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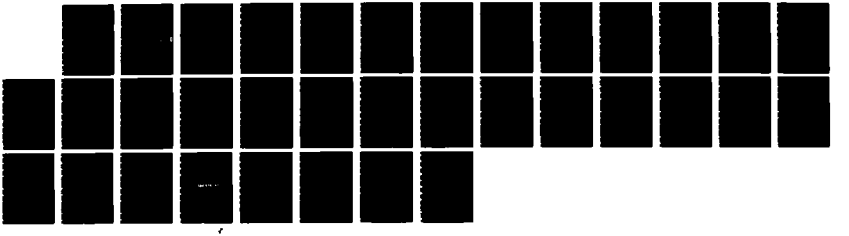

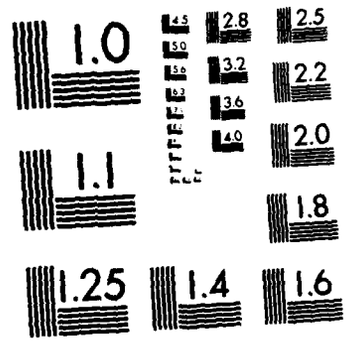

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STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS (U)

Annual Summary Report

1 September 1983

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-82-C-2237

Maurille J. Fournier and Thomas L. Mason
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Banks of cloned Japanese encephalitis virus cDNA and monoclonal antibodies specific to virus structural proteins are being used to characterize the structure and expression of the flavivirus genome. Fragmented RNA has been used as template for initial cDNA synthesis in an effort to produce a statistically complete bank of cDNA in a single round of cloning. For ease in establishing physical and functional maps of the genome,

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intact RNA is also used but in successive rounds of primer-extended synthesis to clone the genome in a few overlapping fragments. The genomic bank derived from the fragmented RNA consists of 40 clones with inserts ranging from 0.2 - 1.6 kb with an average length of about 1 kb. Cloning of cDNA transcribed from the 3'-terminus of the intact RNA has yielded an insert that is somewhat more than 10% of the genome. A biological expression system designed to detect protein coding regions in cloned DNA has been established. The system involves cloning of open-reading frame DNA (ORF) into an out-of-phase β galactosidase gene to successfully restore lacZ function. JEV cDNA fragments have been subcloned into the ORF vector and screening for V3 protein production by E. coli transformants is in progress. To this end we have developed a rapid and effective immunoblot screening procedure for detecting V3-lacZ fusion proteins in ORF$^+$ recombinants and an immunoabsorption procedure for the rapid isolation of V3-lacZ fusion proteins.

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SUMMARY

Banks of cloned Japanese encephalitis virus cDNA and monoclonal antibodies specific to virus structural proteins are being used to characterize the structure and expression of the flavivirus genome. Two strategies based on using either fragmented or intact genomic RNA have been used to develop banks of cloned viral cDNA. Fragmented RNA has been used as template for initial cDNA synthesis in an effort to produce a statistically complete bank of cDNA in a single round of cloning. For ease in establishing physical and functional maps of the genome, intact RNA is also used but in successive rounds of primer-extended synthesis to clone the genome in a few overlapping fragments. The genomic bank derived from the fragmented RNA consists of 40 clones with inserts ranging from 0.2 - 1.6 kb with an average length of about 1 kb. Assuming random fragmentation of the RNA a bank of DNA inserts of this size need have only 27 members for a 90% probability that any given genomic sequence is present. In the second strategy cloning of cDNA transcribed from the 3'-terminus of the intact RNA has yielded an insert that is somewhat more than 10% of the genome. A biological expression system designed to detect protein coding regions in cloned DNA has been established. The system involves cloning of open-reading frame DNA (ORF) into an out-of-phase βgalactosidase gene to successfully restore lacZ function. JEV cDNA fragments have been subcloned into the ORF vector and screening for V3 protein production by E. coli transformants is in progress. T this end a rapid and effective immunoblot screening procedure has been established for detecting V3-lacZ fusion proteins in ORF+ recombinants. A lacZ immunoabsorption procedure which will allow rapid isolation of V3-lacZ fusion proteins has also been developed. Finally, in anticipation of antigenic mapping a high pressure liquid chromatography method has been established for fractionating viral proteins and a screening method developed for detecting individual determinants with antibody probes.

FOREWORD

In conducting the research described in this report, 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 (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. INTRODUCTION-SIGNIFICANCE AND RATIONALE

The flaviviruses constitute one of the four genera in the recently defined family of togaviruses (i.e. Togaviridae). Before reclassification, the flaviviruses corresponded to the group B arboviruses; some new members are non-arboviruses. Group classification is currently based on immunological relatedness although there are other important features which distinguish each genus. Like all togaviruses the flaviviruses are enveloped, have a single-stranded RNA genome and possess a nucleocapsid with icosahedral symmetry.

The flaviviruses account for some two-thirds of the ninety or so known togaviruses and are important disease-causing agents in man and domestic animals. Examples of clinical diseases caused by these viruses include yellow fever, dengue fever and encephalitis. Because they occur world-wide in both tropical and temperate zones, the flaviviruses constitute a significant threat to global health.

The general properties of the viral genome and the encoded polypeptides appear to be similar among several flaviviruses examined (see reviews by Westaway, 1980 and Russell et al., 1980). All mature virions contain a large envelope protein V3 which is usually glycosylated (51-59 kd), a small nonglycosylated envelope protein V1 (7-8 kd), and a nucleocapsid protein V2 (13 kd). In addition the viral genome codes for 5-6 poorly defined nonvirion (NV) proteins which are detected in infected cells but are not found in the mature virus.[+]

Analyses of extracted genomic RNA have shown it to be an infectious single-stranded molecule with a molecular weight of about 4.2×10^6 daltons and sedimentation coefficient of 44S. Because it is infectious the viral RNA can be considered to be 'plus' stranded; by this convention 'plus' RNA has the same base sequence as messenger RNA. The viral RNA is apparently replicated via a 20S double-stranded intermediate. At least some flavivirus RNAs, including DEN-2, are capped at the 5' terminus. Unlike the alphaviruses, the flavivirus genome lacks or only has a small poly A tract at the 3' terminus. With regard to translational activity it seems reasonably clear that the virus-specified proteins are derived from individual translational events rather than being produced by proteolytic processing of polyprotein precursors (Shapiro et al., 1972; Westaway, 1977; Westaway and Shew, 1977). The theoretical informational capacity of the viral RNA (1.2×10^4 bases or 4×10^3 amino acids) is sufficient to encode all nine of the viral-induced proteins (combined molecular weight of 3×10^5 daltons or about 3×10^3 amino acids) with a surplus corresponding to about 25% of the genome.

Despite a large body of excellent research on the flaviviruses, some lines of investigation have proven to be quite intractable and many important questions remain unanswered. These gaps in knowledge represent serious barriers to a complete understanding of the basic biology of these agents and to the development of effective measures for disease control and prevention. In our opinion, the research outlined in this proposal will provide the

[+]The designations V3, V2 and V1 used in this proposal correspond to the E, C and M proteins in the nomenclature proposed by Westaway et al. (1980).

necessary tools for investigators in this field to not only overcome many of the technical problems that have hindered progress, but also to lay the foundation for an alternative, molecular approach to the development of vaccines.

Major obstacles to the elucidation of the molecular events associated with the replication of flaviviruses have been the low yield of virus from cells in culture, the long latent period--often of the order of 10-12 hours, and the fact that host macromolecular synthesis is not switched off during infection (Westaway, 1980). These problems can be readily overcome through the use of specific, sensitive assays with nucleic acid hybridization probes for the viral genome and with immunological probes for the viral proteins. With such probes in hand, it will be relatively straight-forward to detect and quantitate viral RNA and viral proteins in unlabeled extracts from infected cells. Fortunately, recombinant DNA and monoclonal antibody (mAb) technologies now make it possible to generate large quantities of probes suitable for such applications.

These technologies will also provide new approaches to the study of the complex immunopathology of flavivirus infections. A good example of this point can be made by considering what is known about dengue infections in man (see Schlesinger, 1977 and Halstead, 1980). A significant body of information shows that a primary infection by any one of the four dengue serotypes rarely leads to the severe symptoms of dengue hemorrhagic fever-dengue shock syndrome (DHA-DSS). However, a second infection by a different dengue serotype frequently triggers the pathogenetic response associated with DHA-DSS. Interestingly, the sequence of infection may be important with the more virulant infections resulting when the 2 infection is by dengue-2. Thus, previous dengue infections can lead to enhanced viremias during a secondary infection. Enhanced infections have also been induced experimentally in monkeys by the passive transfer of small amounts of anti-dengue antibody to the host shortly before infection. Similar immunological enhancement has been shown for infections of peripheral blood leucocytes in vitro.

When these and many other related observations are considered together, the immune response emerges as a two-edged sword with respect to dengue infections. There is no doubt that the immune response provides significant protection against subsequent infections. For example, there is no evidence for the occurrence of homotypic re-infections. In contrast, it seems equally clear that the severe symptoms of DHA-DSS are directly attributable to the roles played by antibodies and the immune elimination response. While less is known about the immunopathology of JE this situation is also complex. Since the complexity of these immunological parameters must be carefully considered in any strategy for the development of vaccines for DEN, JE and other flaviviruses, it is reasonable and pragmatic to systematically study the relationship between virus structure and the host immune response at the molecular level.

