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DEVELOPMENT OF A GENETICALLY ENGINEERED  
VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE

ANNUAL/FINAL REPORT

DENNIS W. TRENT

APRIL 15, 1991

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Division of Vector-Borne Viral Diseases  
Centers for Disease Control  
Fort Collins, Colorado 80522

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## ABSTRACT

Genomes of the Trinidad donkey (TRD) and TC-83 strains of Venezuelan equine encephalitis (VEE) viruses have been cloned and their nucleotide sequences determined. There are 12 nucleotide differences between the genomes of the TC-83 attenuated virus and its virulent TRD parent strain. One nucleotide substitution and deletion occurred in the 5'- and 3'- noncoding regions of the TC-83 genome, respectively. A conservative Ser to Thr amino acid substitution was identified in nsP1 and an Ala to Asp change in nsP2. Two silent mutations are present, one each in E1 and E2. Two amino acid substitutions are found in E1, and five amino acid substitutions are in E2.

The genome of TRD virus is 11,444 nucleotides long with 5'-noncoding region of 44 nucleotides. The carboxyl-terminal portion of nsP3 contains two peptide segments that are repeated. The open-reading-frame of the nonstructural polypeptide is interrupted by an in-frame opal codon between nsP3 and nsP4, as has been reported for other alphaviruses.

Cloned cDNA encoding the structural proteins of TRD and TC-83 viruses were inserted into vaccinia virus under control of the vaccinia virus 7.5K promoter. Synthesis of the capsid protein and glycoproteins E1 and E2 was demonstrated by immunofluorescence, immunoprecipitation and immunoblotting. Mice immunized with the recombinant VEE/vaccinia virus developed virus-specific neutralizing antibodies and survived intraperitoneal challenge with virulent VEE virus of subtypes IAB, IC, ID and II. Animals immunized with the recombinant, however, did not survive intranasal challenge. T-cell responses of mice and primates to immunization with TC-83 and recombinant vaccinia viruses were serocomplex specific, showing only slight cross reactivity with eastern equine encephalitis virus. Proliferating T-cells secreted interleukin 2 (IL2). Reactivity of primed T-cells with subtype IC, ID, IE and IV viruses was poor.

Horses immunized with the vaccinia/VEE virus recombinant virus developed low ELISA titer and neutralizing antibodies after the first immunization. These animals were reimmunized 92 days after the first immunization to boost the immune response prior to virus challenge. Serum antibody titers seven days following immunization were high, indicating antigenic priming had been accomplished by the initial recombinant virus immunization. Horses were immunized with the recombinant vaccinia TC-5A, wild type vaccinia, and TC-83 vaccine virus and challenged with  $10^4$  PFU of equine virulent VEE virus. Following challenge animals immunized with wild-type vaccinia virus developed a leukopenia, became viremic, febrile, depressed, and were euthanized on the sixth day after challenge. Horses immunized with either TC-83 or vaccinia/VEE recombinant viruses did not become viremic or develop clinical or hematologic signs of VEE disease following challenge. In both groups of immune animals, there was no significant increase in VEE antibody following virus challenge, indicating that immunization had fully protected the horses from neuroinvasion.

Full-length nucleotide-sequence-perfect cDNA clones of VEE TC-83 and TRD viruses have been constructed from fully sequenced subgenomic clones. The full-length clone pVIC-92 is bound at the 5'- end by a unique XbaI site and RNA polymerase T7 promoter. At the 3'- end of the clone there is a poly A tail ( $n=25$ ), and a unique M1 site which facilitates opening of the

plasmid for transcription of full-length genome RNA. RNA transcripts synthesized *in vitro* with T7 RNA polymerase are infectious. Recombinant VEE viruses have been constructed to investigate the contribution of TC-83 specific changes in the virulence of TRD virus. These clones, were constructed by *in vitro* mutagenesis or splicing of TRD specific cDNA fragments into the pVE\IC-92 backbone. Plaque and peripheral virulence phenotypes of the viruses derived from the full-length cDNA clones of TRD, TC-83 clone pVE\IC-92, and recombinant clones pVE\IC-101 through pVE\IC-113 have been constructed and analyzed. Infectious clones 92, 101, 110 and 112 exhibited plaque phenotypes in Vero cell monolayers similar to TC-83 virus. Plaques produced by viruses containing TRD gene substitutions in recombinants 102, 108, 109, 111 and 113 were generally larger than those produced by TC-83. Recombinant virus 104, that contains two amino-terminal changes in E2, and recombinant 108, that contains the TRD 5'-noncoding change, were avirulent. Recombinant 112, containing the 5'-noncoding and nsP3 TRD changes, produces small plaques and is intermediate in its virulence for mice. All of the constructs, except 108 and 110 which contain the TRD 5'-noncoding change, are mouse virulent. These results suggest that virulence resides in a constellation of genetic changes involving the 5'-noncoding region, nsP3 and a specific region in E2.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. <NIH> 78-23, revised 1978).

The investigators have abided by the National Institutes of Health for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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## PROGRESS REPORT

### I. MOLECULAR CLONING AND GENETIC ANALYSIS OF THE TRINIDAD DONKEY AND TC-83 VIRUS GENOMES

Molecular cloning and sequence analysis of the VEE TRD and TC-83 genomic RNA have been completed. The entire nucleotide sequence and deduced amino acid sequence of the VEE TRD genomic RNA are shown in Fig. 1. Excluding the 5'-m7G cap, the TRD RNA genome is 11,444 nucleotides long with a base composition of 28.2%A, 24.5%C, 25.3%G and 22.0%U.

Sequence of the ultimate 5'-nucleotides of the virion RNA was obtained by primer extension sequencing. These studies confirmed the substitution of an A for G at genome nucleotide position 3 in the TC-83 RNA. Excluding the 5'-cap, the 5'-noncoding regions of both TRD and TC-83 viruses are 44 nucleotides in length. The nucleotide sequence at the 3'-terminal region of the viral RNAs was obtained by direct sequencing of denatured plasmid cDNA. These data confirmed the deletion of a U nucleotide in TC-83 virus at genome position 11,405. Sequence analysis of the 26S junction region of the 42S RNA confirmed that the sequence of TRD and TC-83 viruses were identical.

Two in-frame AUG codons at nucleotide positions 12-14 and 45-47 occurred within the 5'-terminal 50 nucleotides of VEE genomic RNA. An out-of-frame AUG codon was present at the 5'-terminus of TRD RNA, but not in TC-83 due to substitution of an A nucleotide at position 3. The AUG sequence at position 45-47 occurred within the consensus sequence CAXXAUGG (where X = any nucleotide) used as initiation codons in eukaryotic mRNA. This sequence was chosen as the initiation codon for the nonstructural VEE virus polyprotein precursor. Translation of the genome was terminated by an ochre (UAA) codon at positions 7524-7526.

The deduced polyprotein is 2493 amino acids long. The open-reading frame of the translated region was interrupted by an opal (UGA) codon at nucleotides 5682-5684. The in-frame opal codon was present in the genomes of TRD and TC-83 viruses, was also present of the genomes of subtypes IAB viruses PTF-39 and 71-180.

Nucleotide sequence data from the cDNA clones indicated that genes encoding nonstructural proteins of VEE TRD and TC-83 viruses differed at nucleotide positions 1696 (C in TRD to A in TC-83), 1873 (T to A), 4698 (A to G), 4809 (T to A), 5877 (G to A), and 7306 (T to C). Independent sequencing of the viral RNAs revealed that only two of these changes were valid: the C to A change at position 1696 and the T to A change at position 4809. These nucleotide changes resulted in an amino acid change at position 16 in rna of Ala to Asp and a Ser to Thr change in nsP3 at position 260. These changes, together with the other changes in the noncoding 3'- and 5'-regions and the structural genes, are shown in Table 1.

The putative cleavage sites used to generate the nonstructural proteins from the polyprotein precursor were identified by comparing the VEE virus amino acid sequence with those of other alphaviruses. The amino acid sequence of the translated nonstructural polyproteins of SIN, SF and VEE virus were aligned to provide maximal homology and sequence in the cognate proteins. The nsP1, nsP2, nsP3 and nsP4 proteins of VEE virus showed 60%-66%, 57%-58%, 35%-44%



and 71%-73% sequence homology, respectively, with cognate proteins of SIN and SF viruses. The 52kd nsP3 proteins of VEE virus were highly conserved when compared to SIN and SF viruses. For example, 94% of 18 Trp amino acids, 73% of 73 Phe, 70% of 61 Cys, 70% of 120 Gly, and 69% of 116 Pro residues were conserved in the aligned nonstructural proteins of VEE, SIN and SF viruses.

The nsP3 polypeptide, which was the least conserved of the four nonstructural proteins, has some interesting features. The amino-terminal half of the protein is highly conserved among the alphaviruses; however, the carboxyl-terminal half of VEE virus nsP3 has virtually no sequence homology with the same regions of the nsP3 proteins of SIN and SF viruses.

The carboxyl-terminal portion of VEE nsP3 contains a short repeated peptide (PXPAPRT, where X = a variable amino acid) and a long 34 amino acid sequence repeat of TPSXXPSPXXSRTSLVSPXPPGVNRVITREEXEZ. These repeated peptide segments were identical in TRD and TC-83 viruses. SF virus nsP3 has a repeated peptide segment similar in sequence to that of SIN virus; this region of SF virus nsP3 lacked 70 and 73 amino acids relative to VEE and SIN viruses, respectively. The in-frame opal termination codon, located six amino acid residues upstream from the carboxyl terminus of VEE nsP3, was aligned in-frame with the opal codon reported in the sequence of SIN virus.

