AD-A171 384 STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS(U) MASSACHUSETTS UNIV AMHERST DEPT OF BIOCHEMISTRY M J FOURNIER ET AL. 04 SEP 84 1/1

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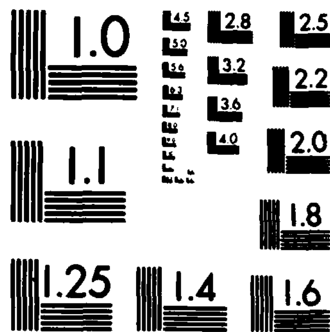

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STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS
ANNUAL SUMMARY REPORT
4 Sept. 84

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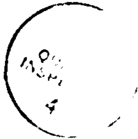

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No. DAMD-17-82-C-2237

STRUCTURE AND EXPRESSION OF GENES FOR FLAVIVIRUS IMMUNOGENS (U)

Annual Summary Report

4 September 1984

Supported by

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

Contract No. DAMD 17-82-2237

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: DAMD-17-82-C-2237

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle): Structure and Expression of Genes for Flavivirus Immunogens

5. TYPE OF REPORT & PERIOD COVERED: Annual Report September 1983 - August 1984

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s): Maurille J. Fournier and Thomas L. Mason

8. CONTRACT OR GRANT NUMBER(s): DAMD-17-82-2237

9. PERFORMING ORGANIZATION NAME AND ADDRESS: Dept. of Biochemistry, University of Massachusetts, Amherst, MA 01003

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A.3M161102BS12.AC.100

11. CONTROLLING OFFICE NAME AND ADDRESS: HQ, USAMRDC, Fort Detrick, Frederick, MD 21701-5012

12. REPORT DATE: 4 September 1984

13. NUMBER OF PAGES: 27

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report): (U)

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report): Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report): Approved for public release; distribution unlimited

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number): Flavivirus, Japanese encephalitis, Dengue, gene cloning, neutralizing antibody, monoclonal antibody, synthetic vaccine.

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

The genome of the Japanese encephalitis virus (JEV) is being cloned for detailed structure-function studies and with a view to the development of a synthetic vaccine. Full-length cDNA transcripts have been obtained via reverse transcription of the 12 kb plus strand RNA genome. Synthesis was from a 3'-specific synthetic primer. Second strand synthesis yielded double-stranded cDNA ranging in size from <500 bp to >10 kb. A variety of plasmids with cDNA inserts of up to 5.0 kb have been developed. At least 8

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kb of unique DNA is contained in three plasmids accounting for 75% of the genome. Much, perhaps all of the remaining sequences will likely be identified in a bank of over 100 cDNA clones currently being characterized. A functional map of the genome is being developed by in vivo expression analyses and DNA sequencing. Protein coding regions are identified by fusion of viral open reading frame (ORF) sequences with the E. coli lacZ gene in plasmid and phage expression vectors. Screening for individual JEV-lacZ hybrid proteins is in progress using antibody probes for specific viral peptides. One cDNA clone containing 1.45 kb has been subjected to extensive sequence and expression analyses. ORF elements of various sizes have been identified by both methods. However, only one of these is thus far considered large enough to accommodate a protein of potential relevance. Inspection of the 1.2 kb sequenced portion of this clone shows stop codons present in all 3 reading frames for both strands. This finding indicates that the entire JEV genome cannot be a single continuous ORF.

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SUMMARY

The genome of the Japanese encephalitis virus (JEV) is being cloned for detailed structure-function studies and with a view to the development of a synthetic vaccine. Full-length cDNA transcripts have been obtained via reverse transcription of the 12 kb plus strand RNA genome. Synthesis was from a 3'-specific synthetic primer. Second strand synthesis yielded double-stranded cDNA ranging in size from 500 bp to 10 kb. A variety of plasmids with cDNA inserts of up to 6.0 kb have been developed. At least 8 kb of unique DNA is contained in three plasmids accounting for 75% of the genome. Much, perhaps all of the remaining sequences will likely be identified in a bank of over 100 cDNA clones currently being characterized. A functional map of the genome is being developed by *in vivo* expression analyses and DNA sequencing. Protein coding regions are identified by fusion of viral open reading frame (ORF) sequences with the *E. coli* *lacZ* gene in plasmid and phage expression vectors. Screening for individual JEV-*lacZ* hybrid proteins is in progress using antibody probes for specific viral peptides. One cDNA clone containing 1.45 kb has been subjected to extensive sequence and expression analyses. ORF elements of various sizes have been identified by both methods. However, only one of these is thus far considered large enough to accommodate a protein of potential relevance. Inspection of the 1.2 kb sequenced portion of this clone shows stop codons present in all 3 reading frames for both strands. This finding indicates that the entire JEV genome cannot be a single continuous ORF.

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 temperature 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 lear 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.

+The goals, strategies and historical background of this program have not changed since the last report. Because the descriptions supplied then are still timely and appropriate the present introduction is taken from the previous report.

++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).

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 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 virulent infections resulting when the 2nd 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 Donald Burke, 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; Peiris *et al.*, 1982; Henchal *et al.*, 1982; Gentry *et al.*, 1982; Minor et al., 1983; Kimura-Kuroda and Yasui, 1983; Kobayaski *et al.*, 1984). 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; Clarke *et al*., 1983). These regions apparently also elicit neutralizing antibodies when they are made biosynthetically in recombinant *E. coli* cells. These results along with the recent progress in the development of a synthetic hepatitis B vaccine from genetically engineered yeast justify the pursuit of 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 generated cDNA clone banks for the JEV genome, established an effective plasmid vector systems 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 second contract period (Sept. 1983 - Nov. 1984) were:

1) To continue the development and banks of cloned cDNA fragments corresponding to the genomes of Japanese encephalitis and dengue-1 viruses.
2) To develop a physical map of the JEV genome.
3) To identify protein coding regions in the JEV cDNA bank with the V3 protein gene having first priority.
4) To develop a functional map of the genome.
5) To develop methods for purifying V3 protein and specific V3 fragments for epitope mapping.