A prerequisite to this approach will be to precisely define the chemical structure of important viral antigens and to obtain sufficient quantities of these antigens so that their immunochemical properties can be critically examined. Accordingly, a primary goal of this proposal is to carefully coordinate the application of recombinant DNA and monoclonal antibody

technologies to provide the necessary information and material for study of the major envelope proteins of medically important flaviviruses.

The focus of this work is on two flaviviruses, the dengue-1 and Japanese encephalitis viruses. There are solid practical and scientific reasons for including both JEV and DEN-1 in the research plan. JE and DHA-DSS share the distinction of being significant problems in global medicine and, while the fundamental importance of immunopathogenic mechanisms for both diseases is generally accepted, very little is known about the molecular bases of these mechanisms. The complexity of the immunological parameters and concern about the safety and effectiveness of whole virus vaccines support the wisdom of pursuing alternative approaches to the development of vaccines for JE and DHS-DSS. In deciding which dengue serotype to study, we initially considered the advantages of DEN-2 with respect to its medical importance and the amount of available background information. However, since the cloning of the DEN-2 genome was already supported by an Army contract and the development of monoclonal antibodies to DEN-2 is proceeding rapidly at WRAIR, we decided to concentrate on DEN-1 in order to avoid unnecessary overlap and redundancy.

Because the strategies and technologies for dealing with the closely related JEV and DEN-1 viruses are identical, it is feasible for us to conduct nearly parallel research efforts with these two viruses. An important feature of our program is the enthusiastic collaborative support of Drs. Joel M. Dalrymple and Connie Schmaljohn, USAMRIID, Fort Detrick, and Drs. Walter E. Brandt, Eric Henchal, and Mary Kathryn Gentry, WRAIR. These colleagues provide important support both intellectually and technically. As established investigators in the field of viral immunology they are in position to continue providing important materials and to assist in various aspects of in vivo testing of the resulting biological products.

There is good reason to have confidence in the research plan proposed here. Similar applications of monoclonal antibody and DNA cloning technologies have been highly successful in recent studies of viral antigens and in the production and preliminary testing of vaccines derived from recombinant microorganisms. Immunodominant sites have been located on the exposed capsid proteins from several viruses (Wild et al., 1969; Lund et al., 1977; Beatrice et al., 1980; Chow and Baltimore, 1982; Emini et al., 1982; Minor et al., 1983; Kimura-Kuroda and Yasui, 1983). In type 3 poliovirus, an analysis of antigenic mutants selected for resistance to neutralizing mAbs led to the precise mapping of an eight-amino-acid tract that may constitute the major antigenic target for neutralizing antibodies (Evans et al., 1983; Emini et al., 1983). A similar antigenic determinant has been found in the VP1 protein of foot-and-mouth disease virus. In this case, several lines of evidence, including sequence information obtained from cloned VP1 cDNA (Kupper et al., 1981; Boothroyd et al., 1981; Kleid et al., 1981), mapping of antigenic peptides derived from isolated VP1 (Bachrach et al., 1979; Strohmaier et al., 1982), and sequence comparison among different serotypes of FMDV (Kupper et al., 1981; Boothroyd et al., 1981; Kleid et al., 1981), formed the basis for the prediction that a region centered on amino acid residues 146-154 was a potential neutralizing determinant (Pfaff et al., 1982). Subsequently, organically synthesized peptides corresponding to the sequence from amino acid residues 141-160 and 144-159 were able to raise neutralizing antibodies in rabbits and induce protection in guinea pigs (Bittle et al., 1982). These regions apparently also elicit neutralizing

antibodies when they are made biosynthetically in recombinant E. coli cells (cited in Brown, 1983). These results along with the recent progress in the development of a synthetic hepatitis B vaccine from genetically engineered yeast (see Newa and Views section - Nature 304, 395, 1983) offer any justification for pursuing similar strategies with other animal viruses. In fact early results from our own work on JEV, detailed below, show that the flaviviruses may be especially well suited for application of these technologies. We have already generated clone banks for the JEV genome, established an effective plasmid vector system for identifying cloned protein coding regions, developed specific assays for detecting JEV-V3 protein in recombinant cells and demonstrated feasibility of our strategy for isolating preparative amounts of viral protein derivatives for both in vitro and in vivo analyses.

II. PROGRESS REPORT

A. Objectives and Period Covered by the Report.

The objectives for the first contract period (Sept. 1982 - Nov. 1983) were:

1) To develop banks of cloned cDNA fragments corresponding to the genomes of Japanese encephalitis and dengue-1 viruses.
2) To identify monoclonal antibodies (mAbs) specific for JEV and DEN-1 specified proteins for use in detecting cloned viral genes and mapping antigenic determinants; antibody probes for the major envelope protein, V3 are of special interest.
3) To identify cloned DNA fragments in the gene libraries which correspond to specific proteins with V3 protein genes having first priority.

All of these aims were addressed during the contract period, however work up to the present has focused almost exclusively on JEV. Research with the two viruses is scheduled to proceed in coordinated fashion with inaugural studies in each phase being conducted on JEV. Cloning of the DEN-1 genome will be initiated during the three remaining months and preliminary characterization of DEN-1 mAbs conducted early in the year will be extended.

Our effort during the first contract year focused on:

1) assembling and training the project research team
2) optimizing the various enzymatic reactions used in the preparation and cloning of viral cDNA
3) cloning the Japanese encephalitis virus genome
4) characterizing the JEV DNA clones by size analysis, restriction mapping and hybridization assays
5) developing an <u>in vivo</u> expression system for the detection of cloned protein coding sequences
6) development of a rapid and sensitive immunological assay for the presence of V3 protein in bacterial transformants
7) establishing an antibody affinity chromatography procedure for isolation of bacterial-produced V3 antigen

B. Research Personnel Comprising the Project Team

The work described was conducted by the personnel listed below. Curriculum Vitae of the Co-P.I.s and postoctoral researchers are attached.

M. J. Fournier--Principal Investigator

T. L. Mason--Co-Principal Investigator

Phyllis C. McAda, Ph.D.--Postdoctoral Fellow; appointed January, 1983

David C. Fritzinger, Ph.D.--Postdoctoral Fellow; appointed February, 1983

Lynn Wharton, B.S.--Research technician; appointed September, 1982

Kathleen Brown, B.S.--Research technician; appointed October, 1982

John Zagorski, B.S.--Graduate Research Assistant (current predoctoral student; appointed September, 1982.

Work was initiated in the first two months of the contract period with two research technicians, one of whom was already in the P.I.'s laboratory. The two postdoctoral workers were recruited soon thereafter but were not able to join the team until January and February. Along with residual course work the graduate assistant devoted his effort during the year to learning basic cloning technology and mastering DNA sequencing.

C. Detailed Progress Report

CLONING OF JEV cDNA AND DETECTION OF PROTEIN CODING REGIONS

1. Cloning strategy. We have adopted two strategies for developing cDNA banks from the viral genome. One approach is to prepare cDNA from randomly fragmented genomic RNA, the second involves reverse transcription of the intact genome initiating at the 3'-terminus. The aim of the first strategy is to achieve a complete collection of small to moderately sized cDNA inserts in a single round of cloning. These cDNAs which range in size from about 0.2 to 1.6 kb represent an excellent start-point for the early identification of individual protein coding regions in an open reading frame expression vector.

The purpose of developing the second cDNA library is to attain a collection of substantially larger cDNA clones in which the entire genome is represented in a relatively small number of overlapping fragments, say 3-5. Banks of this type provide the best source of cDNA for developing physical and functional maps of the genome and for eventually establishing the complete nucleotide sequence.

Development of these latter banks from intact viral RNA requires several cycles of reverse transcription and cloning. The point here is that the cDNA derived from a single cycle will be considerably less then the full-length of 12 kb owing to incomplete transcription, due presumably to slow copying of self-paired domains in the template or interference by RNase which typically contaminates commercial preparations of reverse transcriptase (Efstratiadis and Villa-Komaroff 1979). Despite this constraint cDNA transcripts of 5-6 kb

can be expected (e.g. de Martynoff et al., 1980). A restriction fragment corresponding to an interior region of the viral RNA is then used to prime a second cycle of cDNA synthesis. This process, referred to as primer-extended synthesis, is repeated until the entire genome has been traversed. To ensure that the 5'-distal sequences of the RNA have been cloned, hybridization analyses are performed with 5'-end labeled ^{32}P-genomic RNA (decapped). This strategy was used successfully to prepare and clone poliovirus cDNA (Racaniello and Baltimore, 1981). In that case the full genome of 7.5 kb was represented in three over-lapping cDNA fragments.

Internal initiation of reverse transcription has been shown to occur in cases where viral cDNA synthesis was initiated with an oligo dT primer. In one instance it was possible to obtain a complete set of subgenomic clones of polio cDNA from a single cycle of cloning (van der Werf et al., 1981). Similarly, reverse transcriptions of in vitro polyadenylated Rift Valley virus RNA with oligo dT as primer yielded cDNA transcripts internal to the viral genome (J. Dalrymple and C. Schmaljohn, personal communication). The basis for this unexpected effect is not known but presumably stems from the binding of the oligo dT primer to complementary stretches of A-residues encoded in the RNA or from the polyadenylation of subgenomic RNA fragments arising from spurious nuclease activity.