The nucleotide and deduced amino acid sequence differences between the genomes of VE TRD and TC-83 viruses are summarized in Table 1. TC-83 contained a single nucleotide mutation in the 3'- and 5'-noncoding regions. Additional changes in the sequences of TC-83 and TRD viruses were noted in nsP2, nsP3, E1 and E2.

## II. CONSTRUCTION OF FULL-LENGTH CDNA CLONES OF VEE VIRUSES TC-83 AND TRD

The full-length "infectious" cDNA clone of VEE TC-83 and TRD viruses was constructed from cDNA clones which had been sequenced and fully characterized (Fig. 2; Kinney *et al.*, 1989; Johnson *et al.*, 1986; Kinney *et al.*, 1986). The confirmed differences between the RNA genomes of the TRD and TC-83 viruses are indicated in Table 1. Confirmation of the nucleotide differences between TRD and TC-83 cDNA clones was accomplished by sequence analysis of more than one independent clone for a given virus and/or by direct sequence analysis of the RNA. These clones were spliced together to construct the 11447-bp length cDNA copy of the full-length genome of TC-83 virus. An XbaI restriction site and promoter, recognized by the bacteriophage RNA dependent polymerase T7, were engineered at the 5'-end of the full-length clone. A poly (a-T)<sub>24</sub> tract and an M1 restriction enzyme site were engineered at the 3'-end of the clone. The full-length clone, designated pVEIC-92, and its precursor intermediates were constructed in the pUC18 plasmid (Fig. 3). Portions of two clones, pTRD-26 and pTRD-30, derived from TRD virus RNA which were identical in sequence to TC-83 in these regions, were used in the construction of pVEIC-92. Several splices, including those involving TRD cDNA clones, were performed individually to build up the entire sequence step at a time, thus avoiding errors which might be incorporated in the clone. An extra G nucleotide was mistakenly engineered into the virus sense strand between the T7 promoter and the 5'-terminal A nucleotide of the VEE virus genome. This error has subsequently been removed. Three silent mutations

(A-to C, C-to-A, and T-to-C at nucleotide positions 1613, 1616 and 1619) were engineered into the full-length clone to provide markers for viruses derived from the full-length clone.

The 5'-capped infectious VEE viral RNA transcripts were synthesized *in vitro* from the pVEIC-92 full-length clone opened at the unique M1 site, with T7 RNA polymerase. The transcribed viral RNA product diluted in phosphate-buffered saline containing lipofectin and transfected into slightly subconfluent monolayers of BHK cells. Monolayers were observed for the development of cytopathic effects and the virus containing culture fluids harvested. Transfection of BHK-21 cells with RNA transcripts of the pVEIC-92 and TRD clones resulted in cytopathology and production of viruses having characteristic plaque morphology and antigenic markers (Tables 1 and 2).

### III. VIRULENCE ANALYSIS OF VEEIC RECOMBINANT VIRUSES

Infectious full-length cDNA clones of VEE TC-83 and Trinidad Donkey (TRD) viruses have been assembled in plasmid pUC18 by ligation of previously characterized endonuclease restriction fragments (Fig. 4). Infectious VEE RNA was prepared from the double-stranded full-length cDNA clones by digestion with M1 to open the clone at the 3'-end of the VEE virus genome facilitating transcription of the DNA with T7 DNA dependent RNA polymerase. Transcribed virus RNA was used to transfect BHK-21 cells using lipofectin. Virus specific cytopathic effects were usually evident within 48 hours after transfection, at which time the culture fluids containing virus were harvested. To determine which amino acid changes in the virulent TRD virus genome were responsible for attenuation, recombinant TC-83 viruses with specific TRD sequences were constructed. To construct recombinants TRD, virus-specific cDNA regions were ligated into the VEIC-92 TC-83 virus cDNA backbone or VEIC-92 cDNA modified by *in vitro* mutagenesis. Infectious virus RNA was prepared from each of the recombinant clones and used to transfect BHK-21 cells. Recombinant VEE virus was recovered from the transfected cells 24 hours post-infection.

The virulence of each recombinant virus was analyzed in outbred Swiss mice 21 to 24 days of age. Mice were challenged intraperitoneally with 200 plaque forming units (PFU) of recombinant virus and observed 24 days for signs of encephalitis and/or weight loss (Table 4). Mice challenged with high doses of virulent TRD virus and recombinants VEIC-102, VEIC-103, VEIC-109 and VEIC-113 died of encephalitis within seven days following infection. The average survival time (AST) for mice infected with recombinants VEIC-102, VEIC-103 and VEIC-109, however, was three days longer than for mice inoculated with VEIC-113 or the TRD control. Recombinants VEIC-105, VEIC-107 and VEIC-111 produced fatal infections in 37.5%, 62.5% and 75.0 % of mice, respectively. Animals inoculated with these recombinants had an AST of ten days. VEE recombinants VEIC-92, VEIC-101, VEIC-104, VEIC-108 and TC-83 were avirulent for weanling mice.

The virulence phenotypes of VEE recombinants VEIC-109 and VEIC-113 are similar in both six-week and three-week-old mice (Table 5). The genome of recombinant VEIC-113 is equivalent to wild-type TRD virus except for a sequence error in the TRD-sense gene nsP3 (Fig. 4). Mung bean nuclease was used to remove the single-stranded 5'-overhang sequence from the cDNA template following restriction endonuclease digestion. Viruses obtained after mung bean

nuclease treatment are designated "M" (Table 6). Results of these experiments revealed that the four extraneous 5'-end residues resulting from the M1 digestion did not attenuate virus derived from clones VEMC-109, VEMC-109B, VEMC-113 or VEMC-113B. Survival of mice challenged with the virus derived from the mung bean nuclease cDNA templates were the same as those of the parent viruses. Intraperitoneal challenge of three week-old mice with 10 to 50 PFU of three independently transcribed "IC-109" pVEMC genomes 109, 109B and 109BM or pVEEMC-113 cDNA clones 113, 113B and 113BM resulted in 100% mortality within seven days (Table 3). Viruses VEMC-102, VEMC-103, VEMC-105, VEMC-107, VEMC-111 and VEMC-112 were moderately lethal for three-week-old mice, but were less virulent for six-week-old mice (Tables 4 and 5).

Both VEMC-109 and VEMC-113 recombinants possess the TRD-sense 5'-noncoding region and all of the virus structural genes (Fig. 4). Recombinant virus IC-113 also contains the TRD-sense nsP3 sequence. Presence of the TRD specific nonstructural nsP3 sequence of TRD virus did not enhance the virulence of IC-113 relative to that of IC-109 (Tables 4 and 5). Recombinant virus VEMC-110 containing only the TRD-sense nsP3 region of the virulent TRD virus is avirulent for suckling mice. The combination of the TRD-sense nsP3 and 5'-noncoding regions in recombinant virus VEMC-112 resulted in intermediate virulence with an AST of 11 days for three-week-old mice (Table 6) and a 12.5% mortality with 9-day AST in six-week-old mice (Table 4). The combination of the TRD-sense nsP3 and structural gene regions in recombinant VEMC-111, however, attenuated the virus relative to VEMC-102 which contains only the TRD-sense 26S mRNA region. The relative contribution of the conservative nsP3-260 Ser to Thr mutation to virulence appears unclear at this time.

Virulence of attenuated virus VEMC-92 was not directly affected by the insertion of the TRD-sense 5'-noncoding region in construction of recombinant VEMC-108 which remained avirulent. Insertion of TRD virus structural genes alone, however, clearly enhance virulence. Fifty to one hundred percent of three-week-old mice challenged intraperitoneally with VEMC-102 died 9 to 10 days post-infection. The virulence of IC-102 is influenced by both the age of mice used and the dose of virus used to challenge the animals (Tables 4 and 5).

Recombinants VEMC-103, VEMC-104, VEMC-105 and VEMC-107 were modified to define more precisely the structural gene sequences involved in virus virulence. Virulence of recombinant VEMC-103, which contains the TRD E2 gene, and VEMC-102, which contains both the TRD E1 and E2 genes, is identical. The contribution of genes in the 26S subgenomic region to virulence therefore resides in the E2 envelope glycoprotein. Construction of recombinants VEMC-104, VEMC-105, and VEMC-107 facilitated identification of those loci in the E2 gene that contribute to virulence. The amino-proximal amino acids (K and H) in the TRD E2 sequence do not contribute to virulence as recombinant VEMC-104, which contains these two amino acids, is avirulent. The carboxyl-proximal T-V-T amino acid sequence does, however, influence virulence of the virus as recombinant VEMC-105 is nearly as lethal as recombinants VEMC-102 and VEMC-103 containing all of the TRD E2 gene. Recombinant VEMC-105 killed 62.5% of the three-week-old mice challenged with the virus in approximately nine days (Table 4). Six week-old mice were challenged with recombinants VEMC-102 and VEMC-105 containing the E2 T-V-T sequence and all of the TRD structural genes, respectively (Table 1). The virulence of these two recombinants for three-week and six-week-old mice were identical (Tables 4 and 5). The E2 glycoprotein of TRD virus contains a Thre residue at position 102, which appears to be a dominant but not unique component of the T-V-T triad conferring virulence to

the virus. Substitution of a single amino acid at position 102 in the E2 envelope protein of TC-83 generating recombinant VEIC-107 killed 37.5% of the three-week-old mice challenged (Table 4). However, IC-107 is completely avirulent for six-week old mice (Table 4). It appears that one or both of the other amino acids in the E2 glycoprotein T-V-T triad contributes to the virulence of TRD virus.