All of these aims were addressed during the year. As in the initial start-up year our effort continues to be focussed on the JEV genome. This decision is based on both scientific grounds and limiting personnel. In the first regard, the yields of dengue-1 RNA for cDNA synthesis are typically only 10% or less of that for JEV. Single preparations of DEN-1 RNA corresponds to only a few micrograms, equal to or less than the amount needed for productive cloning under optimal conditions. Because of this limitation the decision was made to optimize the cDNA synthetic and cloning methodology with the more abundant JEV material. Because the genomes of the two viruses are expected be be sequence related, the availability of characterized JEV cDNA clones will make the task of cloning the DEN-1 genome markedly easier. Accordingly, the strategy adopted is to test the extent of sequence homology by hybridization analysis. If the sequences are related, cloning of the DEN-1 genome will be done by primer extended cDNA synthesis using defined and carefully spaced JEV DNA segments. This approach will be much more efficient than using a single synthetic DNA primer at the 3' terminus of the RNA as was required for the JEV cloning. Also contributing to the decision to clone the JEV and DEN-1 genomes in tandem fashion was the availability of only one worker in our group with cDNA cloning skills; the other members of the team have different responsibilities.

Our effort during the past year was concerned with the following specific aims:

1) optimizing the assorted procedures used in the preparation and cloning of viral cDNA
2) completion of the bank of JEV cDNA clones with special interest in achieving large cDNA inserts
3) physical characterization of the JEV DNA clones by size and restriction enzyme analysis
4) identification of cloned protein coding regions, by DNA sequence and _in vivo_ expression analyses
5) evaluation of newly developed ORF expression vectors with potentially important advantages

6) optimizing immunological screening procedures for use with monoclonal antibodies
7) purification of JEV-β-galactosidase fusion proteins and evaluation of their potential for eliciting an immune response against viral-encoded proteins
8) purification of the V3 protein from JEV using reversed phase HPLC

B. Research Personnel Comprising the Project Team

The work described was conducted by the personnel listed below.

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-April 1984

Lynn Wharton, B.S.--Research technician; appointed September, 1982-August, 1984

Judith Partaledis, B.S.--Research technician; appointed January, 1984-September, 1984

Cynthia Clapp, B.S.--Research technician; appointed July, 1984

The research team, consiting of two postdoctorals, two technicians, and part-time student workers reached full strength at about the mid-point of the first contract year. Some personnel turnover has occurred but replacements have been made in an orderly and timely manner. Ms. Linda Niedowicz, a technician highly skilled in DNA sequencing has replaced Ms. Wharton (August) and two excellent postdoctoral workers, Drs. Peter Mason and Reiner Feick, will join us in October to replace Dr. Fritzinger and Ms. Partaledis. The curriculum vitae of the new doctoral workers will be supplied with the next report.

C. Detailed Progress Report

CLONING OF JEV cDNA AND DETECTION OF PROTEIN CODING REGIONS

1. Cloning strategy. Two strategies have been used successfully in developing our library of cDNA clones. One approach utilizes double-stranded cDNA, the other direct cloning of RNA:cDNA hybrids. In both strategies the double-stranded nucleic acid is dC-tailed and incorporated into PstI-cut, dG-tailed pBR322 vector. Reverse transcription in both cases is from a 3'-positioned synthetic primer. The two strategies are shown in schematic form in Figure 1. The sequences of the 3'-terminus of the JEV RNA and synthetic DNA primer are shown in Figure 2. The sequence information was developed and supplied by Drs. Connie S. Schmaljohn and Joel Dalrymple. The colleagues generously continue to supply viral RNA.

2. cDNA synthesis and cloning. We have continued our effort to optimize each of the many procedures involved in the preparation and cloning

of the cDNA. The primary purpose was to obtain as large as possible cDNA products at maximal yield with the limited amount of RNA available. Important and exciting progress has been made with successes well beyond that realized by all but a very few other laboratories utilizing this technology. Major breakthroughs came in both the synthesis and cloning areas.

The largest gain was experiences with the preparation of cDNA. As a consequence of systematically evaluating assorted methods for denaturing the template RNA and inhibiting RNase activity that commonly inhibits commercial preparations, two important benefits were achieved. First was the discovery that template activity was markedly improved when the agent used to denature the RNA was increased five-fold over that recommended in the literature for other RNAs (Maniatis, T. et al., Molecular Cloning, Cold Spring Harbor, 1982). The denaturant methylmercury hydroxide, is used to remove secondary base pairing which limits the progression of reverse transcriptase along the RNA. By using more of this compound (50 mM) improvements were seen in both the overall yield and size distribution of the resulting cDNA. These results were not carefully quantified but the benefits were estimated to be approximately two-fold.

The major gain on this front came from an evaluation of the commercial RNase inhibitor 'RNasin'. Inclusion of RNasin in the reverse transcription reaction resulted in a very dramatic increase in both size and yield of the cDNA Products. An example of this effect is shown in the agarose gel fractionation pattern in Figure 3. The electrophoretic distribution of cDNA made in the absence of RNasin is shown in Lane A. While too faint to be easily observed in photocopies the products ranged in size from 500 bp up to 4-6 kb. The effect of RNasin (two levels) is shown in Lanes B and C. It is readily apparent that the equivalent aliquot of reaction mix contains both more and larger cDNA. To our surprise and great delight a significant amount of full-length cDNA was made. Product cDNA made in the presence of RNasin ranges in size from 500 bp to 12 kb. An arrow marks the position of full-length material. (The size standards correspond to fragments of λDNA cleaved with Hind III.

The improved yields and larger transcripts in the reverse transcription reaction in turn improved the overall quality of the final double-stranded cDNA. Figure 4 shows the pattern of resulting double-stranded cDNA before and after treatment with the single-strand specific S1-nuclease (this step cleaves the loop caused by self-priming and trims the termini to yield two blunt ends).

Finally, the improved yields made it possible to size fractionate the cDNA prior to incorporation into the cloning vector. Here, it was determined that the molecular seive, Sephacryl S-1000 could be used effectively to eliminate cDNAs smaller than 500 bp in length. This step increases the eventual yield of large cDNA inserts and significantly reduces the high background of recombinants possessing small cDNA of questionalbe value.

In a recent cloning effort, 4 μg of JEV genomic RNA yielded approximately 100 ng of unfractionated ds cDNA. A Sephacryl S-100 gel filtration colyumn was used to fractionate 50 ng of this material into two fractions of cDNA greater 500 bp. Although there is overlap, fraction (1

(8ng) contains the largest cDNA and fraction 2 (10 ng) contains a higher percentage of smaller cDNA.