In developing our banks of large cDNAs we have opted to use as first primer a synthetic 12-base DNA fragment complementary to the 3' terminus of the RNA. The use of this homologous oligomer should ensure that reverse transcription initiates only at the 3' end of the RNA and also enhances the efficiency of reverse transcription. In this last regard all viral RNAs possess a primer-complementary sequence but not all RNA molecules become tailed in an in vitro poly A reaction.

2. Preparation of biological materials for cDNA cloning and characterization of enzymatic reactions. In preparation for cloning and expression analysis, milligram quantities of the E. coli plasmid vectors pBR327 and pMR100 were prepared and a substantial effort was devoted to optimizing the many enzymatic reactions involved in cloning cDNA (Soberon et al., 1980; Gray et al., 1982). In order to conserve valuable viral RNA, the various reaction conditions for cDNA synthesis and construction of hybrid plasmids were first developed using more readily available globin messenger RNA (Rougeon et al., 1975; Villa-Komaroff et al., 1978; Cooke et al., 1980; Bothwell et al., 1981; Land et al., 1981). Because homopolymer tailing of both plasmid and cDNA is central to the particular cloning strategy selected an in-depth characterization of the DNA terminal transferase enzyme was carried out. Reaction conditions were developed for good control of tailing efficiency and tail length, both of which are essential for high cloning efficiency (Reychoudhury and Wu, 1980; Rowekamp and Firtel, 1980). Conditions were defined for oligo (dC) or oligo (dG) tailing of blunt-ended and 3'-extended DNAs to yield 3' tails of 20-25 residues with an overall efficiency of about 80%. In addition, conditions were defined for efficient 3'-dephosphorylation of RNA fragments to yield a free 3'-hydroxyl and for adding a polyadenylic acid tract of 75-150 bases to these 3' termini with poly A polymerase (Sippel, 1973). This procedure makes it possible to prime

and reverse transcribe from the once internal 3' ends of fragmented RNA.

3. Cloning the JEV RNA genome. Plus-stranded genomic RNA prepared from purified virions (Repik et al., 1976, 1983) was supplied to us by Drs. Joel Dalrymple and Connie Schmaljohn at USAMRIID. Inspection of the RNA by electrophoretic analysis on agarose gels (Peacock and Dingman, 1968) revealed that one preparation corresponded to intact full-length RNA while a second was extensively fragmented. The latter preparation consisted of a population of fragments ranging from 200 to 6000 bases (1-50% of full length). On discovering this we decided to also prepare cDNA from this material in an effort to develop a complete bank in a single round of cloning. With this strategy it would not only be possible to obtain a complete gene bank early but the resulting cDNA sizes would be well suited for the planned ORF analysis.

Dr. Schmaljohn provided us with the sequence of the first 25 nucleotides at the 3' terminus of the JEV RNA. This information enabled us to adopt the strategy of using an homologous synthetic primer to initiate cDNA synthesis rather than by the more usual poly A tailing and priming with oligo dT. A 12-base DNA primer was synthesized for us by Dr. Robert Cedergren of the University of Montreal. The sequences of the JEV RNA and DNA primer are both shown in Figure 1 in the section titled Figures and Tables (p.17).

The strategies used to prepare and clone cDNA from both the intact and fragmented RNA preparations are shown in Figures 2 through 4. cDNA synthesis from the fragmented RNA was carried out by poly A tailing of the 3' ends, previously dephosphorylated with polynucleotide kinase, (Cameron and Uhlenbeck, 1977) and primed with oligo dT. After removal of the RNA by alkaline hydrolysis, the single-stranded cDNA was recovered and 3'- oligo (dC) tailed with terminal transferase. An oligo (dG) primer was used to initiate second strand synthesis by DNA polymerase I and reverse transcriptase (Efstratiadis and Villa-Komaroff, 1979). Size analysis of the resulting double-stranded cDNA showed a population of fragments ranging from 0.2 - 4 kb in length.

The first round of cDNA synthesis from the intact RNA was carried out using the 3' complementary synthetic primer. After removal of the RNA the cDNA was recovered and converted to double-stranded DNA by self-priming "snapback" synthesis. As before, second strand synthesis was effected with DNA polymerase I and reverse transcriptase. The hairpin loop generated from the self-priming reaction and the ragged termini were cleaved with S1 nuclease to yield double-stranded, blunt-ended DNA (Efstratiadis et al., 1976). Agarose gel analysis of the cDNA revealed a distribution of fragments ranging from 0.2 to 6 kb in length.

In each case the resulting cDNA products were inserted into the vector pBR327. This plasmid, a smaller derivative of the standard cloning vector pBR322, (Bolivar et al., 1977 a,b) harbors antibiotic resistance markers for tetracycline and ampicillin (Soberon et al., 1980). The plasmid was prepared for cloning by linearization at the PstI restriction site of the ampicillin gene and oligo (dG) tailing of the 3' ends with terminal transferase. After oligo (dC) tailing of the 3' termini, the cDNA fragments were incorporated into the vector by annealing of the complementary homopolymer tails (Land et al., 1983). Construction of hybrid plasmids in this manner generates a PstI

site at each end of the cDNA insert (Villa-Komaroff et al., 1978). The presence of this site enables the insert to be easily recovered. Following transformation of E. coli, colonies were screened for resistance to tetracycline and ampicillin (Mandel and Higa, 1970; Dagert and Ehrlich, 1979). Transformants which were able to grow on tetracycline but not on ampicillin were selected for further characterization.

4. Characterization of JEV clones. Pst I restriction analysis of plasmids containing DNA from the intact RNA template showed that the cloned inserts ranged in size from 0.4 to 1.45 kb. The presence of JEV-specific sequences was demonstrated by hybridization analysis (Kafatos et al., 1979) using 5'- ^{32}P-labeled fragmented viral RNA as probe. The largest insert represents somewhat more than 10% of the genome, however it was substantially smaller then the larger cDNA fragments generated (up to 6 kb). Because of the bias toward cloning of the smaller DNA inserts, subsequent cycles of cloning will now include a size fractionation of the cDNA prior to annealing with the vector. This step should enable us to establish a bank of larger cDNAs in the size range of 3-6 kb. New rounds of cDNA synthesis and cloning are currently in progress using both the synthetic primer and a 5'-proximal fragment from the already cloned 1.45 kb DNA. Comparative restriction analysis of the plasmid carrying the 1.45 kb insert, plasmid pJEV-1 (shown in Figure 5) and other smaller recombinants has identified a unique 50 bp Pvu II-Hinc II fragment suitable for use as a primer. A partial restriction map of pJEV-1 is shown in Figure 5.

Generation of cDNA clones from the fragmented RNA resulted in 49 positive transformants of which 40 (85%) contained inserts ranging from 0.2 - 1.6 kb. The average size of the inserts is about 1 kb. Assuming random fragmentation of the RNA genome a statistically complete bank of genomic sequences of this size requires only 27 recombinants; the probability of any given sequence being represented in such a bank is 90%. Thus, there is an excellent probability that all viral coding sequences including that for protein V3 are contained within this population of 40 cloned cDNAs. The expression used to predict cloning probability and bank size is given in Figure 6 (Clarke and Carbon, 1976). The results obtained thus far with the two cloning strategies are summarized in Table I.

5. Identification of open reading frame DNA. Subcloning of viral cDNA for expression of protein coding sequences is being carried out in the open reading frame (ORF) vector developed by Gray et al (1982). As shown in Figure 7, this vector contains a hybrid gene constructed from a bacterial promoter and the amino terminus of the bacteriophage λcI gene fused to an amino-terminally deleted derivative of the lacZ gene which codes for β-galactosidase. As constructed the lacZ reading frame is out of phase relative to the upstream cI region. Insertion of an ORF DNA sequence with the correct phasing will restore the reading frame and result in production of an active β-galactosidase fusion protein (see scheme in Figure 8). The ORF insertion is at a unique cloning site between lacZ and the upstream translational start point. After transformation of an E. coli host deleted in the lacZ gene, colonies producing β-galctosidase are screened for lactose utilization on MacConkey indicator plates and detected as red colonies. Transformants which fail to express functional lacZ remain white. We have verified that the system works as described by analyzing a restriction digest of plasmid pBR322 for the occurrence of ORF sequences. As reported by Gray and coworkers a Hae III digest yielded three ORF$^+$ DNA fragments.

We are now in the process of testing the cloned JEV DNA for ORF sequences. The strategy involves 1) removal of the cDNA inserts from the pBR327 vector by Pst I digestion, 2) size reduction of the larger inserts with restriction enzymes to yield subgenic segments of 0.2-0.6 kb, 3) Bal 31 treatment to randomize the ends of the cDNA fragments (to achieve all 3 possible reading phases), 4) ligation of the fragments into the ORF expression vector, 5) transformation of the lacZ deleted host strain and 6) screening for restored lacZ activity on lactose MacConkey plates.

6. Preparation of immunological probes for detection of viral gene products. Specific immunological probes are required for the identification of cloned viral genes. Since V3 and other viral polypeptides expressed in recombinant E. coli will have physical and chemical properties different from the mature polypeptides found in infected cells and in virions, it is especially important to obtain antibodies with high affinity for antigenic determinants retained in denatured or fragmented polypeptides. Accordingly, a major goal of the current contract period has been to obtain antibodies to JEV-encoded polypeptides that recognize "linear" determinants, as indicated by their ability to bind to polypeptides that have been resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter paper, i.e., western blot analysis (Towbin et al., 1979; Burnett, 1981; Howe and Hershey, 1981). To this end, the laboratories at UMass and at WRAIR have coordinated efforts in the production of JEV mAbs. Immunizations in our laboratory have been restricted to peparations of inactivated infected cell lysates and purified virion whereas immunizations at WRAIR have been with live virus. A total of six cell fusions have been performed with these immunized mice, three at WRAIR and three at UMass. Our progress in producing and characterizing JEV mAbs can be summarized below.