To determine the contribution which the TRD 5'-noncoding region makes to virulence and the synergism between this region and the TRD-specific E2 glycoprotein sequences, the TRD 5'-specific noncoding regions were substituted for the cognate region of recombinant viruses VEIC-101, VEIC-103, VEIC-104, VEIC-105 and VEIC-107. These reconstructions produced constructs VEIC-114, VEIC-115, VEIC-116, VEIC-117 and VEIC-118 (Fig. 4). Recombinant VEIC-109 had previously been constructed as the cognate of VEIC-102 (Fig. 4). The results of virulence testing of these viruses in three-week-old mice are shown in Table 5. The TRD-specific 5'-noncoding region in VEIC-115 (TRD-sense 5'-noncoding plus the TRD E2 genes), VEIC-117 (TRD-sense 5'-noncoding plus the E2 glycoprotein amino acid triad T-V-T) and VEIC-118 (TRD-sense 5'-plus the E2 amino acid change at position 120 to Thr) enhanced virulence relative to the recombinants which did not have the TRD 5'-noncoding regions VEIC-103, VEIC-105 and VEIC-107, respectively. Recombinant VEIC-104, which contains the E2-amino-proximal K-H amino acid residues of TRD virus, was not synergistically enhanced by the addition of TRD-specific 5'-noncoding region (VEIC-116).

The weight of individual animals was determined following primary virus challenge to determine if illness and weight loss could be correlated with virus virulence (Tables 4 and 5). Only those mice which received 50 to 1,000 PFU of virulence or moderately virulent virus were observed to lose weight or show signs of illness. All mice which survived primary challenge with any of the recombinant VEIC viruses developed protective antibody responses as shown by survival following secondary challenge with VEE TD virus 23 to 33 days after primary challenge (Tables 4 to 5). Those animals which survived primary challenge with recombinant virus developed VEE virus specific antibody, as indicated by ELISA testing of pooled mouse sera (Table 7). ELISA titers following secondary TRD virus challenge were similar to those observed after primary VEIC virus challenge. The lack of boost in ELISA titer was interpreted to indicate that the mice were immunized by the primary VEIC virus challenge and that neutralizing antibody had prevented replication of TRD virus following secondary challenge.

The results of peripheral virulence testing of VEIC viruses in three-week-old mice are summarized in Table 8. The VEIC viruses are listed in descending order of virulence. The structural gene contribution to virulence appears to be localized in the TRD E2 amino acid triad T-V-T (E2 glycoprotein amino acid positions 120-192-296). All constructs containing this triad are at least moderately virulent, as are recombinants with the TRD-specific 5'-noncoding region and the nsP3 noncoding change. The TRD K-H (E2 amino acid positions 7-85) diad, on the other hand does not appear to participate in determining virulence even in presence of the TRD 5'-noncoding region (VEIC-116). E2 glycoprotein position 120 (T) appears to be an important virulence moiety as this single TRD structural protein specificity, in conjunction with the TRD 5'-noncoding region (VEIC-118), confers full or nearly full TRD like virulence to the virus. However, the TRD E2-120 Thr specificity encodes only moderate virulence in the absence of the TRD 5'-noncoding region (VEIC-107), while the T-V-T triad (VEIC-105) confers more virulence to the virus phenotype. Recombinant VEIC-102, VEIC-103 and VEIC-105 virus challenges in mice resulted in similar AST. A combination of two, or perhaps all three, residues

of the E2 glycoprotein T-V-T triad comprise the critical structural elements to determine VEE virus virulence.

In summary, the molecular determinants of VEE TRD virulence are encoded in constellations of genetic determinants dominated by the 5'-noncoding region and the T-V-T amino acid triad in the E2 envelope glycoprotein. The TRD-specific E2 structural component(s) confers moderate virulence in the absence of the 5'-noncoding TRD sequence specificity. The converse, however, is not true. The TRD 5'-noncoding specificity alone (VEIC-108) is avirulent. The mechanism which confers virulence *in vivo* to VEE virus is probably dual in nature. Structural determinant(s) may be involved in host cell tropism, interaction with the immune system, and/or virus maturation. The TRD 5'-noncoding specificity may modulate virus replication and enhance the virulence potential of any downstream TRD amino acid specificities. Differential levels of virus-specific RNA synthesis during the early stages of TRD and TC-83 virus replication have been previously reported (Mecham and Trent, 1983). Since the average survival time of mice challenged with recombinants VEIC-109 or VEIC-113 are longer relative to that observed with type viruses, it is possible that amino acid differences in nsP3 (position 260) or nsP2 (positions 16) may contribute to the virulence phenotype of the virus.

Recombinant VEIC-101, identical to VEIC-102 except for the incorporation of a cDNA "artifact" at E2 position 209 (a Glu to Lys error in the TRD cDNA clone), has contributed to our understanding of virulence. First, the location of the artifact further defines the E2 glycoprotein T-V-T triad region as a biologically important locus, as indicated by virulent VEIC-102 and completely avirulent VEIC-101. Secondly, VEIC-101, which may be considered as an attenuated vaccine candidate virus, clearly demonstrates that the virulence-potentiating effect of the 5'-noncoding region must be abrogated in development of a vaccine candidate. Recombinant VEIC-101 contains the attenuating mutation artifact which directly affects virulence by changing biological functions of the E2 glycoprotein. Addition of the TRD sense 5'-noncoding region to virus VEIC-101 created recombinant VEIC-114, which was virulent for 75% of three-week-old mice with an average survival time of 9 days (Table 5).

#### **IV. COMPARISON OF THE NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES OF THE ENZOOTIC AND EPIZOOTIC VEE VIRUS STRUCTURAL GENES**

The 26S RNA regions of the genomic RNAs of VEE viruses 71-180 (VEE subtype-variant I-AB, Texas, 1971), P676 (I-C, Venezuela, 1963), 3880 (I-D, Panama, 1961), Everglades Fe3-7c (EVE, II, Florida, 1963), and Mena II (MENA, Panama, 1962) have been cloned and sequenced. Sequences of the structural genes of these five viruses are summarized in schematic form in Figure 5. The VEE TC-83 vaccine virus has been included for comparison. All nucleotide substitutions that occurred relative to the TRD virus sequence were confirmed by sequence analysis of more than one cDNA clone and/or by primer extension sequencing of the genomic RNA.

One of the most important results obtained by sequencing the structural genes of these VEE viruses is the close genetic relationship of VEE virus 71-180 and TRD viruses, isolated in Texas in 1971 and Trinidad in 1943, respectively. The nucleotide sequence of the 71-180 and TRD

virus 26s mRNAs encoding the structural genes of these two viruses differ by only 19 nucleotides. This means that there are only ten more nucleotide differences between TRD and 71-180 than there are between TC-83 and TRD virus. Even more significant is the fact that 71-180 and TRD virus structural proteins differ by only 4 amino acids -- two less than observed in comparing the TC-83 and TRD viruses. For these reasons it is highly unlikely that the 71-180 isolate is a natural IAB VEE virus variant. It is more likely that 71-180 is a recent derivative of TRD virus. Early VEE vaccines used throughout South and Central America consisted of formaldehyde-inactivated virulent TRD virus. Presence of residual live virus in such "inactivated" VEE virus vaccines has been previously documented. It is therefore highly probable that the 1969-1971 VEE epidemic resulted from multiplication of residual live virus in equines immunized with improperly inactivated TRD virus vaccine. Furthermore, other IAB associated epidemics have likely originated in the same manner. Serology, oligonucleotide fingerprint analysis, and tryptic peptide maps demonstrate that the IAB strain PTF-39, a 1969 isolate from Guatemala, is nearly identical in genome and structural phenotype to TRD virus. In light of the data now available, it seems particularly significant that no VEE IAB epizootic/endemic have occurred since the introduction of the live, attenuated TC-83 vaccine and cessation in use of the formaldehyde-inactivated virus preparations. It seems correct to conclude that VEE virus IAB epizootic/epidemics have resulted from introduction of live TRD virus into the ecosystem and that the IAB subtype virus has not evolved from enzootic IC, ID or IE viruses by mutation or genetic selection.

The sequence data describing the virus structural genes reflect the antigenic relationships established among these viruses. EVE and MENA viruses, both enzootic virus strains not associated with VEE epidemics, are distantly related to the epizootic I-AB and I-C viruses. In particular, the 26S mRNA of MENA virus shares only 77.5% to 78.5% nucleotide sequence identity and 88.6% to 90.4% amino acid sequence identity with the 26S RNAs of TRD, P676, 3880, and EVE viruses. Clearly, epizootic strains of VEE virus have not evolved by mutation and selection from either of these two enzootic subtype viruses.

Most of the nucleotide substitutions in the structural gene coding sequence of 71-180, P676, 3880, EVE, and MENA 26S RNAs are silent. Ratios of nucleotide to resulting amino acid substitutions in the translated region of the 26S mRNA were 4.0, 6.4, 9.3, 6.7, and 6.3, respectively, relative to the TRD reference virus. The E1 envelope glycoprotein genes of P676 and 3880 viruses, in particular, contained a large number of silent changes. In the E1 protein of P676, 35 nucleotide substitutions resulted in a single amino acid substitution, while 62 nucleotide substitutions resulted in 3 amino acid changes in the E1 of 3880 virus. This high degree of genetic variability, in association with minimally altered primary amino acid phenotype in the E1 glycoprotein of VEE viruses and other alphaviruses, indicates that conservation of the primary sequence, which determines the secondary and tertiary structure of the E1-glycoprotein, is critical to the structure of the protein spike and its role in the biology of the alphaviruses.

