) protective potential to lethal challenge of virus.

4) identify E and NS1 protein domains that define sites of antibody recognition, ie. epitopes. Strategies involve antibody binding assays with mutant variants of recombinant proteins and chemically synthesized peptides.

5) to develop cDNA and RNA probes for use in research and clinical diagnosis.

All of the aims have been addressed during the contract period. The major thrust of the program is vaccine development, and the objectives most closely related to this goal have received priority.

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 NS1.' The paper describes the sequence of 3745 nucleotides at the 5' end of the DEN-1 genome.

Efforts to complete the sequencing of the genome continue, primarily through the work of visiting scholars; first through the efforts of Dr. Koh Chong-lek and Dr. Deepak Gadkari and more recently Dr. Zhang Yong-he and Dr. Xiao Ze-shuai have contributed to this phase of the project. 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 encephalitis 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 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.

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

Expression of JE cDNA in *E.coli* using the bacteriophage lambda-gt11 vector led to the identification of two separate regions of the E protein that were efficiently expressed as JE-beta-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 protein, 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. 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, 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. We used deletion analysis to define the minimal sequences necessary to express the antigenic determinants for these antibodies. 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. 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).

6. Recombinant E and NS1 Immunogens: E. coli products.

Fusion proteins. - Experiments were initiated at WRAIR to examine the potential of three different trpE-JE fusion proteins as protective immunogens in mice. Disappointingly, none of the recombinant proteins appeared to elicit an immune response capable of affording protection against virus challange.

Non-fusion proteins - The major focus of our effort during the reporting period has been directed at producing a new generation of recombinant proteins in *E.coli*. A new strategy has been initiated that features synthesis of proteins that contain essentially only flavivirus protein elements. This approach is in contrast to that used to produce our first generation immunogens. Heretofore, our recombinant proteins were produced as fusion proteins, with segments of *E. coli* beta-galactosidase (lacZ) or trpE proteins at the amino terminus. The fusion derivatives have been very effective in a variety of applications - including the placement of individual coding sequences on the genomic map, epitope mapping of E and NS1, and development of novel antibody reagents, but they have not proven to be effective in generating a protective immune response (see above). We have therefore designed and constructed vectors for the expression of viral antigens as non-fusion

derivatives. This effort is motivated by the desire to fully evaluate the potential of *E.coli* as an expression system. *E.coli* offers tremendous advantages over insect and mammalian systems for the inexpensive production of large quantities of recombinant proteins for use as subunit vaccines. Our plan is to test the protective potential of non-fusion proteins developed for one virus before initiating similar constructions for the second. To this end, efforts have been in progress to develop non-fusion variants of the E and NS1 proteins of DEN-1. In this strategy, proteins are expressed from a vector in which viral cDNA is fused to a plasmid segment containing a bacterial ribosome binding element, a translational start signal and codons for six or seven 'foreign' amino acids. The sequences expressed include the major epitopic domains of each immunogen expressed in *E.coli*, as determined by our earlier epitope mapping analyses. The E protein variants also contain a segment that corresponds well with a 9-kDa proteolytic fragment of the tick borne encephalitis virus E protein previously shown to elicit neutralizing antibodies in mice.

Good expression results have been obtained thus far for the E protein vectors. Under inducing conditions, the viral protein, designated delta-34, comprises upwards of 15% of the total protein in the recombinant organisms and purification schemes were developed for producing biochemical quantities of the delta-34 protein. The procedure is simple, involving: i) disruption of the bacterial cells and concentration of the recombinant protein by centrifugation (the protein product self-associates to form an insoluble aggregate), ii) solubilization of the protein in the presence of a denaturing agent followed by extraction in an acid solution condition, iii) dialysis in urea and a sulfhydryl reducing agent - to cleave possible intermolecular disulfide bonds, iv) purification by chromatography on a carboxymethyl cellulose ion exchange resin, and v) dialysis to remove urea and reducing agent - to permit protein renaturation. This procedure has been scaled up successfully to yield about 50 milligrams of protein per five liters of culture. The purified Q34 protein was detyermined to contain one disulfide bond, analogous to the situation that obtains for the corresponding domain in authentic DEN-1 E protein. The immunogenic potential of our delta-34 antigen will be evaluated at WRAIR.

A variant of this antigen is being constructed. In this derivative, the potential for wrong disulfide bond formation, which could lead to loss of immunogenic activity, will be eliminated by site-directed mutagenesis. In the new derivative, a cysteine to serine substitution will remove the cys residue that is not involved in formation of antigenic domain II.