At UMass, twelve Balb/c mice were subjected to a series of three injections with inactivated extracts from JEV infected suckling mouse brain. Six mice received native material; the other six received antigen that had been denatured by heating in the presence of 0.2% SDS and diluted to 0.05% SDS prior to injection. The injection schedule included a interperitoneal primary injection with Freunds complete adjuvant followed by i.p. injections without Freunds at 30-day intervals. The mice were bled from the tail vein 7 days after the third injection and the serum was tested for reactivity to virion proteins on western blots. The sera from each of the 6 mice that had been immunized with native antigen displayed strong reactivity to V3 in this test; the mice injected with denatured antigen were uniformly negative. It was celar from these results that the denatured preparation was ineffective in eliciting antibodies suitable for immunoblot analysis whereas the native antigen, perhaps because it was generally more immunogenic, produced a detectable level of antibodies capable of binding to denatured V3. Thus we were reasonable confident that mAbs to "linear" determinants on V3 could be obtained using the native mouse brain extract as the immunogen.

At this point, however, mAbs in culture fluids and ascitic fluids from 30 JEV-specific hybridoma lines (prepared from the J1 fusion at WRAIR) were tested for binding to SDS-denatured virion and non-virion proteins on western blots. The mice used in this fusion had been immunized with a primary injection of JEV-infected mouse brain extract and a booster injection with the same material. Antibodies from 20 of the 30 clones were reactive to polypeptides on blots of a JEV-infected Vero cell lysate. However, none of

these mAbs were reactive in the same analysis when virion proteins were used as the binding target. Since at least two of the antibodies tested are known to bind to native V3 in immunoprecipitation reactions (Dr. C. Schmaljohn, personal communication), these results indicated to us that the major immunogenic determinants on native V3 are probably lost upon denaturation and suggested that there would be little chance of obtaining JEV mAbs suitable for our purposes unless it was possible to further stimulate the production of antibodies to the denatured form of V3. We reasoned, therefore, that boosting the previously immunized mice with denatured virion would enhance the probability of recovering mAbs to determinants exposed on denatured V3.

All three fusions at UMass were carried out using the mice originally immunized with native antigen, and the X63-Ag8 6.5.3 myeloma line as the fusion partner. The fusion protocol has been described earlier (Kimura et al., 1982). The first and second fusions were performed after 2 and 3 booster injections, respectively, with a SDS-denatured virion preparation. Both of these fusions were technically successful and produced over 150 independent hybridomas that were scored as positives (5 x the minus antigen and minus antibody control values) in ELISA solid phase binding assays with infected Vero cell lysates as the binding target. Ninety culture supernatants were examined by western blot analysis for the presence of antibodies to V3; all were negative. Furthermore, many of the supernatants were later found to contain antibodies to determinants present in uninfected Vero cells. These disappointing results led us to conclude that the virion preparations must have contained small amounts of highly immunogenic Vero cell proteins that overwhelmed the contribution of the more abundant but poorly immunogenic denatured virion proteins. In an attempt to circumvent this problem, a fusion was performed with the two remaining mice after they were boosted with the original native antigen preparation. A total of 30 hybridomas were selected for subcloning on the basis of a differential signal in the ELISA favoring infected over uninfected Vero cell lysates as the binding target. Only one of the resulting antibodies, an IgM, appears to react with V3 on western blots of virion proteins. In summary, although the possibility remains that some of the mAbs we have generated bind to native V3, there is little doubt that we have not been able to produce "linear" or "sequence" specific mAbs to V3.

Two additional fusions were performed at WRAIR with mice that were injected first with infected mouse brain extract, but in this case, instead of receiving a booster of the same material, the survivors were injected intravenously with either purified virion (fusion J2) or a partially purified envelope fraction (fusion J3).

Ascitic fluids from 24 of the resulting hybridomas were analyzed on western blots of purified virion and infected Vero cell lysate. As shown in Fig. 9a, 10 of the 24 reacted strongly with polypeptides present in the purified virion; 9 of these bound to the 55 kDa V3 polypeptide and one, J2-2F1-2, bound to a polypeptide of 10 kDa, probably V1 (Russell et al., 1980). Nine additional mAbs displayed weak, but detectable, binding to V3 in blots of purified virion.

A similar pattern of reactivity was found for the 9 V3-specific mAbs when they were used to probe blots of infected Vero cell lysates (Fig. 9b). Surprisingly, however, the putative V1-specific mAb bound to a 20-25 kDa

polypeptide rather than the 10kDa species detected in the virion prep (Fig. 9b,c). One tentative interpretation of this result is that V1, which does not accumulate in infected cells (Russell et al., 1980), is derived from a larger precursor polypeptide during maturation or shedding of the virus. It has been postulated previously that NV2, an intracellular glycoprotein, is a precursor of V1 (Shapiro et al., 1972), but attempts to demonstrate a chemical relatedness between V1 and larger flavivirus-specified polypeptides by peptide mapping have been inconclusive (Wright and Westaway, 1977; Westaway et al., 1977). Thus, the J2-2F1-2 mAb may provide a powerful experimental tool for defining an important precursor-product relationship in viral replication.

Of greatest signficance with respect to the goals of this project is the identification of 9 mAbs that appear to recognize "linear" epitopes. Of additional importance is the fact that 4 of these mAbs (see Table II) are also positive in in vitro virus neutralization tests. A comparison of the functional properties of these 9 mAbs suggests that they may represent 3-4 different binding sites on V3 with perhaps two of these sites being involved in virus neutralization (Dr. J. Dalrymple, personal communication). This means that monoclonal antibody probes such as J3-10E1-4 and J3-14E6-1 will be useful not only in cloning V3 but also in the definition of neutralizing antigenic determinants. Furthermore, if the neutralizing mAbs are in fact binding to "linear" determinants, there is reason to be excited about the prospects for a synthetic vaccine for JEV. In this regard, the preliminary results discussed here already suggest that neutralizing determinants on the V3 polypeptide of JEV, and probably of other flaviviruses, will be more amenable to molecular analysis than has been the case for poliovirus. For example, the vast majority of neutralizing mAbs antibodies for poliovirus apparently do not bind to the isolated VP1 polypeptide (Emini et al., 1982, 1983; Blondel et al., 1983) and thus have restricted application for direct mapping of the corresponding determinants.

In summary, several mAbs are now available for the sensitive detection of V3-ORF sequences in transformed E. coli. Some of these will permit the rapid identification of nucleic acid sequences that correspond to neutralizing determinants of V3. One mAb may be useful for cloning the gene coding V1 (or its precursor). Polyclonal hyperimmune ascitic fluids are available as less specific probes for other virion and non-virion proteins. In addition, further characterization of the JEV mAbs already isolated at WRAIR should expand the repertoire of probes for JEV-specified polypeptides.

Our emphasis in the current contract period has been on immunological probes for JEV, but we have also initiated testing of culture fluids from 54 DEN-1-specific hybridoma lines (Henchal et al., 1982) for immunoreactivity on western blots of DEN-1-infected mosquito cell lysates. Two of these showed binding to a polyepeptide with an apparent mol. wt. similar to that of V3. These mAbs have not yet been used to probe blots of purifed virion.

The research plan we have proposed requires the development of an effective method for the rapid purification of a wide variety of JEV-lacZ fusion proteins. We have decided to adopt a general scheme that utilizes an anti-βgalactosidase immunomatrix for the affinity purification of all fusion proteins on the basis of their common lacZ determinants. Accordingly, we have prepared mAbs to E. coli β-galactosidase (Table III).

7. Detection of V3 cDNA. An immunological screening procedure has been established for detecting V3 coding DNA among the resulting ORF-positive transformants with this method, described in Methodology below. We are optimistic that V3 clones for JEV will be identifed during the current contract year and that DNA sequencing and immunological studies can be initiated. Testing of the DEN-1 gene bank for V3 coding sequences will be done first by simple colony hybridization analysis with V3 cDNA from JEV. The rationale here is that the V3 genes for the two viruses may contain homologous sequences. If this should prove to be true then the corresponding DEN-1 region can be easily detected and isolated without need for the ORF and immunological screening steps. Of course, confirmation and definition of the coding region will require these assays. If the JEV V3 DNA does not show detectable homology with DEN-1 DNA then it will be necessary to resort to the less-direct immunological screening strategy.

D. Anticipated Publications

During the next several months we expect to extend our current findings to the point where first, major publications can be prepared. Likely titles for these planned works include:

1. Physical and Partial Functional Map of the Japanese Encephalitis Virus Genome.
2. Structure of the Major Envelope Protein Gene from Japanese Encephalitis Virus.
3. Identification of Major Antigenic Domains in the V3 Envelope Protein of Japanese Encephalitis Virus.

IV. LITERATURE CITED

Bachrach, H. L., Morgan, D. O., and Moore, D. M. (1979) Intervirol. 12, 65.

Beatrice, S. T., Katze, M. G., Zajac, B. A. and Crowell, R. L. (1980) Virol. 104, 426.

Bittle, J. L., Houghten, R. A., Alexander, H. A., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowland, D. J. and Brown, F. (1982) Nature 298, 30.