After dG-tailing and annealing of the cDNA into a dG-tailed vector, transformation with fraction 1 (4ng) yielded 112 clones with inserts ranging from 500 bp to 6 kb. Of these, 20% contained inserts larger than 2 kb with half of these larger than 3 kb. Fraction 2 yielded another 150 clones that have not yet been characterized for insert size.

3. Development of a complete genomic bank of JEV cDNA. The cloning attempts made during the first year yielded reasonable numbers of recombinants however, the cDNA inserts were in the range of only a few hundred bp to 1.5 kb (see last annual report). While this is typical for cDNA cloning in general and satisfactory for some applications, products of this size make difficult the task of developing a complete bank for a genome the size of JEV. Additionally, the inability to achieve full-length primary transcripts necessitates subsequent rounds of cDNA synthesis using internally positioned DNA primers obtained from earlier attempts. Unless random transcriptional initiation can be arranged, several cycles of cDNA synthesis and cloning would be required to reach the 5'-terminus of the RNA template.+

4. Physical characterization of the JEV DNA clones. Restriction maps have been developed for three unqiue cDNAs. The mapping results are shown in Figure 5. The pattern of restriction sites appears to be unique in each case indicating that the three segments are unrelated. Taken together, the three cDNAs account for 8 of the 12 kb of the genome. Thus, at least 75% of the viral genome has been cloned. Because full-length cDNA was observed in both the single- and double-stranded DNA preparations it seems assured that additional unique segments will be discovered in the recent bank. Sizing of the cloned inserts made by the new methods has thus far shown cDNA products ranging from about 500 bp to 6 kb. Characterization of this collection is still in progress, however, with this pattern, the bank could actually be complete. Additional restriction analyses and hybridization assays will be carried out to construct the genomic structure and determine the relative position of the assorted cloned segments.

5. Identification of viral protein coding regions. cDNA segments containing translational open reading frames (ORF) are being identified with two different classes of expression vectors. Both depend on in-frame fusion of ORF fragments with vector-encoded *E. coli* β-galactosidase (*lacZ*). The two strategies are outlined in Figure 6. One class of vector consists of a plasmid containing a fusion of DNAs from *lacZ* and a second protein gene--the cI protein in one case (Gray et al., 1982, Proc. Nat. Acad. Sci. USA 79:6598-6602) and the *E. coli* *ompF* protein in the other (Weinstock, G. M. et al. 1983, Proc. Nat. Acad. Sci. USA 80:4432-4436). ORF cloning sites have

+A quirk of fate last year allowed us to generate a collection of random small cDNA clones. As described in the previous report, one preparation of viral RNA was highly fragments, presumably in random fashion. Tailing of this RNA with polyA and using an oligo-dT primer yielded a statistical mix of cDNAs. Additional characterization of these clones revealed the average insert to be too small (< 200 bp) to make the 40 clone bank complete. This approach was abandoned when subsequent RNA preparations proved to be largely intact.

been placed at the junction of the two coding sequences. In both cases, the lacZ fusion results in its being out of frame. Insertion of an ORF sequence in the correct frame restores translation of the downstream lacZ region to yield functional β-galactosidase activity. Thus, ORF sequences can be identified by plating of transformants on lacZ indicator plates.

The alternate strategy involves the use of λgt11 expression vector newly developed by Young and Davis (1983, Proc. Nat. Acad. Sci., USA 80:1194-1198). In this case cloning of the cDNA segments is into the distal portion of lacZ resulting in an inactive viral lacZ fusion protein.

Experience with both the pMR100 vector of Gray et. and the λgt11 system during this past year has revealed each to possess unique features important to the success of our program. Accordingly, we anticipate continuing to use both types of vector. Several JEV ORF sequences have been successfully identified with the pMR100 plasmid vector but it appears that only ORF segments of a few hundred bases will yield a stable tribrid protein with lacZ activity (see below). The λgt11 vector on the other hand has been used successfully to link gene-size ORF sequences to lacZ large fusion proteins with good stability (Young and Davis, 1983). Use of this vector in our own laboratories has born out this observation. In this last regard, we are now convinced that the λgt11 vector provides the best method for the identification of cloned genes for which we already possess specific immunological probes. Since available monoclonal antibodies and hyperimmune ascitic fluids have combined reactivities with polypeptides that account for over 80% of the coding capacity of the JEV genome, we are now developing a bank of JEV cDNA in λgt11 for expression analysis. In the event that unidentified ORFs remain after we have exhausted the potential of the phage expression system, we will use the DNA sequence information obtained from these regions to engineer their expression in the plasmid vectors and to select potential antigenic regions of the ORF for production of synthetic peptide antigens. Antibodies raised against the polypeptides expressed in vivo and those obtained by solid-state synthesis will be used to determine whether or not the unidentified ORFs are expressed during viral replication in infected cells.

6. DNA sequence and ORF analyses of JEV cDNA. One of the first sizeable cDNA clones isolated, pJEV has been subjected to extensive sequence and ORF analyses. All but about 200 bases of the 1.45 kb fragment has been sequenced by the M13-dideoxy method. Inspection of the sequence revealed hypothetical coding regions in all three reading frames for both DNA strands. The positions of these regions is shown in Figure 7.

Analysis of segments of pJEV-1 DNA in the expression vector pMR100 has also revealed the occurrence of ORF elements. Here a pool of assorted restriction fragments were treated with the exonuclease Bal31 before subcloning to randomize the ends--thereby assuring fusion of the cDNAs in all possible reading frames. Subsequent restriction and protein analyses of the ORF-positive clones confirmed the occurrence of DNA and protein fusions. The positions of three of the ORF sequences yielding JEV-lacZ fusion proteins are shown in Figure 7. The electrophoretic patterns of the fusion proteins are shown in Figure 8.

Comparison of the ORF maps generated by the sequence and expression assays reveals an interesting situation. Only one of the three ORFs identified in the expression analysis is completely within an ORF region detected in the sequence analysis. The other two ORF+ restriction fragments do not correspond to a single continuous ORF in any reading frame. Interestingly, the gel pattern of the hybrid proteins resulting from these fusions are consistent with this observation. In both cases the resulting fusion products are too small to correspond to a fully translated ORF. While the basis for the small increase in size over the control cI-lacZ fusion is not clear, it is reasonable to suggest that translation may have initiated within the viral cDNA cloned into the pMR100 vector.