The E2-glycoprotein of VEE virus elicits both neutralization and hemagglutination-inhibition antibodies and is largely responsible for attachment of virus particles to the cellular plasma membrane. The amino acid sequence of this protein is highly variable among the serologically distinct alphaviruses. Although EVE and MENA VEE viruses contain a large number of amino acid substitutions in the E2 protein relative to TRD virus, the E2 glycoprotein of 71-180, P676, and 3880 viruses contained only 2, 7, and 8 amino acid substitutions in the ectodomain portion

of the protein. Thus, the protein sequence phenotype of E2, and entire 26S polyprotein precursor, of 3880 virus and the epizootic strains TRD and 71-180 are very similar.

Shared amino acid differences relative to the reference TRD virus are shown in Fig. 5. All amino acid positions that were identical in at least two of the non-reference virus sequences are shown. P676 and 3880 amino acid residues at positions C-76+100, E2-57, and E1-9+103+112+142+347 were identical to the VEE IAB residues at the corresponding positions, while those at C-62 and E2-75+147 were identical to the cognate residues of EVE and MENA. P676 residues E2-192+320 and E1-14 were identical to IAB, while the cognate residues of 3880 virus were identical to EVE and MENA. P676 and 3880 residue E2-214 was identical to MENA. The E2 transmembrane domains of both P676 and 3880 viruses were more similar to those of EVE and MENA than to those of IAB viruses. EVE and MENA viruses were identical in 18 of the 23 amino acid positions shown in Fig. 5. Thus, the epizootic strain P676 and enzootic 3880 viruses show complex relationships with subtype VEE IAB enzootic strains and enzootic II and IE strains. To evaluate the biological significance of specific nucleotide and amino acid differences in the ID virus 3880, the structural region of the virus could be cloned into the VEEIC full-length TC-83 clone and mutated to alter amino acids E2-75, 147, 192, 214 and E1-14 to determine the effect(s) of these amino acid changes on the biology of ID virus.

An Asn-to-Tyr mutation in the E2 protein of EVE virus resulted in the loss of the potential glycosylation site at amino acid position TRD E2-291 (Table 1). Amino acid substitutions in the E2 envelope glycoproteins of P676, 3880, and MENA viruses have resulted in the loss of the TRD E2-212 glycosylation site. Although 3880, EVE, and MENA virus E2 proteins contained an amino acid substitution at the TRD E2-318 glycosylation site, the substitution was in all three cases a conservative Thr-to-Ser substitution that preserved the Asn-linked glycosylation site (Asn-X-Ser or Thr, where X = any amino acid).

The amino acid sequence of the capsid protein region comprising amino acids 51 to 117 is highly variable (Fig. 3). The hydrophilic, Pro-Lys-Arg-Gln-Gly rich region is thought to be intimately associated with the genomic RNA in the mature nucleocapsid. Similarly, conservative amino acid substitutions that preserve the general domain character are readily permitted in the transmembrane and cytosol domains of the E2 envelope glycoprotein (Fig. 1).

## V. EXPRESSION OF VEE VIRUS STRUCTURAL GENES BY RECOMBINANT VACCINIA VIRUS

Three vaccinia\VEE recombinant viruses have been constructed: VAAC\TC-5A, expressing the structural genes of TC-83, and two vaccinia TRD recombinant viruses, VACC\TRD-1A and VACC\TRD-20A, both expressing the structural genes of TRD virus. To confirm insertion of VEE cDNA into the vaccinia thymidine kinase gene, DNA purified from wild-type or recombinant vaccinia virus was digested with HIND III for southern blot hybridization. The 5 kb Hind III J fragment of vaccinia virus was not present in the recombinant virus DNA; however, a new band of about 9 kb was present in the gel pattern. This new fragment is of the predicted size and mobility of the HIND III J fragment containing the VEE cDNA fragment plus the 7.5 K promoter from the chimeric plasmid.

Immunoblotting of VACC\TC-5A, VACC\TRD-1A and VACC\TRD-20A virus infected cells lysate clearly showed polypeptide bands which comigrate with the VEE virus capsid protein. Lysate of cells infected with the three recombinant viruses also contained polypeptides that comigrated with the E1 and E2 envelope glycoproteins. From these experiments it was concluded that the structural proteins of VEE virus were synthesized and processed in cells infected with the recombinant viruses.

Expression of VEE proteins in recombinant virus-infected cells was also demonstrated by fluorescent antibody (FA) analysis. VEE virus antigens were detected in acetone-fixed VEE TC-83 or recombinant VACC\TC-5A virus infected cells. The level of VEE structural proteins expression in the recombinant virus-infected cells was usually lower than in cells infected with VEE virus.

Surface expression of VEE virus antigens demonstrated by FA analysis of unfixed cells was also lower in recombinant infected cells than on the surface of cells infected with wild type VEE virus.

Antiserum prepared against vaccinia virus showed positive FA only with vaccinia and VACC\VEE virus-infected cells. Polyvalent anti-TC-83 virus ascitic fluid reacted with cells infected with VEE virus or VACC\VEE recombinant viruses (Table 9). The reactivity of monoclonal antibodies with different E1 and E2 epitope reactivities was investigated. Monoclonal E2 antibodies generally reacted similarly with cells infected with VEE or VACC\VEE virus (Table 9). In particular, the TC-83 specific antibody 5B4D-6 (epitope E2a) reacted more strongly with recombinant VACC\TC-5A infected cells than with VACC\TRD-1A infected cells. Monoclonal antibodies 1A4A-1 (E2c), 1A4D-1 (E2f) and 1A3A-9 (E2g) showed positive FA reactivities with cells infected with both VEE virus and the recombinants. Monoclonal antibody IA3A-5 (E2e) reacted poorly with recombinant virus infected cells. Antibody 1A6C-3 (E2d), which reacted poorly with cells expressing TC-83, TRD or VACC\TRD-1A virus antigens, failed to react with VACC\TC-5A infected cells. The A3B-7 (E2h) antibody failed to react with VACC\TRD-1A recombinant-infected cells, while reacting strongly with cells infected with VACC\5A, TC-83 or TRD virus.

Of the four E1 glycoprotein epitopes identified on VEE virus, only E1b and E1d epitopes were detected in recombinant virus-infected cells (Table 9). Although the E1a and E1c epitopes were detected in VEE TC-83 and TRD virus-infected, acetone-fixed cells, these epitopes were not evident by FA in recombinant VACC\TC-5A or VACC\TRD-1A infected cells.

## **VI. MURINE HUMORAL AND CELL MEDIATED IMMUNE RESPONSES TO IMMUNIZATION WITH RECOMBINANT VACCINIA\VEE VIRUS**

### **A. Humoral responses to vaccination.**

Responses of AJ mice to immunization with recombinant VACC\TC-5A virus are shown in Table 10. Mice were immunized i.p. with  $10^4$  or i.d. with  $10^8$  PFU of VACC virus or  $10^5$  to  $10^7$  recombinant vaccinia virus VACC\TC-5A. Animals were bled 3 weeks later to determine prechallenge antibody titers and again two weeks after virulent TRD



virus challenge. Only those mice receiving TC-83 or VACC\TC-5A virus vaccine were protected from virulent TRD virus challenge. Mice which received  $10^5$  or  $10^7$  PFU of VACC\TC-5A virus developed similar levels of prechallenge HI or ELISA antibody. Animals which were given  $10^5$  PFU of VACC\TC-5A, however, developed no measurable or reduced HI or ELISA antibody. Reliable immunity to VEE virus was obtained only in those animals given a VACC\TC-5A dose of  $1-7$  PFU. Animals given the TC-83 attenuated virus developed antibody titers which were much higher than those observed in animals given the recombinant virus. Although post-challenge VEE virus viremias were not determined, post-challenge antibody titers showed a dose-related boost in the VACC\TC-5A immunized animals that was much greater than that shown in the TC-83 mouse (Table 10). This suggested that a lower level of immunity in recombinant-immunized mice probably was less effective in inhibiting replication of the challenge virus.

Prechallenge PRNT antibody titers in individual animals receiving various doses of recombinant vaccine showed a dose-related response (Table 11). In all cases, the response to the vaccinia recombinant was lower in animals which received the TC-83 vaccine. Three mice immunized with the TC-5A recombinant did not develop PRNT antibody and did not survive virus challenge.

AJ, mice immunized with  $10^7$  PFU of VACC\TC-5A virus, were bled at 18, 33 and 86 days post-immunization to determine the duration of VEE virus immunity (Table 12). Neutralizing antibody titers elicited by the VACC\TC-5A vaccine were stable for at least 3 months.

PRNT antibody titers were determined for both Swiss NIH and C3H mice which received  $10^8$  PFU of VACC\TC-5A virus by the i.d. route. The recombinant VACC\TC-5A vaccine elicited a weaker immune response in outbred Swiss NIH mice than in the inbred C3H or AJ mice.

#### **B. Cross-protection afforded mice immunized with the recombinant TC-5A virus.**

High-titered mouse anti-TC-83 or anti-VACC\TC-5A sera were pooled and tested for their ability to neutralize viruses representing different antigenic subtypes of VEE virus (Table 13). The PRNT cross reactivities of anti-TC-83 and anti-VACC\TC-5A were similar. Both sera neutralized epizootic VEE viruses IAB and IC efficiently. Subtype ID, IE and type II viruses were neutralized only at high antibody concentration. Anti-VACC\TC-5A serum PRNT titers were lower than those of the anti-TC-83 serum. Neither TC-83 nor VACC\TC-5A antiserum neutralized VEE subtype IV or western equine encephalitis virus.