Blondel, B., Akacem, O., Crainic, R., Couillin, P. and Horodniceanu, F. (1983) Virology 126, 707.

Bolivar, F., Rodriguez, R. L., Betlach, M. C. and Boyer, H. W. (1977) Gene 2, 75.

Bolivar, F., Rodriguez, R. L., Green, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. H. and Falkow, S. (1977) Gene 2, 95.

Boothroyd, J. C., Highfield, P. E., Cross, G. A. M., Rowlands, D. J., Lowe, P. A., Brown F. and Harris, T. J. R. (1981) Nature 290, 800.

Bothwell, A., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K., and Baltimore, D. (1981) Cell 24, 625.

Brown, F. (1983) Nature 304, 395.

Burnett, W. N. (1981) Anal. Biochem. 112, 195.

Cameron, V. and Uhlenbeck, O. C. (1977) Biochem. 16, 5120.

Chow, M. and Baltimore, D. (1982) Proc. Natl. Acad. Sci., U.S.A. 79, 7518.

Clarke, L. and Carbon, J. (1976) Cell 9, 91.

Cooke, N. E., Coit, D., Weiner, R. I., Baxter, J. D. and Martial, J. A. (1980) J. Biol. Chem. 255, 6502.

Dagert, M. and Ehrlich, S. D. (1979) Gene 6, 23.

de Martynoff, G., Pays, E. and Vassart, G. (1980) Biochem. Biophys. Res. Comm. 93, 645.

Efstratiadis, A., Kafatos, F. C., Maxam, A. M. and Maniatis, T. (1976) Cell 7, 279.

Efstratiadis, A. and Villa-Komaroff, L. (1979) in Genetic Engineering, Vol I, Setlow, J. K. and Hollander, A. (eds.) Plenum Press, N.Y., pp. 15-35.

Emini, E. A., Jameson, B. A., Lewis, A. J., Larsen, G. R. and Wimmer, E. (1982) J. Virol. 43, 997.

Emini, E. A., Jameson, B. A. and Wimmer, E. (1983) Nature 304, 699.

England, T. E. and Uhlenbeck, O. C. (1978 Nature 275, 560.

Evans, D. M. A., Minor, P. D., Schild, G. S. and Almond, J. W. (1983) Nature 304, 459.

Gray, M. R., Colot, H. V., Guarente, L., and Rosbash, M. (1982) Proc. Natl. Acad. Sci., U.S.A. 79, 6598.

Halstead, S. B. (1980) in the Togaviruses, Schlesinger, R. W., ed., Academic Press, New York, pp. 107-173.

Henchal, E. A., Gentry, M. K., McCown, J. M. and Brandt, W.E. (1982) Am. J. of Trop. Med. and Hyg. 31, 830.

Howe, J. G. and Hershey, J. W. B. (1981) J. Biol. Chem. 256, 12836.

Kimura, K., Mason, T. L., and Khorana, H. G. (1982) J. Biol. Chem. 257, 2859.

Kafatos, F. C., Jones, C. W. and Efstratiadis, A (1979) Nucl. Acids Res. 7, 1541.

Kimura-Kuroda, J. and Yasui, K. (1983) J. Virol. 45, 124.

Kleid, D., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H. and Bachrach, H. L. (1981) Science 214, 1125.

Kupper, H., Keller, W., Kurz, C., Forss, S., Schaller, H., Franze, R., Strohmaier, K., Marquordt, O., Zaslavsky, V. G., and Hofschneider, P. H. (1981) Nature 289, 555.

Land, H., Grez, M., Hauser, H., Lindenmaier, W. and Schutz, G. (1981) Nucl. Acids Res. 9, 2251.

Lund, G. A., Ziola, B. R., Salmi, A. and Scraba, D. G. (1977) Virol. 78, 35.

Mandel, M. and Higa, A. (1970) J. Mol. Biol. 53, 159.

Peacock, A. C. and Dingman, C. W. (1968) Biochem. 7, 668.

Pfaff, E., Mussgay, M., Bohm, H. O., Schulz, G. E. and Shaller, H. (1982) EMBO. J. 1, 869.

Racaniello, V. R. and Baltimore, D. (1981) Proc. Natl. Acad. Sci., U.S.A 78, 4887.

Repik, P., Flamand, A. and Bishop, D. H. L. (1976) J. Virol. 20, 157.

Repik, P. M., Dalrymple, J. M., Brandt, W. E., McCown, J. M. and Russell, P.K. (1983) Am. J. Trop. Med. Hyg. 32, 577.

Reychoudhury, R. and Wu, R. (1980) Methods in Enzymol. 65, 43.

Rougeon, F., Kourilsky, P. and Mach, B. (1975) Nucleic Acids Res. 12, 2365.

Rowekamp, W. and Firtel, R. A. (1980) Dev. Biol. 79, 409.

Russell, P. K., Brandt, W. E. and Dalrymple, J. M. (1980) in the Togaviruses: Biology, Structure, Replication, Schlesinger, R. W. (ed.) Academic Press, New York, pp. 503-529.

Schlesinger, R. W. (1977) Dengue Viruses, Springer-Verlag, New York.

Shapiro, D., Brandt, W. E. and Russell, P. K. (1972) Virol. 50, 906.

Sippel, A. (1973) Eur. J. Biochem. 37, 31.

Soberon, X., Covarrubias, L. and Bolivar, F. (1980) Gene 9, 287.

Strohmaier, K., Franze, R. and Adam, K.-H. (1982) J. Gen. Virol. 59, 295.

Towbin, H. T., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci., U.S.A. 76, 4350.

van der Werf, S., Bregegere, F., Kopecka, H., Kitamura, N., Rothberg, P. G., Kourilsky, P., Wimmer, E. and Girard, M. (1981) Proc. Natl. Acad. Sci., U.S.A. 78, 5983.

Villa-Komaroff, L., Efstratiadis, A., Broome, S., LoMedico, P., Tizard, R., Naber, S. T., Chick, W. L. and Gilbert, W. (1978) Proc. Natl. Acad. Sci., U.S.A 75, 3727.

Westaway, E. G. and Shew, M. (1977) Virol. 80, 309.

Westaway, E. G. (1977) Virol. 80, 320.

Westaway, E. G. (1980) in the Togaviruses: Biology, Structure, Replication, Schlesinger, R. W. (ed.) Academic Press, New York, pp. 531-538.

Westaway, E. G., Schlesinger, R. W., Dalrymple, J. M. and Trent, D. W. (1980) Intervirol. 14, 114.

Wild, T. F., Burroughs, J. N. and Brown, F. (1969) J. Gen. Virol. 4, 313.

Wright, P. J., Bowden, D. S. and Westaway, E. G. (1977) J. Virol. 24, 651.

Wright, P. J. and Westaway, E. G. (1977) J. Virol. 24, 662.

V. FIGURES AND TABLES

Fig. 1 Sequence of JEV Synthetic Primer

JEV RNA 5'-GAUGUGUAUAUAUAACACAGGAUCU$_{OH}$-3'

Synthetic Primer 3'-TTGTGTCCTAGA-5'