The longest ORF identified in the sequence analysis spans the expression-positive clones #12 and #18 (see Fig. 7) and accounts for about 400 bp of the 1.45 kb insert. This region could theoretically code for a protein of about 130 amino acids or 14 kDal. In view of its size it is possible that this ORF might actually correspond to a viral protein gene. The possibility that this and other ORF sequences do encode proteins is being assessed by immunological procedures (see below).

The finding of translational stop signals in all possible reading frames in the DNA sequence indicates that the viral genome cannot be a single, continuous ORF. However, since the genomic position of the pJEV-1 DNA is not yet known it is still possible that some or all of the viral proteins could be derived from a polyprotein precursor(s).

7. Immunological screening with monoclonal antibodies. Our strategy for the identification of cloned viral genes relies on the ability of immunological probes to detect the expression of viral sequences in *E. coli*. Depending on the expression vector used, the screening is performed either on lysed *E. coli* colonies or on plaques formed by the lysis of *E. coli* after infection with phage lambda. In either case the proteins released by cell lysis are trapped on nitrocellulose filter paper prior to reaction with the immunological probes. The published reports on these methods all describe the use of polyclonal antisera, which contain antibodies that typically bind to several different sites on a polypeptide. Since many of our best probes are monoclonal antibodies, we considered it important to optimize conditions for the use of single monoclonal antibodies, which can be expected to bind to only one epitope per polypeptide chain. As our test system we used monoclonal antibodies that we have prepared against *E. coli* β-galactosidase to probe expression vector banks that have been prepared using total genomic DNA from yeast. Since both of these vectors, pMR100 and λgt11, produce lacZ fusion proteins, a monoclonal against β-galactosidase can be expected to give a signal similar in intensity to one elicited by a monoclonal to a polypeptide specified by a cloned ORF.

Strong, specific signals have been obtained with monoclonal antibodies to β-galactosidase in both colony and plaque screening. Typical results obtained with plaque screening are shown in Fig. 9.

As additional preparation for cloning JEV cDNA into the phage lamda expression systems, we have also optimized *in vitro* phage packaging reactions for use with recombinant λgt11 DNA.

8. Purification and immunological characterization of a JEV-β-galactosidase fusion protein. As a first approach to the identification of ORFs in the genome of JEV, we decided to purify the fusion protein produced in E. coli cells transformed with plasmid pMR100-18 (Fig. 8) and to evaluate it as an immunogen for raising antibodies against a viral-encoded polypeptide. Two approaches were taken for purification of the protein. In the first, standard procedures were followed for the isolation of native β-galactosidase; in the second, the fusion polypeptide was isolated in denatured form after electrophoresis in polyacrylamide gels containing SDS.

The isolation of the fusion polypeptide in the form of active β-galactosidase was not successful. It was possible to isolate active β-galactosidase from the transformed cells, but at the final stage of purification none of the lacZ polypeptide was in the form of a fusion product. We suspect that the abnormal tribrid fusion protein is an ideal target for proteolysis and that the extensions at the amino terminal end of the polypeptide are selectively removed by proteolytic cleavage. In future attempts to isolate native fusion polypeptides we will need to minimize proteolysis and develop more rapid procedures for processing the cell extracts. We anticipate that HPLC will be an advantage in this regard.

The denatured form of the fusion polypeptide was gel-purified from a protein preparation obtained by extracting the E. coli cells in the presence of SDS. The fusion protein was electrophoretically eluted from the gel onto nitrocellulose filter paper, the band corresponding to the fusion protein was localized by staining a strip of the filter with amido black, and this band was cut from the filter and used for immunization. For injection into mice the filter containing the protein was dissolved in a minimal volume of DMSO and then mixed with an equal volume of Freund's complete adjuvent. This mixture was used for primary injections administered intraperitoneally; booster injections were prepared with incomplete adjuvent.

Serum obtained after the first and second boosters was tittered for reactivity against β-galactosidase and against proteins from JEV-infected and noninfected Vero cells. Although there was a clear response to β-galactosidase in an ELISA, western blot analysis of the Vero cell lysates failed to reveal a convincing reaction against polypeptides that were specific to the infected cells. At this point we cannot distinguish the possibility that the fusion protein was a poor immunogen for the JEV-specified sequence from the possibility that this sequence is either simply not expressed or is expressed at very low levels in the infected cells.

This work was carried out by Michael Lu, a first year graduate student who spent three months in the laboratory on a rotation project. Our efforts on this aspect of the project will increase dramatically when a new post-doctoral associate, Dr. Reiner Feick, joins our group in October.

9. Purification of the V3 polypeptide by reversed phase HPLC. An important overall goal of this project is to obtain a molecular definition for the major epitopes on the V3 polypeptide that are responsible for the neutralization of the virus. While the critical analysis on this subject will not begin until we have cloned the V3 coding region, we considered it worthwhile to explore the immunological properties of peptides derived from

native V3. The first step in this approach is to isolte V3 from preparations of the virion.

A sample of cobalt-irradiated JEV (provided by Dr. Connie Schmaljohn) was used as the starting material in these analyses. Chromatography was performed with a Synchropak RP-300 C-18 octadecasilyl silica column which was eluted with either a gradient of isopropanol in 60% formic acid, 0.1 M NaClO4 or an acetonitrile gradient in 0.1% trifluoracetic acid. The eluent was monitored at 214 nm and 280 nm. Fractions were collected and analysed for the presence of V3 by immunological reactivity and by SDS-polyacrylamide gel electrophoresis followed by silver staining.

We were unable to reproducibly detect V3 in the fractions eluted with acetonitrile. This was attributed to the hydrophobic nature of V3 and the inability of acetonitrile to dissociate it from the C-18 matix. A more defined pattern was obtained upon elution in the presence of formic acid (see Fig. 10). A peak was tentatively identified as being V3 on the basis of the electrophoretic analysis of fractions from across the profile. Surprisingly, the eluted polypeptide was immunologically inactive with several monoclonal antibodies, which we have shown previously to be highly reactive with either the native or SDS-denatured form of V3. There was, however, a low level of immunoreactivity when a polyclonal hyperimmune ascitic fluid was used as the probe. Control experiments showed that this loss of antibody binding activity was the result of the exposure of V3 to high concentrations of formic acid. Furthermore, the formic-acid treated V3 exhibited an increased electrophoretic mobility in SDS gels that corresponded to a decrease in the apparent mol. wt. of 8-10 kDa.