7. Recombinant E and NS1 proteins: Non-bacterial expression systems.

Efforts have been initiated to assess the potential of producing effective recombinant immunogens in organisms other than *E.coli*. Of special interest are eukaryotic cells where maturation and folding of the virus protein products may occur more naturally. The alternate expression systems include insect and animal cells. Some of these efforts are being initiated solely by us and some are being carried out in collaboration with other groups.

Baculovirus mediated gene expression in insect cells - A collaborative program was established during the reporting period to assess the potential

of producing recombinant E and NS1 proteins in insect cells using the potent baculovirus expression vector. This effort is being conducted by the commercial firm, MicroGeneSys, (located in West Haven, Connecticut) in cooperation with investigators at Walter Reed and our own group at the University of Massachusetts. Our role is to supply appropriate E and NS1 segments of JE and DEN-1 cDNA, to participate in evaluation of product proteins and serve as consultants as needed.

Viral cDNAs encoding the E and NS1 proteins have been delivered to MicroGeneSys and that group has successfully expressed the proteins of interest for JE. We will be especially interested in the the extent to which the recombinant antigens behave like authetic antigens from flavivirus-infected cells.

Expression of JE and DEN-1 proteins in mammalian cells - Work is in progress to study production of recombinant immunogens in mammalian cells. The approaches include the use of vectors developed from SV40, adenovirus and vaccinia virus. The SV40 and adenovirus vector systems are being evaluated in our laboratory. Work with vaccinia vectors will be started here, but the major effort will be in collaboration with the group of Dr. Enzo Paoletti at the Virogenetics company in Albany, New York. We have devoted a portion of our effort to engineering the transient expression of DEN and JE proteins using SV40 vectors in COS cells. Our immediate objective is to characterize the immunological quality of proteins made in the animal cells and assaying reactivity with the panel of flavivirus antibodies.

8. Improvement of viral diagnostic procedures.

An initiative was launched during the reporting period to evaluate new non-radioactive methods of labeling diagnostic nucleic acid probes. This work was initiated by a UMASS graduate student working under Dr. Gadkari's direction and is being extended by a new guest worker from Bangkok. The new colleague is Dr. Watcharee Attatippahohkun, Instructor in Clinical Chemistry at Mahidol University. (Dr.Watcharee Attatippahohkun is supported by a one year Fogarty International Fellowship).

The aim here is to establish protocols for the production of highly sensitive nonradioactive RNA or DNA hybridization probes for use in detecting and discriminating between virus nucleic acid in test materials. This will be of special importance for technical workers in the field and researchers and clinical personnel.

We have completed our first side-by-side comparisons of DEN-1 probes tagged with biotin by three different enzymatic methods: i) *in vitro* transcription of cDNA using T7 and T3 phage RNA polymerases; ii) nick-translation of cDNA fragments; iii) random primer labeling of cDNA. For labeling of RNA transcripts, allylamine-UTP was used as a substrate, and the RNA was biotinylated in a second reaction with CAB-NHS (caproylamidobiotin-N-hydroxysuccinamide ester). Biotinylation of the DNA probes was accomplished by incorporation of biotin-21-dUTP, a dTTP analog with biotin attached to the pyrimidine ring by a 21-atom spacer arm. Side-by-side comparison of the probes in dot blot assays using alkaline-phosphatase-conjugated avidin for detection showed that the RNA probes were the most sensitive, followed by random primer labeled DNA and nick-translated DNA, in that order.

Hybridization analyses were carried out with a variety of target nucleic acids including total RNA extracted from uninfected and DEN-1 infected C6\36 cells and DEN-1 RNA purified from virions. Although our analysis is still in progress, results developed to date indicate that the biotinylated RNA probes are capable of detecting less than 5 pg of purified DEN RNA and of producing a positive signal with less than 50 pg of RNA extracted from infected cells. There was no background signal with a 10,000-fold excess of the RNA from uninfected cells. Based on these promising preliminary results, we are extending the analysis in a systematic manner to include testing of clinical and other biological samples.

9. Virus culture facility. We have recently established a P2 containment laboratory for the growth and analysis of dengue virus and for our work with viral expression vectors including baculovirus, vaccinia and adenovirus. We have acquired expertise in the following areas: i) titering, growth and purification of DEN-1; ii) isolation of full-length DEN-1 RNA in excellent yield; iii) PRNT assays; iv) immunofluoresence assays; and v) isolation of recombinant viral expression vectors. While these are not research accomplishments, they provide much of the the technical foundation for the broadened scope of the experimental work we have proposed for the next contract period.

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 viral antigens expressed in *E.coli* and eukaryotic cells.
6. Continue the development of sensitive diagnostic assays for DEN-1.