Fig. 2

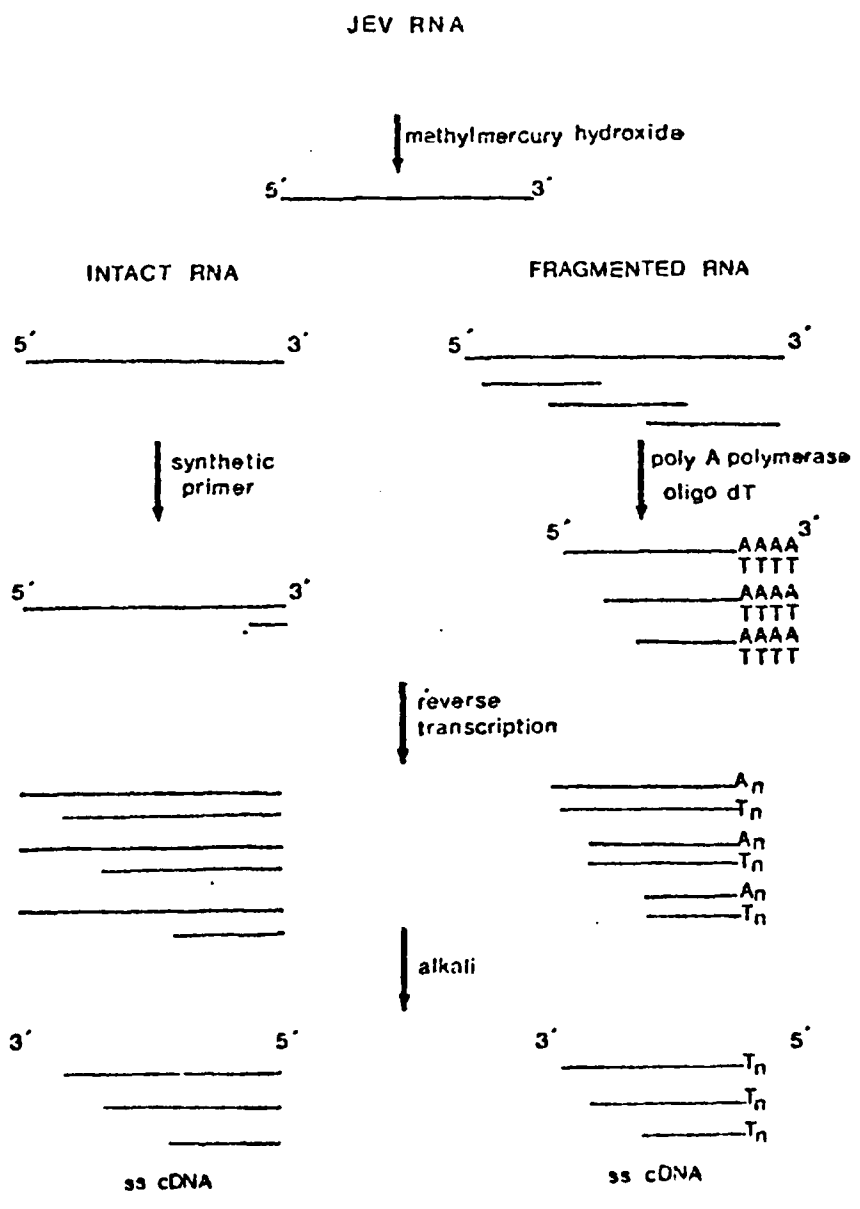


Fig. 3

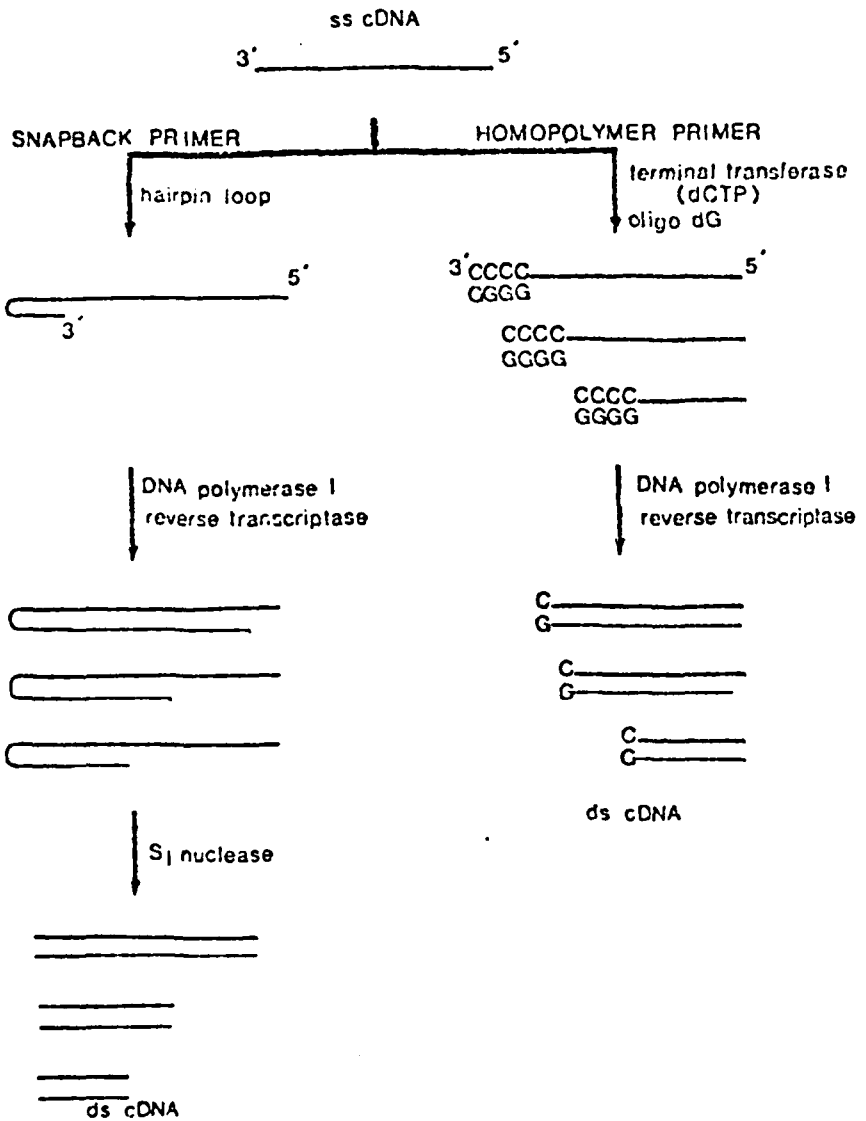

SECOND STRAND SYNTHESIS
ss cDNA
3'
5'
SNAPBACK PRIMER
HOMOPOLYMER PRIMER
hairpin loop
terminal transferase
(dCTP)
oligo dG
3'CCCC
CGGG
CCCC
GGGG
CCCC
GGGG
DNA polymerase I
reverse transcriptase
DNA polymerase I
reverse transcriptase
ds cDNA
S1 nuclease
ds cDNA

Fig. 4

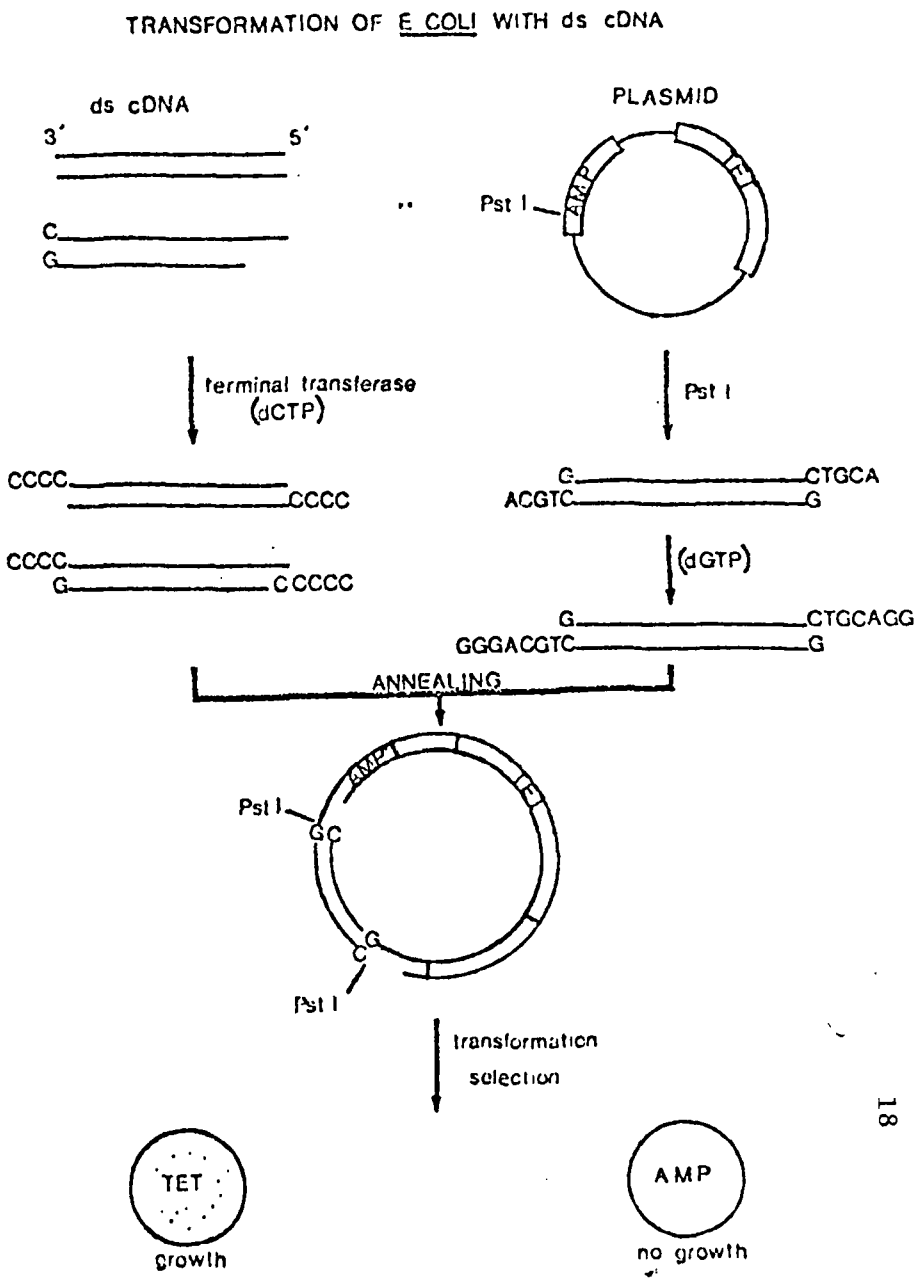
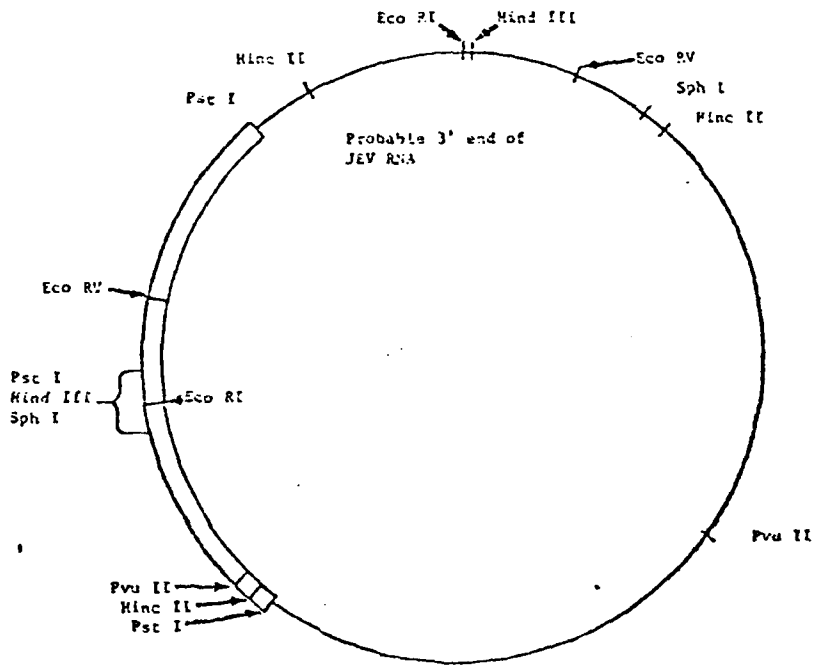