The concomitant loss of immunoreactivity and an apparent shift in the mol. wt. of the V3 polypeptide suggests that formic acid treatment alters the structure of this protein, perhaps by cleavage of labile peptide bonds. Aspartyl-prolyl bonds are good candidates for such a cleavage. In fact mild treatment in formic acid has been used as a means of inducing specific cleavage at asp-pro bonds (Landon, 1977). As a working hypothesis to explain these preliminary results, we propose that the major antigenic determinants on V3 are clustered near the amino terminus of the molecule and that this domain (8-10 kDa) can be released by cleavage with formic acid. If this hypothesis can be confirmed, we will have localized the major neutralizing epitopes to approximately 20% of the polypeptide chain.

This work was carried out by Keith Kruithoff as part of his research for a senior honors thesis in the Department of Biochemistry. His work will be continued by Drs. Reiner Feick and Peter Mason.

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IV. FIGURES AND TABLES

Figure 1

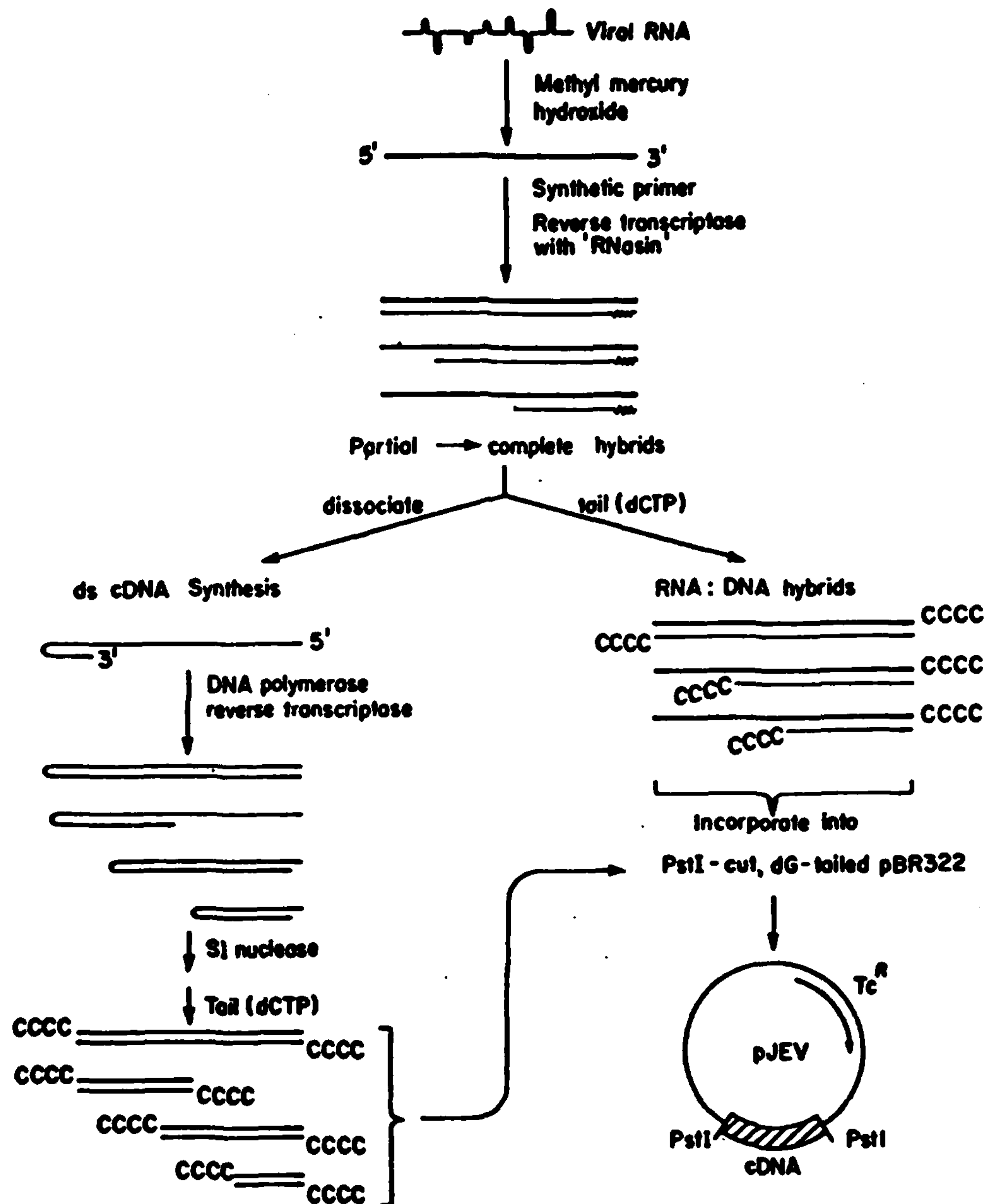


Figure 2

SEQUENCE OF JEV SYNTHETIC PRIMER

JEV RNA

└→ 5'-GAUGUGUAUAUAUAACACAGGAUCU$_{OH}$-3'

• • • • • • • • • • • • •

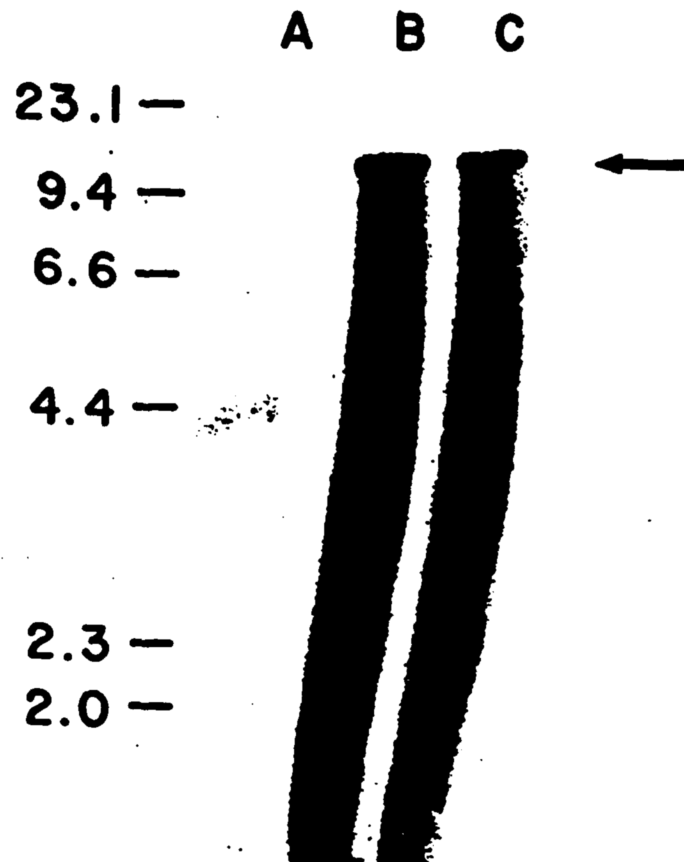
TTGTGTCCTAGA-5'