In vitro neutralization test information indicated that the recombinant VACC\TC-5A vaccine should protect mice from challenge with subtype IA-D viruses (Table 13). To evaluate this, C3H and NIH Swiss mice were immunized with TC-83 or the vaccinia recombinant VACC\TC-5A and challenged with TRD virus (Table 14). Both TC-83 and VACC\TC-5A vaccines induced protection in mice from lethal infection with IAB, IC and ID viruses. Although protection of Swiss NIH mice against infection with subtype

II virus challenge was ambiguous, the C3H mice were clearly protected by both vaccines from challenge with subtype II virus. High doses of TRD and subtype IC viruses caused three fatalities in recombinant immunized mice, but none in the TC-83 immunized Swiss mice (Table 14).

To determine the level of protection afforded by immunization with recombinant vaccinia virus to aerosol challenge with TRD virus, mice were immunized with VACC\TC-5A virus and challenged with virulent TRD virus. The VACC\TC-5A recombinant effectively protected mice against peripheral infection but not intranasal challenge with TRD virus. However, the TC-83 vaccine protected mice from TRD virus challenge when given by either route (Table 15). Five of twenty TC-83 immunized C3H mice succumbed to intranasal TRD virus challenge. The six VACC\TC-5A recombinant immunized mice which survived intranasal challenge became ill after challenge, but showed no signs of paralysis. The six AJ survivors recovered slowly and were not normal even after three weeks post-challenge.

### C. Murine helper T-cell responses to VEE vaccine candidates.

Studies of cell-mediated immunity to alphaviruses have defined the limits of genetic restriction for cytotoxic T lymphocytes (CTLs) within the murine major histocompatibility complex (HMC) (Mullbacher and Blanden, 1978), recognized the cross-reactive nature of the alphavirus CTL (Mullbacher *et al.*, 1979; Wolcott *et al.*, 1982) and found that protection of mice from virulent virus after adoptive transfer of immune splenocytes is T-cell dependent (Rabinowitz and Adler, 1973; Peck *et al.*, 1975). Indirect evidence for the importance of T-helper (Th) cells in the immune response to alphaviruses was reported by Burns *et al.* (1975). In this study, Sindbis (SIN) virus elicited only a transient T-cell-independent IgM response in nude mice; however, only T-cell-competent, normal litter mates produced IgG and mounted a secondary immune response.

Lymphocyte transformation assays with SIN and VEE virus indicate that T-cells are necessary to induce antigen-specific proliferation (Adler and Rabinowitz, 1973; Griffin and Johnson, 1973; Marker and Ascher, 1976). A virus-specific proliferative response was noted in limited studies with Getah, Ross River, and SIN viruses (Asakov *et al.*, 1983). We have investigated the antigen-elicited T-cell proliferation by virus-primed T lymphocytes incubated *in vitro* in the presence of syngeneic stimulator cells previously exposed to virus. Our results indicate that after inoculation of mice with VEE virus, T-cells of the Th cell phenotype which secrete interleukin-2 (IL-2) are generated against the dominant epitopes that represent virus-specific amino acid sequences and provide help to virus-specific B cell epitopes.

Antigen processing cells (APC) were infected with various concentrations of VEE TC-83 virus to determine the optimal amount of antigen required to induce maximum virus-specific lymphoproliferation. Maximum stimulation of VEE TC-83 virus-primed T cells occurred between 5 and 50 micrograms of virus for ten million splenocytes. Nylon wool (NW) enriched T cells from virus-primed mice proliferated in the presence of phytohemagglutinin to levels comparable to optimal induction levels by purified virus.

Virus stimulated the proliferation of T cells in the absence of irradiated APC, at reduced levels, indicating that NW chromatography did not remove important APC.

To determine the phenotypes of T cells involved in proliferation, NW enriched T cells from VEE TC-83 or SIN virus-primed mice were treated with T cell marker antisera plus complement (Table 16). Only complement control or cells treated with anti-Lyt-2.1 antibodies proliferated in response to VEE or SIN virus stimulation. Similar results were obtained when measuring the proliferative response of cells to VEE virus after a culture period of 5 days. Depletion procedures indicated the cell populations involved in proliferation were of the Th cell phenotypes THy-1(+), Lyt 1(+), 2(-), and L3T4(+). Flow immunocytometry confirmed that most of the Thy-1 positive cells were of the L3T4 phenotype (Table 17).

Specificity of the cellular and humoral response to VEE virus was evaluated at various times post-infection using VEE, western equine encephalitis (WEE), and eastern equine encephalitis viruses (EEE) (Fig. 6). Lymphoproliferative assays were done to evaluate mice primed to any one virus. For VEE virus-primed cells, the antigen stimulated proliferative response was specific for VEE virus. The maximum response occurred at day 14, along with a cross-reactive response to EEE virus. At day 54 post-immunization, there was a good memory response to the VEE virus, but the EEE response of the cells had disappeared. Th cells in the assay did not respond to WEE virus throughout the entire period of observation. The response of Th cells from EEE and WEE immunized mice was virus-specific (Fig. 6).

When sera from all mice used in the proliferation studies were examined by ELISA, the results paralleled those seen in the proliferation assays. There was a significant homologous antibody response to all viruses used for immunization with a low level cross reactivity seen between EEE and VEE viruses. The WEE virus B cell response was virus-specific, and antibodies in mice immunized with EEE or VEE virus did not cross react with WEE virus.

To analyze further the blastogenic response among more closely related alphaviruses, VEE subtype viruses were used to stimulate VEE TC-83 virus-primed cells (Table 18). The magnitude of the Th cell response correlated well with the B cell antigenic relatedness except for Everglades virus (subtype II). However, Everglades virus is neutralized well by antisera prepared to the purified E2 glycoproteins of TC-83 (IA) and PTF-39 (IB) viruses (Kinney et. al., 1983).

Elevated levels of IL-2 were used as an indication of antigenic stimulation of Th cells (Table 4). Supernatants from proliferation assays were harvested 72 hours after antigenic stimulation and assayed for IL-2 activity with CTLL-2 cells. Levels of IL-2 were elevated only when virus-primed T cells were stimulated with homologous virus. In general, there was a direct correlation between a positive lymphoblastogenic response and elevated levels of IL-2.

## VII. PRIMATE HUMORAL RESPONSES TO IMMUNIZATION WITH VEE VIRUS CANDIDATE VACCINES

Two experimental vaccine trials in primates have been completed. Animals in the first group were inoculated intradermally with  $10^5$  PFU of recombinant VACC\TC-5A virus. Control animals were immunized with TC-83 or C-84 VEE vaccines or vaccinia virus. Immunized animals were monitored for antibody production and peripherally challenged with  $10^5$  PFU of virulent subtype VEE virus 71-180. Animals in the second group were similarly immunized, but challenged intranasally with  $10^3$  PFU of 71-180 virus. Four of the six animals immunized with TC-5A vaccine developed neutralizing antibody titers. When these animals were challenged with virus, 1 of 3 peripherally challenged animals demonstrated a low-level transient viremia, while 1 of 3 intranasally challenged animals developed a significant viremia. Viremia titers correlated with the lack of neutralizing antibody following VACC\TC-5A immunization. Leukopenia was observed only in the intranasally challenged viremic animals.

All animals immunized with control vaccines (TC-83 and C-84) developed neutralizing antibodies. One C-84 immunized animal developed significant leukopenia and viremia following intranasal challenge. All TC-83 immunized animals were solidly protected. All TC-5A and vaccinia immunized controls developed vaccinia neutralizing antibodies. All vaccinia immunized and non-immunized control animals developed significant viremia and leukopenia following VEE virus challenge.

Because VEE virus challenge by the subcutaneous and intranasal routes is non-lethal, post-challenge antibodies were monitored. Four of 6 VACC\TC-5A immunized animals demonstrated an anamnestic response to VEE virus following virus challenge. Two of the animals had stable antibody titers. Three of the six C-84 immunized animals demonstrated an anamnestic response. The anamnestic responses could be due to low levels of virus replication following challenge; however, there was no correlation between low-level pre-challenge neutralization antibody titers and subsequent secondary antibody response. None of the TC-83 immunized animals demonstrated an anamnestic response to VEE virus challenge. The lack of a secondary antibody response in TC-83 immunized animals was probably due to limited virus replication following challenge.

In an attempt to identify neural invasion in intranasally challenge animals, we have analyzed the IgM and IgG antibody levels in cerebrospinal fluid. VEE virus-specific IgM antibody could be detected only in the vaccinia immunized control animals following virulent virus challenge. IgM antibody was detected in the cerebrospinal fluids of 2 of the three 3 TC-5A vaccinated animals and 1 of 3 animals immunized with C-84 vaccine. IgM and IgG class antibodies to VEE virus were present in all animals immunized with TC-83, C-84 or the VACC\TC-5A vaccines.

## VIII. THE IMMUNE RESPONSE OF EQUINES TO IMMUNIZATION WITH TC-83 AND RECOMBINANT VACCINIA\VEE TC-5A VIRUSES

### A. Humoral responses to immunization and virus challenge.

A time line showing the immunization and challenge schedule for equines immunized with TC-83, wild-type vaccinia, and the vaccinia recombinant TC-5A are shown in Figure 7. All horses were initially immunized on December 21, 1988. Four horses were reimmunized with TC-5A on March 22, 1989, 91 days after the initial immunization. All animals were challenge with virulent VEE virus strain 71-180 on April 12, 1989, 112 days after the initial immunization.