TRANSFORMATION OF E COLI WITH ds cDNA
ds cDNA
PLASMID
Pst I
AMP
TET
terminal transferase
(dCTP)
Pst I
CCCC
CCCC
CCCC
G
CCCCC
G
CTGCA
ACGTC
G
(dGTP)
G
CTGCAGG
GGGACGTC
G
ANNEALING
AMP
TET
Pst I
GC
G
C
Pst I
transformation
selection
TET
growth
AMP
no growth

Fig. 5

Restriction Map of pJEV1

Total Size 5.9kbp

Size of Insert 1.45kbp



No sites in insert for the following enzymes: Bam HI, Cla I, Sal I, Aha III.

TABLE I

SUMMARY OF JEV cDNA SYNTHESIS AND CLONING

	Genomic Template	
	Intact (12 kb)	Fragmented (0.2-6 kb)
cDNA Size (kb)	0.5 - 6+	0.2 - 4+
No. Characterized Clones	3	40
Size of Cloned cDNA (kb)	0.4 - 1.45	0.2 - 1.6
Percent of Genome	> 10	statistically complete (27 required)

Fig. 6

Library Size Needed to Represent Entire JEV Genome (90% Probability)

$$N=\frac{\ln(1-P)}{\ln(1-f)}$$

N= Needed number of clones

P= Desired probability

f= Fraction of genome in average clone

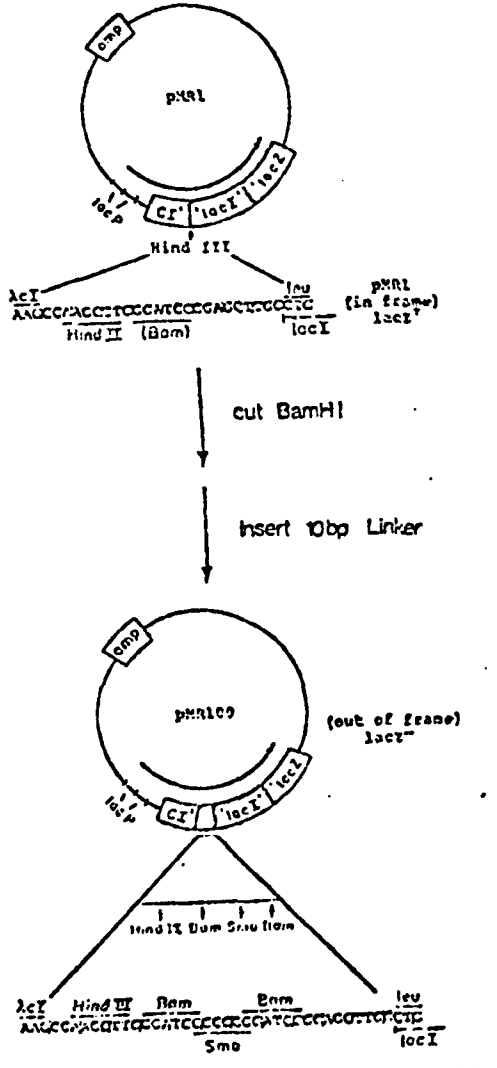
To calculate the library size needed to represent the entire JEV genome, with a probability of 90%, and assuming that the average insert size is 1kb(or 1/12 of the genome):

$$N=\frac{\ln(1-0.9)}{\ln(1-0.083)}=\frac{\ln(0.1)}{\ln(0.917)}=\frac{-2.3}{-0.087}=26.5$$

Clarke, L. & Carbon, J., (1976). Cell 9, 91-99.

Fig. 7

CLONING VECTOR CONSTRUCTION



Gray, M., et al. (1982) Proc. Natl. Acad. Sci. USA 79, 6598-6602.

Fig. 8

IDENTIFICATION of ORF cDNA

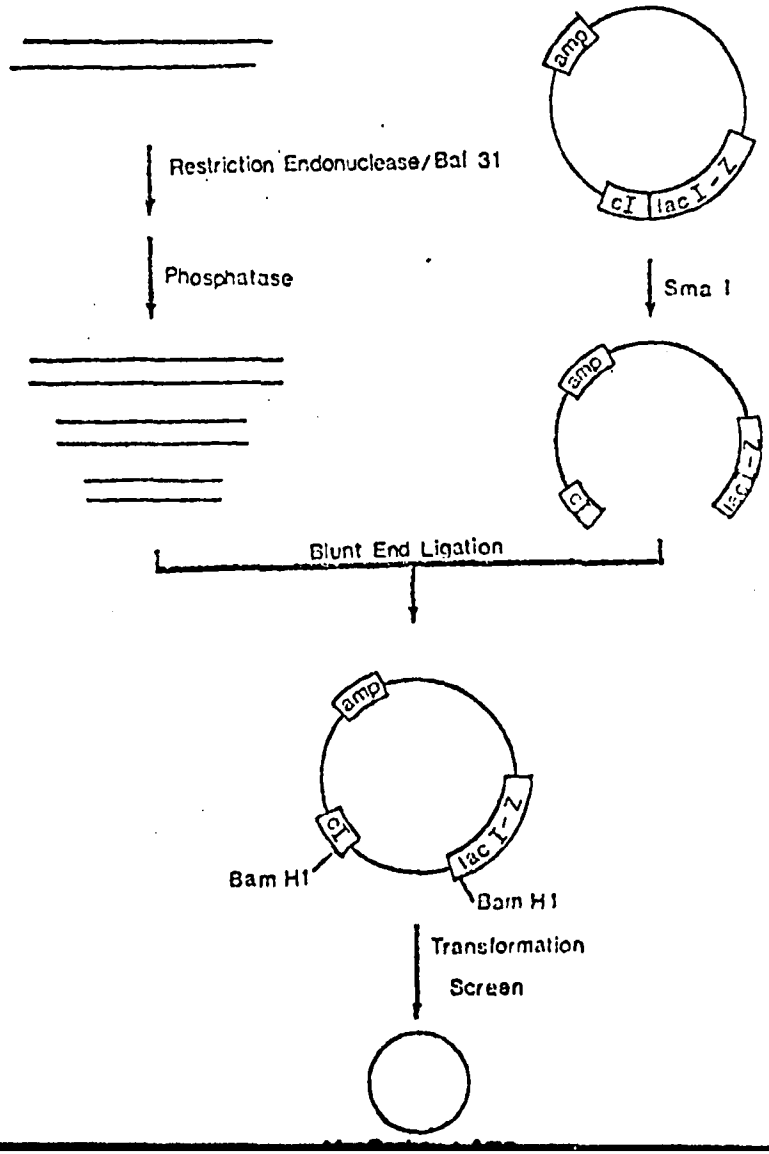


Fig. 9 Western blot analysis of monoclonal antibodies to JEV.