┌→

Synthetic Primer

Figure 3

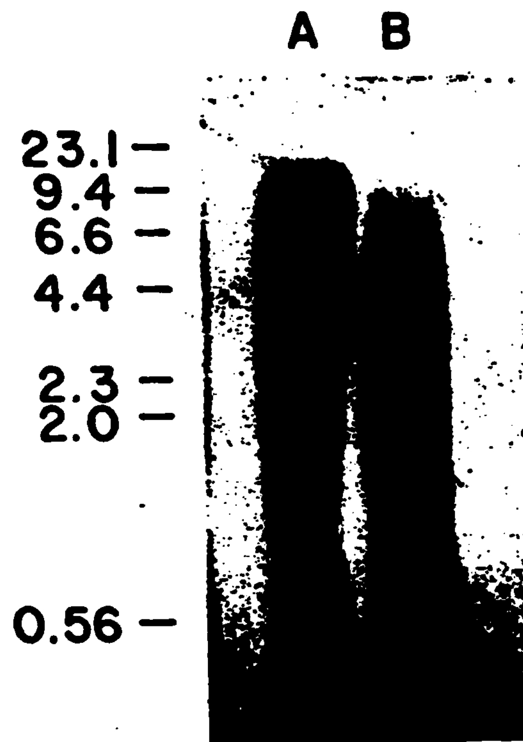
EFFECT OF THE RNase INHIBITOR RNasin ON THE SYNTHESIS OF JEV-cDNA



The electrophoretic patterns of reverse transcribed cDNA products are shown for reactions carried out in the presence and in the absence of RNasin: Lane A: no RNasin; Lanes B and C: 200 units/ml and 400 units/ml of RNasin.

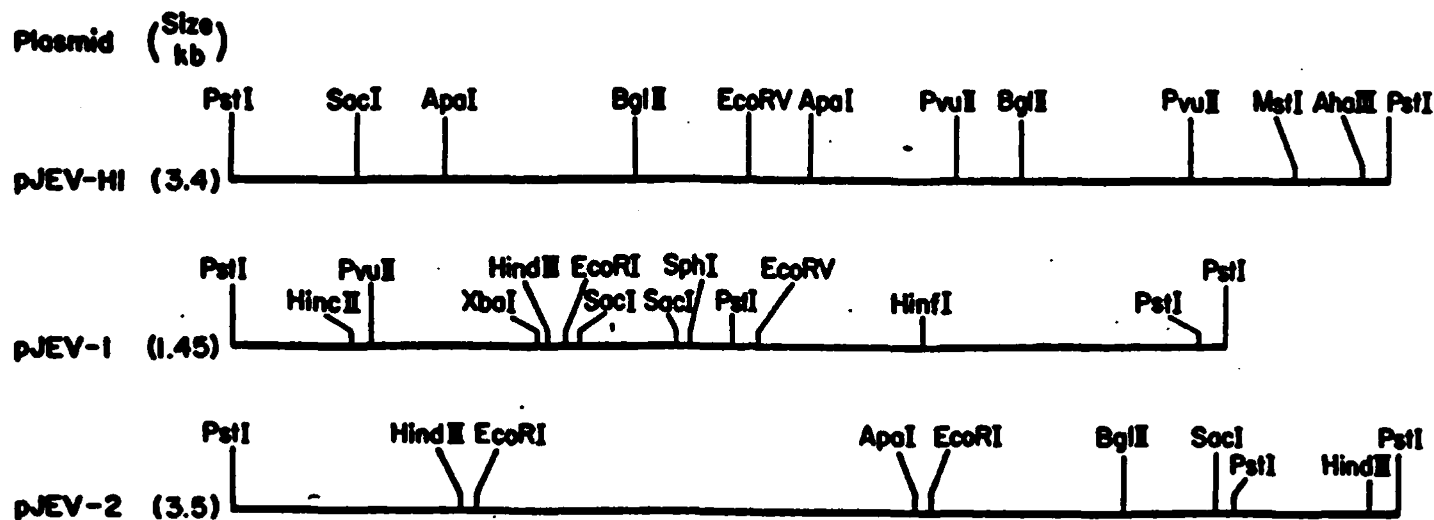
Figure 4

SECOND STRAND SYNTHESIS OF JEV-cDNA



The gel patterns shown are for double-stranded JEV-cDNA before (A) and after (B) treatment with S1-nuclease.

Figure 5



pJEV-H1 resulted from direct cloning of RNA:cDNA hybrids. The other clones were developed from double-stranded cDNA.