The immune response of horses to immunization with TC-83 and recombinant vaccinia virus TC-5A prior to virulent virus challenge has been analyzed in detail. Horses were immunized with  $1 \times 10^8$  plaque forming units (PFU) of wild-type or recombinant vaccinia viruses by intradermal route. All animals given TC-83 or recombinant TC-5A developed neutralizing and ELISA reactive antibodies after the primary immunization (Table 19). Serum from both horses (animals No. 70 and 182) which received TC-83 vaccine had very high ELISA titers and neutralization titers of 64. The four horses immunized with TC-5A (No. 14, 77, 134, and 160) developed low ELISA antibody titers and neutralization titers of 8 to 16. Neutralizing antibody titers of sera from these same horses to the virulent VEE virus 71-180 and vaccinia virus were determined (Table 20). Sera from the TC-83 immunized horses immunized VEE virus 71-180 at a lower titer than TC-83 virus (Table 5) and did not neutralize vaccinia virus. Antibodies in sera from the two horses immunized with vaccinia virus (No. 84 and 86) neutralized only vaccinia virus. Vaccinia antibody neutralization titers of sera from the TC-5A immunized horses ranged from 64 to  $\geq 128$ ; however, the sera did not neutralize the equine virulent VEE virus. Following secondary immunization with TC-5A, all horses developed significantly higher ELISA and neutralization antibody titers, indicating that the primary immunization had sensitized the animals to VEE virus antigen (Tables 20 and 21).

Kinetics of the equine immune response following immunization was analyzed, revealing that the two TC-83 immunized animals had VEE reactive antibodies seven days after immunization. Antibodies to vaccinia virus were first detected in sera from animals immunized with wild-type vaccinia or TC-5A viruses on day seven (data not shown). Seven days following secondary immunization with TC-5A recombinant virus, antibodies to VEE were demonstrated in all animals. By day 14, ELISA titers had reached levels of 6,000.

The cross-reactivity of antibodies in sera of horses immunized with VEE-TC-83 and TC-5A with other subtype VEE viruses, WEE, and EEE viruses is shown in Table 21. Horse 70, immunized with TC-83 virus, was negative to all alphavirus antigens prior to immunization; however, horse 182 was positive to both WEE and EEE viruses by ELISA. This animal had tested negative to VEE, WEE, and EEE by serum dilution neutralization prior to TC-83 immunization. After TC-83 immunization, serum from horse 182 reacted by ELISA with VEE subtype viruses I to IV. Animal 70, initially negative to all alphaviruses tested, reacted with all of the subtype VEE viruses tested and

EEE virus. The four horses immunized with recombinant virus TC-5A were negative prior to immunization and following immunization were positive to all subtype VEE virus antigens tested. Thus, the cross-reactivity of ELISA antibodies following immunization with the recombinant antigen and TC-83 were similar. Horse No. 134, whose sera was positive by ELISA to WEE prior to immunization with TC-5A, developed antibodies to EEE virus after immunization.

The horses were challenged with 10,000 plaque forming units of virulent VEE 71-180 virus and observed 21 days. Antibody titers to VEE virus after challenge remained stable in the TC-83 immunized animals. This indicated TC-83 immunization provided solid immunity to virus challenge and that insufficient antigen was produced following challenge to stimulate the immune response. The antibody titers in horses immunized with TC-5A showed modest increases following challenge. The two horses which received wild-type vaccinia virus (No.84 and 86) developed ELISA reactive antibodies by the sixth day following challenge; however, their sera did not neutralize VEE virus. When kinetics of the antibody response to VEE were examined in detail, it was determined that the ELISA antibody titers from the TC-83 and TC-5A animals were stable over the 21-day period following virulent virus challenge.

#### **B. Cell mediated responses to immunization and virus challenge.**

Equine bloods were collected in heparin, and the peripheral blood mononuclear cells (PBMC) were separated over ficoll-paque. Blastogenic assays were set up with  $2.5 \times 10^8$  cells. Purified virus at a concentration of 1 microgram per well was used to stimulate lymphocytes. Assays were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  and pulsed with  $^3\text{H}$ -thymidine and harvested at different time intervals over a three- to five-day period.

Representative proliferative response results for all of the horses in this study with homologous and heterologous alphaviruses are shown in Table 22. The CPM data have been converted to stimulation indices for comparative purposes. PBMC from VEE\TC-83 virus immunized horses were positive after the primary immunization. Two of the four vaccinia recombinant TC-5A immunized horses (No. 77 and 134) were positive after a single inoculation of virus. After secondary immunization with TC-5A, horse number 14 became positive; however, horse 160 did not convert until challenge with virulent virus. Both of the vaccinia control horses showed a positive proliferative response on day six following virus challenge. Proliferation results with VEE virus 71-180 correlated with the TC-83 virus stimulation data confirmed a close antigenic relationship between the two viruses. The heterologous responses to WEE virus may indicate that either cross-reactive Th-cell epitopes are involved, or that the horses had been exposed to WEE virus or WEE virus vaccine some time prior to their immunization with VEE virus antigens.

**IX. CHALLENGE OF EQUINES IMMUNIZED WITH RECOMBINANT VEE/VAC, VEE-TC-83, AND WILD-VACCINIA VIRUS WITH EQUINE VIRULENT VEE/71-180**

**A. Clinical and pathological responses.**

The two mares immunized with wild-type vaccinia virus and challenged with VEE 71-180 virus became depressed and anorectic beginning approximately 30 hours after challenge. Except for a brief period of appetency and heightened awareness on day three, the mares became progressively depressed, reluctant to move and ataxic. Six days following challenge, these animals were standing, but severely somnolent and minimally responsive and were euthanized. These clinical symptoms and signs of disease reflected changes in body temperature, hematologic parameters, and development of a viremia (Fig. 24). Both mares underwent a biphasic increase in body temperature that peaked in the evenings of days 1 and 4, and both mares were viremic on days 1 through 3 following inoculation of challenge virus. During the period following virus challenge, both vaccinia-immunized mares had significant decreases in total numbers of blood leukocytes, erythrocytes and platelets. The only abnormalities observed in serum chemistries were terminal elevations in glucose and aminotransferase, particularly evident in one mare.

Gross lesions were not observed in tissues of the two vaccinia-immunized mares after VEE virus challenge (Table 24). Histologically, each displayed severe, nonsuppurative meningoencephalitis characteristics of infection with VEE virus. Brains of both animals had widespread lymphocytic perivascular cuffs, hyperemia and focal hemorrhage, endothelial cell swelling, areas of focal and diffuse gliosis (some with central necrosis), and mild lymphoid meningitis. Significant lesions were not observed in liver, kidney, pancreas or spleen. Lymph nodes displayed varying degrees of lymphoid hyperplasia, and the bone marrow in both animals was moderately to markedly cellular, without evidence of necrosis.

In contrast to the vaccinia-immunized control animals, none of the four mares immunized with VACC/TC-5A nor the two mares immunized with TC-83 virus vaccine displayed any signs of disease during the three weeks following challenge, with the exception of a slight elevation in body temperature on day four in two of the VACC/TC-5A immunized mares. VEE virus was not isolated from these animals at any time following challenge, and aside from sporadic fluctuations in a few hematologic parameters, none of them displayed abnormal hematologic or serum chemical abnormalities during the entire challenge period. Tissues collected at necropsy (21-23 days after challenge) revealed focal areas of mild perivascular and/or meningeal lymphoid infiltration in the brain of each horse. In the liver, mild portal lymphoid cell infiltrates were noted in all but one horse. Lesions attributable to VEE virus infection were not observed in the pancreas, kidney, spleen or bone marrow. A variable degree of lymphoid hyperplasia was observed in lymph nodes from each of these animals, consistent with recent antigenic stimulation.

## X. ANTIGENIC ANALYSIS OF THE VEE VIRUS E1 AND E2 GLYCOPROTEINS

### A. Definition of the VEE virus neutralization domain.

Stable neutralization escape variants of VEE virus have been selected with anti-E glycoprotein monoclonal antibodies (Mabs) that neutralize viral infectivity, block viral hemagglutination, and passively protect mice (Johnson et al, 1990). Nucleotide sequence analysis of the E1, E2 and E3 genes of four variants revealed a clustering of single nucleotide mutations in a domain of E2 spanning amino acids 182 to 207. The conformation of this short linear sequence affects antigenicity of the neutralization domain as reduction and alkylation of the virus disrupts binding of some E2 neutralizing Mabs. Serologic evidence for the interaction of neutralizing Mabs which define E2 epitopes using peptides from regions of the E2 protein has been obtained. Mutations in the neutralization domain of VEE virus did not alter the kinetics of binding to Vero cells. However, some of the neutralization escape variants were less virulent for mice than the parent TC-83 virus.

### B. Antigenic mapping of VEE virus constructs with MAb.

The antigenic structure of the virus derived from the pVE/IC-92 derived infectious clone viruses and viruses derived by Dr. Robert Johnston have been analyzed. Viruses grown in either Vero or CV-1 cells were tested for reactivity with a pertinent set of MABs by indirect immunofluorescence. All MAB reactivities with the recombinant viruses were as predicted. Of interest was the ability to place the E2<sup>a</sup> epitope within the first 120 amino acids of the E2.

The Johnston variant at amino acid RJ 209 lost reactivity with the VEE virus complex reactive MAB 1A3B-7 (E2<sup>b</sup>). This was not unexpected because a neutralization escape variant to this MAB had an amino acid substitution at E2 residue 207.