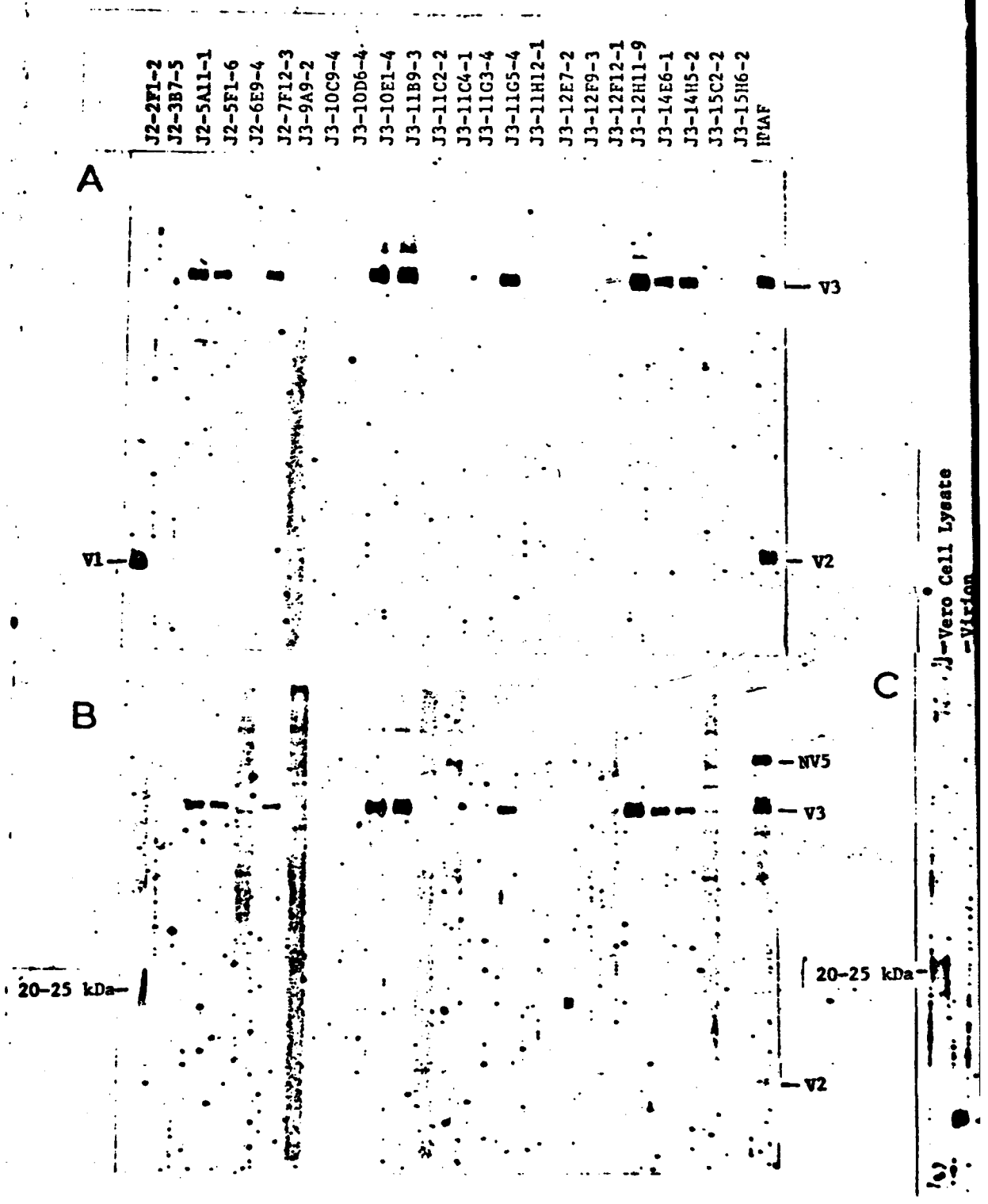


Fig. 10 Analysis of lacZ fusion proteins in E. coli lysates.

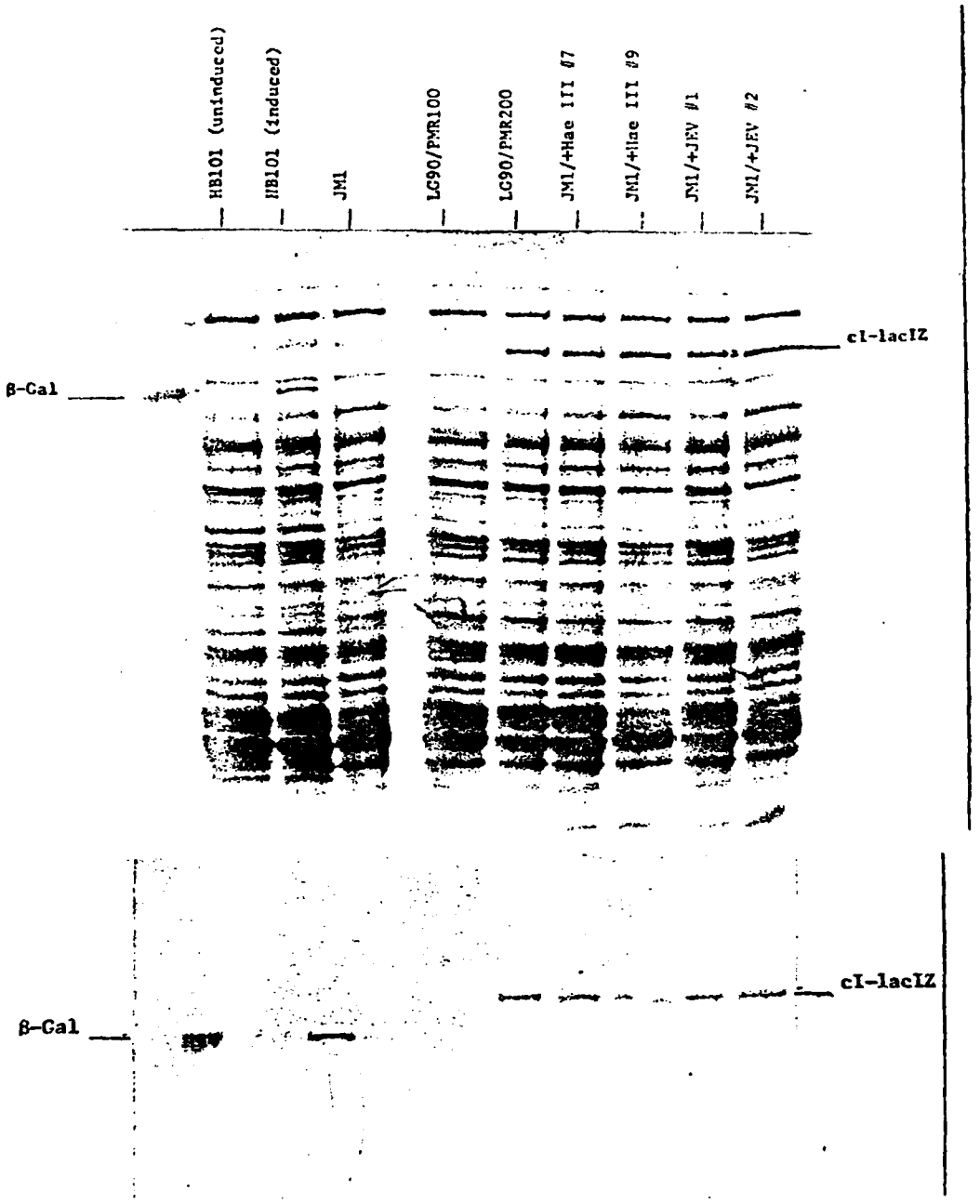


Table II

Properties of "Sequence Specific" JEV Monoclonal Antibodies

Hybridoma	Isotype	Specificity	Neutralization
J2-2F1-2	-	V1	-
J2-5A11-1	$IgG2_a$	V3	+
J2-5F1-6	$IgG2_a$	V3	+
J3-10E1-4	IgG1	V3	+
J3-11B9-3	$IgG2_a$	V3	-
J3-11G5-4	-	V3	
J3-12H11-9	$IgG2_a$	V3	-
J3-14E6-1	$IgG2_a$	V3	+++
J3-14H5-2	$IgG2_a$	V3	-

Table III

Properties of Monoclonal Antibodies to E. coli β-Galactosidase

		Reactivity		
Hybridoma	Isotype	ELISA	Western Blot	Protein A Binding
G12H10	IgM	+	+	-
G5F8	IgM	+	+	-
4C4E9F3G6	IgM	+	+	-
2B3	$IgG2_a$	+	+	+
2B10A4	IgA	+	+	-
2D1B7	$IgG2_b$	+	-	+
2B2C1	$IgG2_a$	+	-	+
D9	$IgG2_b$	+	-	+

VI. FIGURE LEGENDS (Figures 9 and 10)

Fig. 9. Immunoblot analysis of JEV mAbs. In order to prepare nitrocellulose strips with immobilized JEV antigens, either purified virion or JEV-infected Vero cell lysate in SDS-sample buffer was layered uniformly across the top of 12.5% polyacrylamide slab gels. The polypeptides were resolved by electrophoresis and then electrophoretically transferred to sheets of nitrocellulose filter paper. The sheets were then cut into narrow strips for incubation with different mAbs. A strip representing purified virion (panel A) and one of the infected cell lysate (panel B) were incubated in pairs with diluted ascitic fluids from 24 representative hybridomas from the J2 and J3 fusions (performed at WRAIR). Also included are strips incubated with hyperimmune ascitic fluid (HMAF). Antibody binding was detected by decoration with ^{125}I-goat anti-mouse Ig and autoradiography. The distortion in the band for J2-2F1-2 in B is the result of a gel artifact; this strip was cut from the edge of the gel. The incubations with J2-2F1-2 were repeated with new strips in order to confirm the reactivity toward a 20-25 kDa polypeptide in infected cells (panel C). Note that the low mol. wt. polypeptides, V1 and V2, have been tentatively identified on the basis of the following observations: 1) the two bands designated as V1 and V2 are not immunologically related and must, therefore, be separate polypeptides; 2) the electrophoretic mobility of V1 is greater than V2; 3) V2 is present in virion and in infected cells; V1 is absent in infected cells.

Fig. 10. Analysis of lacZ fusion proteins in E. coli lysates. The upper panel shows the polypeptide profile of E. coli extracts prepared according to Gray et al. (1982). The polypeptides were separated by SDS-PAGE in a 7.5% slab gel and stained with Coomassie Blue. The samples include: HB101 grown with and without the inducer IPTG; JM1, a lacZ deleted strain; LG90, a lacZ deleted strain, carrying either the out-of-frame ORF expression vector (pMR100) or the in-frame vector (pMR200) which produces a cI-lacZ fusion protein; JM1 transformed with pMR100 carrying ORF sequences from Hae III digestion of pBR322 (cf. Gray et al., 1982); JM1 transformed with pMR100 carrying putative JEV-ORF sequences. The postions of lacZ and cI-lacZ are indicated. There is no additional increase in the mol. wt. of the fusion proteins in the four samples of JM1 with pMR100 because of the small size of the inserts. The lower panel is a western blot of a similar gel loaded with 10-fold less protein. The binding of the anti-β-galactosidase mAb, 2B3, was visualized with perioxidase conjugated anti-mouse IgG.

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