Figure 6

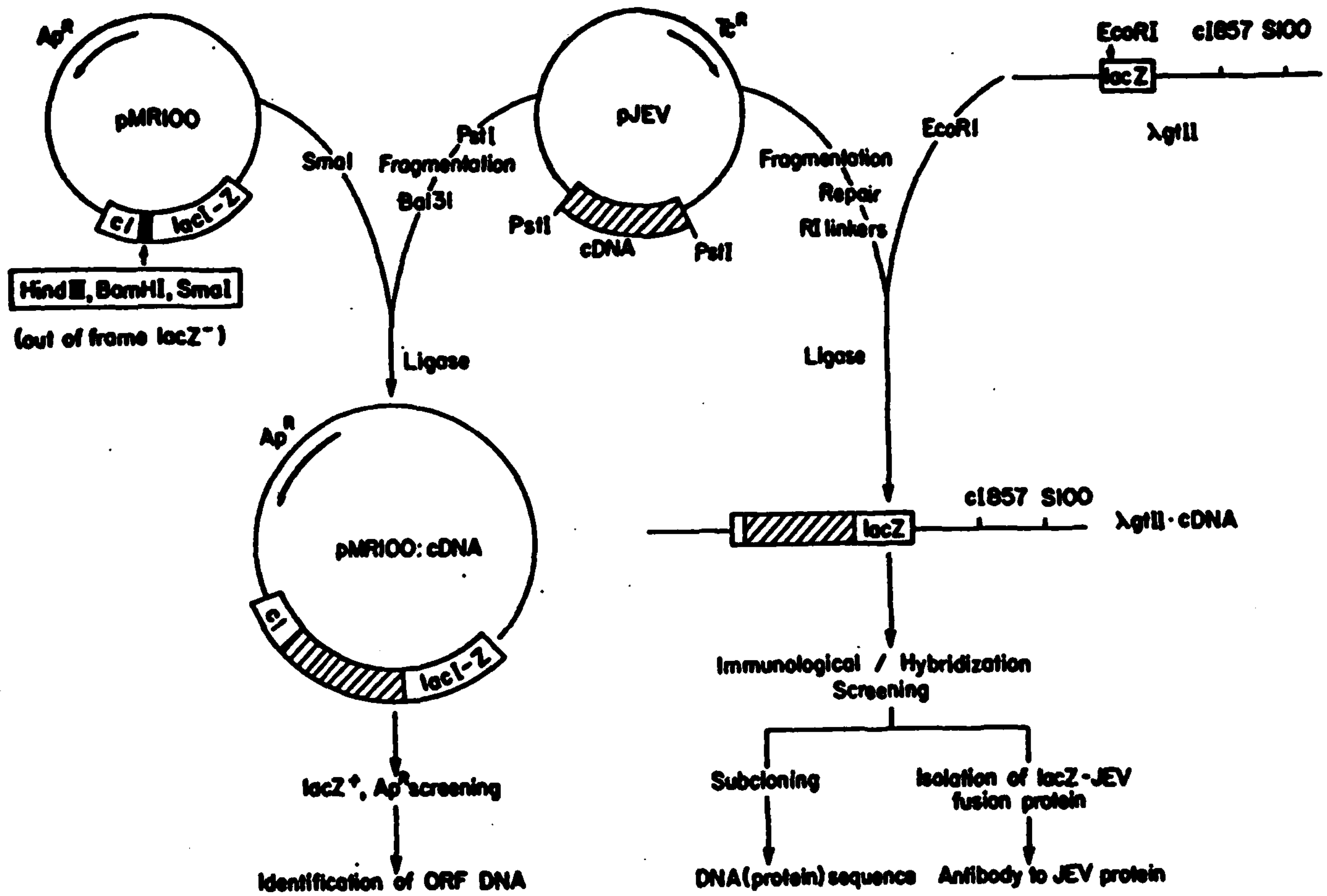


Figure 7

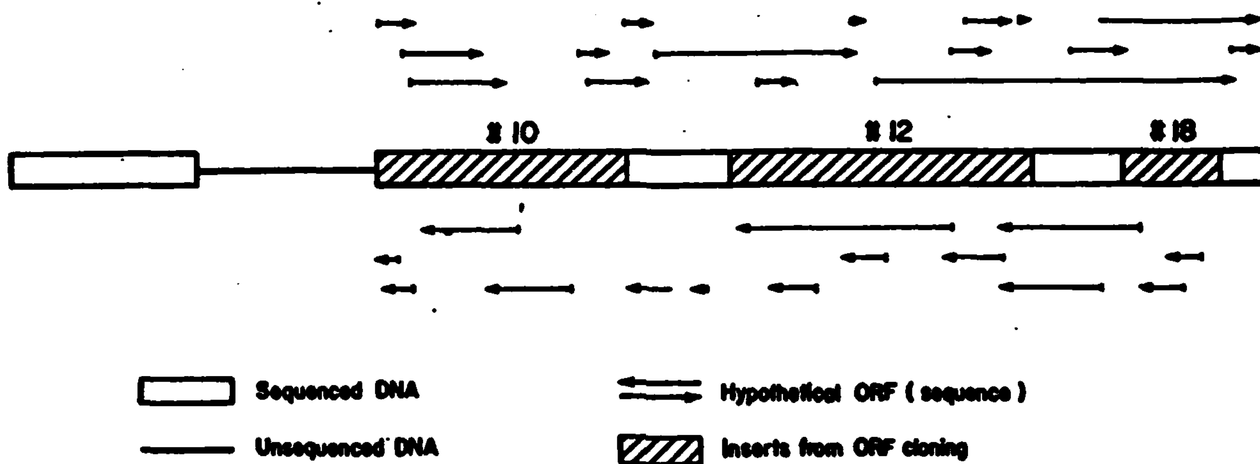
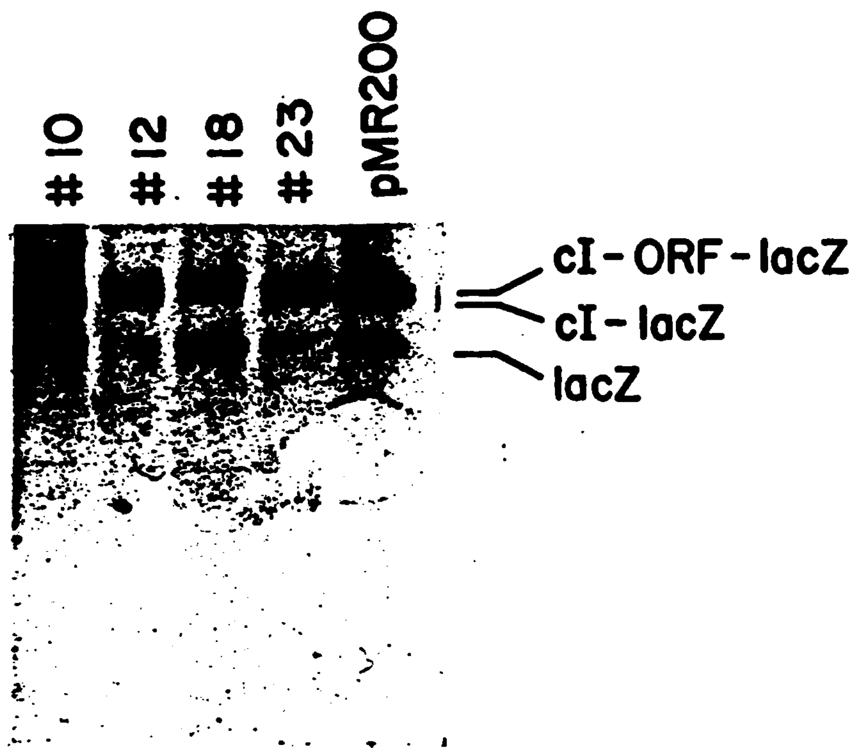