### C. Identification of type-specific epitopes.

We have previously identified a unique epitope (E2<sup>a</sup>) on the E2 surface glycoprotein of TC-83 virus using monoclonal antibodies. Unfortunately, we were unable to identify a reciprocal antibody reagent capable of unambiguously identifying the wild-type TRD virus parent. A subsequent study identified a MAB capable of differentiating epizootic subtype viruses (1AB and 1C) from enzootic subtype viruses (1D, 1E and 1F). In our studies with synthetic peptides derived from the deduced amino acid sequences of the envelope proteins (E1 and E2), we observed that peptides derived from the amino terminal 25 amino acids of TC-83 and TRD viruses (VE2pep01) were able to elicit virus specific responses in peptide immunized BALB/c mice. Upon subsequent sequencing of the E2 amino-terminus, it was observed that the TRD sequence was retained for subtype 1C, 1D and 1E viruses. This predicted that the TRD E2 amino-terminal antipeptide might react with all naturally occurring VEE viruses. This was indeed the case. We were able to isolate a monoclonal antipeptide antibody capable of differentiating all naturally occurring prototype VEE viruses from the TC-83 VEE virus vaccine derivative. The synthetic peptide antigen used in preparing this MAB



corresponded to the amino terminal 19 amino acids of the deduced sequence of the TRD virus E2 glycoprotein. The sequence of this peptide is STEELFKEYKLTRPMARC. The sequence of the same region on TC-83 virus differs only at residue number 7 (K->N substitution). The MAb, 1A2B-10, secreted by this hybridoma did not neutralize virus infectivity, block viral hemagglutination, or fix complement (data not shown). The MAb subclass was determined to be IgG1 in an isotyping ELISA. The specificity of this reagent is shown in Tables 8 and 9. These results indicated that MAb 1A2B-10 was not only reactive with TRD virus, but it also reacted with prototype VEE viruses representing all other naturally occurring subtypes and varieties. A similar analysis using IFA on virus infected Vero cells also demonstrated TRD virus specificity of MAb 1A2B-10. The use of a synthetic peptide derived from a region on the E2 of TRD virus known to differ in sequence from the TC-83 vaccine derivative has enabled us to derive a wild-type VEE virus reactive MAb. This MAb, when used in conjunction with the previously characterized TC-83 specific MAb, 5B4D-6, should permit rapid ELISA or IFA differentiation of VEE vaccine from wild-type strains for the first time.

#### **D. Reactivity of anti-clone sera with diagnostic VEE peptides.**

To test the specificity hypothesis of the E2 amino terminus, sera from animals challenged with the recombinant viruses were tested in ELISA with these peptides to determine their reactivity profiles. These results indicated that animals immunized with clones containing the TRD E2 amino terminal sequences (pVE/IC-102, 103 and 104) reacted specifically ( $\geq 4$ -fold titer difference) with the TRD peptide. Those animals challenged with clones containing the TC-83 E2 amino terminal sequences produced cross-reactive antibody and are consistent with previous observations. This approach may assist in modifying vaccine candidate viruses to contain marker sequences that can readily differentiate wild-type VEE infection from immunization with infectious clone derived virus vaccine.

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**XII. APPENDIX****A. Tables****B. Figures****C. Figure legends**

**Table 1. Nucleotide and amino acid sequence differences between VEE TRD and TC-83 viruses.**

<u>Position</u>		<u>Nucleotide</u>		<u>Amino Acid</u>	
<u>Nucleotide</u>	<u>Amino Acid</u>	<u>TRD</u>	<u>TC-83</u>	<u>TRD</u>	<u>TC-83</u>
3	5'-Noncoding	g	a		
1696	nsP2 - 16	C	A	Ala	Asp
4809	nsP3 - 260	U	A	Ser	Thr
8584	E2 - 7	G	U	Lys	Asn
8816	E2 - 85	C	U	His	Tyr
8922	E2 - 120	C	G	Thr	Arg
9138	E2 - 192	U	A	Val	Asp
9397	E2 - 278	U	C	Phe	Phe
9450	E2 - 296	C	U	Thr	Ile
10481	E1 - 161	U	A	Leu	Ile
10633	E1 - 211	A	U	Ser	Ser
11409	3'-Noncoding	u	-		

Table 2. Antigenic maps of the infectious clone derived viruses.

Virus	E2 <sup>a</sup>	E2 <sup>c</sup>	E2 <sup>f</sup>	E2 <sup>g</sup>	E2 <sup>h</sup>	E2 <sup>i</sup>	E1 <sup>a</sup>	E1 <sup>b</sup>	E1 <sup>d</sup>	Control
TC-83	+	+	+	+	+	-	+	+	+	-
TRD	-	+	+	+	+	+	+	+	+	-
RK-92	+	+	+	+	+	-	+	+	+	-
-102	-	+	+	+	+	+	+	+	+	-
-103	-	+	+	+	+	+	+	+	+	-
-104	-	+	+	+	+	+	+	+	+	-
-105	+	+	+	+	+	-	+	+	+	-
-107	+	+	+	+	+	-	+	+	+	-
-108	+	+	+	+	+	-	+	+	+	-
-109	-	+	+	+	+	+	+	+	+	-
-110	+	+	+	+	+	-	+	+	+	-
-111	-	+	+	+	+	+	+	+	+	-
-112	+	+	+	+	+	-	+	+	+	-
-113	-	+	+	+	+	+	+	+	+	-
RJ-76	-	+	+	+	+	n.d.	+	+	+	-
-120	-	+	+	+	+	n.d.	+	+	+	-
-209	-	+	+	+	-	n.d.	+	+	+	-

Table 3. Plaque size phenotype of cDNA-derived viruses.

Virus	Plaque ~ ~ Size (S.E.)		TRD/TC-83 Sequence Specificity ~							
			5'NC	nsP3	E2					E1
					2	6	7	8	9	
			3	0	7	5	0	2	6	1
TRD	3.8	(1.0)	g	S	K	H	T	V	T	L
TC-83	2.2	(0.2)	a	T	N	Y	R	D	I	I
VE/IC-92	2.1	(0.3)	•	•	•	•	•	•	•	•
VE/IC-101 <sup>^</sup>	1.5	(0.3)	•	•	K	H	T	V	T	L
VE/IC-102	4.2	(0.8)	•	•	K	H	T	V	T	L
VE/IC-103	4.2	(1.0)	•	•	K	H	T	V	T	•
VE/IC-104	3.1	(0.5)	•	•	K	H	•	•	•	•
VE/IC-105	3.0	(0.7)	•	•	•	•	T	V	T	•
VE/IC-107	4.3	(0.8)	•	•	•	•	T	•	•	•
VE/IC-108	3.0	(0.7)	g	•	•	•	•	•	•	•
VE/IC-109	5.3	(0.5)	g	•	K	H	T	V	T	L
VE/IC-110	2.0	(0.4)	•	S	•	•	•	•	•	•
VE/IC-111	3.7	(0.7)	•	S	K	H	T	V	T	L
VE/IC-112	2.6	(0.5)	g	S	•	•	•	•	•	•
VE/IC-113	4.4	(0.6)	g	S	K	H	T	V	T	L

~ = Dot indicates TC-83 amino acid or nucleotide  
 Upper case = amino acid  
 Lower case = nucleotide

~ ~ = Average diameter (mm) of 10 plaques in Vero cells

<sup>^</sup> = VE/IC-101 is identical to VE/IC-102 except for the incorporation of a cDNA "error" at amino acid position E2-209 in VE/IC-101

**Table 4. Survival of 6-week-old white male Swiss NIH mice following PRIMARY intraperitoneal challenge with 200 PFU of VEE TRD, VEE TC-83, or cDNA-derived VE/IC virus and SECONDARY intraperitoneal challenge 23 days post-PRIMARY challenge with 40 PFU of VEE TRD virus.**

<u>Virus</u>	<u>PRIMARY Survivors/ Total</u>	<u>AST (SE)<sup>a</sup></u>	<u>Weight Loss/ Total</u>	<u>SECONDARY Survivors/ Total</u>
BA-1 <sup>b</sup>	8 / 8	- <sup>c</sup>	0 / 8	0 / 8
TC-83	8 / 8	-	0 / 8	8 / 8
TRD	0 / 8	6.0 (0.0)	8 / 8	-
VE/IC- 92	8 / 8	-	0 / 8	8 / 8
VE/IC-101	8 / 8	-	0 / 8	8 / 8
VE/IC-102	5 / 8	14.7 (1.9)	5 / 8	5 / 5
VE/IC-103	7 / 8	16.0 (0.0)	1 / 8	7 / 7
VE/IC-104	8 / 8	-	0 / 8	8 / 8
VE/IC-105	5 / 8	13.3 (1.9)	3 / 8	5 / 5
VE/IC-107	8 / 8	-	0 / 8	8 / 8
VE/IC-108	8 / 8	-	0 / 8	8 / 8
VE/IC-109	0 / 8	6.0 (0.0)	8 / 8	-
VE/IC-110	8 / 8	-	0 / 8	8 / 8
VE/IC-111	8 / 8	-	1 / 8	8 / 8
VE/IC-112	7 / 8	9.0 (0.0)	1 / 8	7 / 7
VE/IC-113	0 / 8	6.6 (0.2)	8 / 8	-

[a] Average survival time (standard error) in days

[b] Diluent

[c] Not applicable or no change

**Table 5. Survival of 21- to 24-day-old white male Swiss NIH mice following PRIMARY intraperitoneal challenge with 200 PFU of VEE TRD, VEE TC-83, or cDNA-derived VE/IC virus and SECONDARY intraperitoneal challenge 24 days post-PRIMARY challenge with 40 PFU of VEE TRD virus.**