Figure 8

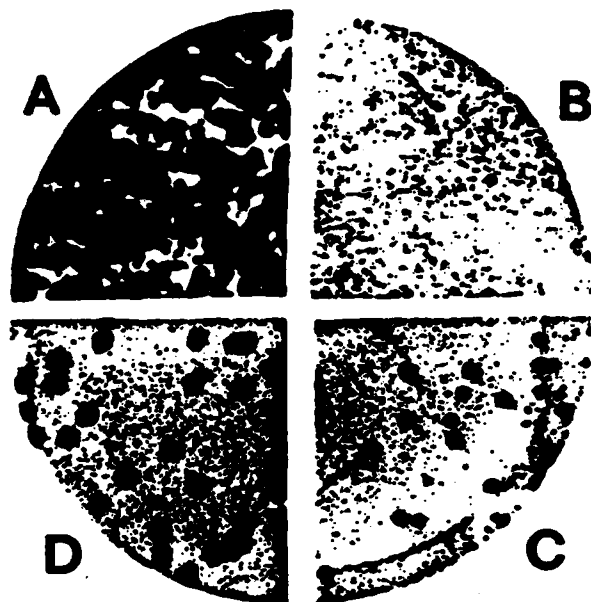
DETECTION OF JEV-β-GALACTOSIDASE FUSION PROTEINS BY IMMUNOBLOT ANALYSIS WITH A MONOCLONAL ANTIBODY TO β-GALACTOSIDASE



Polypeptides extracted from transformed E. coli were resolved by electrophoresis in a 7.5% polyacrylamide gel containing SDS. The polypeptides were then transferred electrophoretically to nitrocellulose filter paper and probed with a monoclonal antibody to β-galactosidase. Antibody binding was visualized with ^{125}I-labelled anti-mouse IgG and autoradiography. The positions of the mature lacZ polypeptide, the CI-lacZ fusion protein produced by the in-frame vector pMR200, and the slightly larger proteins generated by plasmids carrying JEV-ORF inserts are indicated.

Figure 9

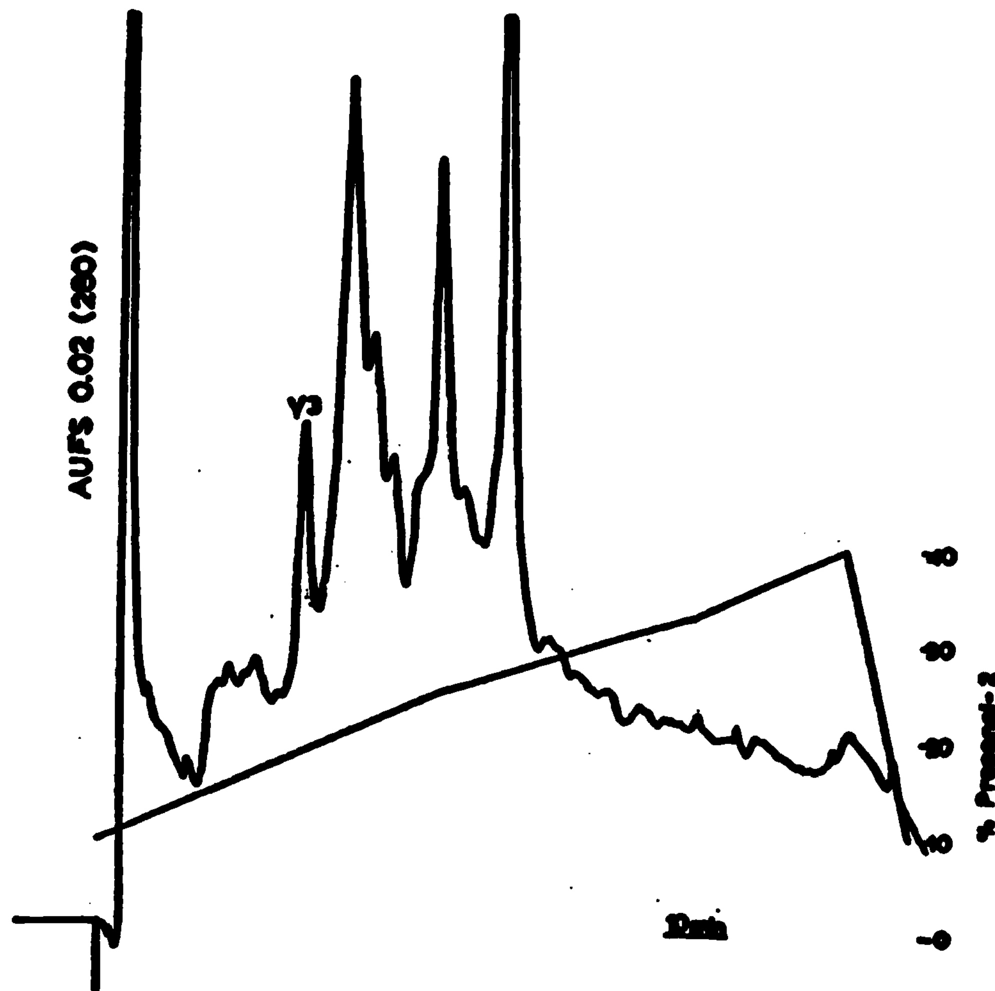
PLAQUE SCREENING WITH MONOCLONAL ANTIBODIES



Autoradiograms of four filters are shown: (A) phage λgt11 plaques detected with a monoclonal antibody against E. coli β-galactosidase; (B) control filter in which no positive plaques are present; (C and D) recombinant phage λgt11 plaques isolated from a yeast genomic DNA bank by screening with monoclonal antibodies to two different yeast proteins. These filters show the reaction after partial purification of the recombinant phage.

Figure 10

ISOLATION OF THE V3 POLYPEPTIDE BY REVERSE PHASE HPLC



Cobalt-irradiated JEV was dissociated in 70% formic acid, applied to a Synchropak RP-P column and eluted with a gradient of 10-40% propanol-2 over 119 min. The identity of the peak corresponding to the V3 polypeptide was established by analysis of the fractions on SDS-polyacrylamide gels. The unlabelled peaks did not contain any protein and are probably due to nonproteinaceous contaminants in the sample.

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