<u>Virus</u>	<u>PRIMARY</u> <u>Survivors/</u> <u>Total</u>	<u>AST (SE)<sup>a</sup></u>	<u>Weight</u> <u>Loss/</u> <u>Total</u>	<u>SECONDARY</u> <u>Survivors/</u> <u>Total</u>
BA-1 <sup>b</sup>	8 / 8	- <sup>c</sup>	0 / 8	0 / 8
TC-83	8 / 8	-	0 / 8	8 / 8
TRD	0 / 8	5.6 (0.2)	8 / 8	-
VE/IC-92	8 / 8	-	0 / 8	8 / 8
VE/IC-101	8 / 8	-	0 / 8	7 / 8 <sup>d</sup>
VE/IC-102	1 / 8	9.4 (0.6)	8 / 8	1 / 8
VE/IC-103	0 / 8	9.4 (0.3)	8 / 8	-
VE/IC-104	8 / 8	-	0 / 8	8 / 8
VE/IC-105	3 / 8	9.8 (0.4)	8 / 8	3 / 3
VE/IC-107	5 / 8	10.7 (0.3)	8 / 8	5 / 5
VE/IC-108	8 / 8	-	0 / 8	8 / 8
VE/IC-109	0 / 8	6.0 (0.0)	8 / 8	-
VE/IC-110	8 / 8	-	0 / 8	7 / 8
VE/IC-111	6 / 8	9.5 (1.5)	5 / 8	6 / 6
VE/IC-112	4 / 8	11.5 (2.2)	8 / 8	4 / 4
VE/IC-113	0 / 8	6.5 (0.3)	8 / 8	-
VE/IC-114	2 / 8	8.5 (1.0)	7 / 8	1 / 2 <sup>d</sup>
VE/IC-115	0 / 8	7.4 (0.5)	8 / 8	-
VE/IC-116	8 / 8	-	0 / 8	5 / 8 <sup>e</sup>
VE/IC-117	0 / 8	7.9 (0.5)	8 / 8	-
VE/IC-118	0 / 8	6.6 (0.2)	8 / 8	-

[a] Average survival time (standard error) in days

[b] Diluent

[c] Not applicable or no change

[d] One mouse died within one day of SECONDARY virus challenge

[e] Mice died on days 9, 10, or 11 following SECONDARY virus challenge.

All other post-TRD challenge deaths occurred within 7 days.



**Table 6. Survival of 21-day-old white male Swiss NIH mice following PRIMARY intraperitoneal challenge with low doses (10 or 50 PFU) of VEE TRD, TC-83, or cDNA-derived VE/IC virus and SECONDARY intraperitoneal challenge with 40 PFU of VEE TRD virus.**

<u>Virus</u>	<u>PFU Dose</u>	<u>PRIMARY Survivors/ Total</u>	<u>AST (SE)<sup>a</sup></u>	<u>SECONDARY Survivors/ Total</u>
Ba-1 <sup>b</sup>	- <sup>c</sup>	8 / 8	-	0 / 8
TC-83	50 (56) <sup>d</sup>	8 / 8	-	8 / 8
TRD	50 (40)	0 / 8	4.1 (0.5)	-
TRD-Transf <sup>e</sup>	50 (14)	0 / 8	5.4 (0.5)	-
VE/IC-102	50 (54)	0 / 8	9.6 (0.9)	-
VE/IC-102B <sup>f</sup>	50 (56)	0 / 8	10.9 (0.6)	-
VE/IC-102BM <sup>g</sup>	50 (56)	4 / 8	9.3 (0.8)	4 / 4
VE/IC-109	50 (36)	0 / 8	6.5 (0.4)	-
VE/IC-109B	50 (38)	0 / 8	6.8 (0.2)	-
VE/IC-109BM	50 (56)	0 / 8	5.9 (0.2)	-
VE/IC-113	50 (102)	0 / 8	6.6 (0.3)	-
VE/IC-113B	50 (56)	0 / 8	6.9 (0.1)	-
VE/IC-113BM	50 (78)	0 / 8	6.9 (0.1)	-
TC-83	10 (13)	8 / 8	-	8 / 8
TRD	10 ( 5)	0 / 8	4.6 (0.3)	-
TRD-Transf	10 ( 5)	0 / 8	6.2 (0.3)	-
VE/IC-102B	10 ( 8)	4 / 8	10.0 (1.2)	4 / 4
VE/IC-109B	10 ( 7)	0 / 8	5.8 (0.3)	-
VE/IC-113B	10 ( 8)	0 / 8	7.0 (0.0)	-

[a] Average survival time (standard error) in days

[b] Diluent

[c] Not applicable or no change

[d] Virus inoculum frozen for virus titration. Values in parentheses are doses as measured by titration of the challenge inoculum

[e] TRD virus from cells transfected with TRD viral RNA

[f] Second, independent virus derived by RNA transfection

[g] Linearized cDNA treated with mung bean nuclease

**Table 7. ELISA titers of pooled mouse (3-week or 6-week-old white male Swiss NIH) sera from survivors of PRIMARY intraperitoneal challenge with 200 PFU of TC-83, TRD, or VE/IC virus and SECONDARY intraperitoneal challenge with 40 PFU of VEE TRD virus.**

Virus	3-Week-Old Mice			6-Week-Old Mice	
	Mice per Pool	3 Weeks Post-PRIMARY Challenge	2 Weeks Post-SECONDARY Challenge	Mice per Pool	3 Weeks Post-PRIMARY Challenge
BA-1 <sup>a</sup>	8	0	-	8	0
TC-83	8	10240	10240	8	5120
TRD	<sup>b</sup>	-	-	-	-
VE/IC-92	8	5120	10240	8	5120
VE/IC-101	8	10240	5120	8	5120
VE/IC-102	1	2560	10240	5	5120
VE/IC-103	-	-	-	7	5120
VE/IC-104	8	5120	640	8	2560
VE/IC-105	3	2560	1280	5	10240
VE/IC-107	5	640	1280	8	5120
VE/IC-108	8	5120	640	8	5120
VE/IC-109	-	-	-	-	-
VE/IC-110	8	5120	640	8	5120
VE/IC-111	6	10240	1280	8	10240
VE/IC-112	4	10240	2560	7	10240
VE/IC-113	-	-	-	-	-
VE/IC-114	2	20480	N.D. <sup>c</sup>	N.D.	N.D.
VE/IC-115	-	-	-	N.D.	N.D.
VE/IC-116	8	640	N.D.	N.D.	N.D.
VE/IC-117	-	-	-	N.D.	N.D.
VE/IC-118	-	-	-	N.D.	N.D.

[a] Diluent

[b] Not applicable (no survivors)

[c] Not done (post-SECONDARY challenge sera not collected)

**Table 8. Relative virulence of cDNA-derived VE/IC viruses by intraperitoneal inoculation of 3-week-old white male Swiss NIH mice.**

<u>Virus</u>	<u>Percent Mortality</u>	<u>AST<sup>a</sup></u>	<u>Sequence Phenotype<sup>b</sup></u>
TRD	100	4.1- 6.2	g-A-S-----K H T V T--L----
VE/IC-109	100	5.8- 6.5	g-----K H T V T--L----
VE/IC-113	100	6.5- 7.0	g-.S-----K H T V T--L----
VE/IC-118	100	6.6	g-----T-----.
Ve/IC-115	100	7.4	g-----K H T V T-.-----
Ve/IC-117	100	7.9	g-----T V T-.-----
VE/IC-103	100	9.4	-----K H T V T-.-----
VE/IC-102	50-100	9.4-10.9	-----K H T V T--L----
VE/IC-114	75	8.5	g-----K H T V.T--L----
VE/IC-105	62	9.8	-----T V T-.-----
VE/IC-112	50	11.5	g-.s-----.
VE/IC-107	38	10.7	-----T-----.
VE/IC-111	25	9.5	---.s-----K H T V T--L----
VE/IC-116	0	N.A. <sup>c</sup>	g-----KH-----.
VE/IC-110	0	N.A.	---.s-----.
VE/IC-108	0	N.A.	g-----.
VE/IC-104	0	N.A.	-----KH-----.
VE/IC-101	0	N.A.	-----K H T V.T--L----
VE/IC- 92	0	N.A.	-----.
TC-83	0	N.A.	a-D-T-----N Y R D I--I----

[a] Average survival time in days

[b] The relevant nucleotide (lower case) and amino acid (upper case) residues are indicated. In the VE/IC schematics, only TRD virus-specific residues are indicated. Dashes in the relevant positions indicate identity with the TC-83 genotype. Dots indicate known "errors" in the cDNA.

[c] Not applicable (no mortality)

Table 9. Indirect immunofluorescence of acetone-fixed CV-1 cells 24 h post-infection.

<u>Antibody*</u>	<u>Specificity</u>	<u>Virus</u>					
		<u>Unin- fected</u>	<u>Vaccinia</u>	<u>VACC/ TC-5A</u>	<u>VACC/ TRD-1A</u>	<u>TC-83</u>	<u>TRD</u>
Mouse Serum	Vaccinia	-	3†	3	3	-	-
MHIAF	VEE TC-83	-	-	3	3	4	4
5B4D-6	E2 <sup>a</sup> TC-83	-	-	3	1	4	1
1A4A-1	E2 <sup>c</sup> PTF-39	-	-	3	3	4	4
1A6C-3	E2 <sup>d</sup> EVE	-	-	-	2	2	2
1A3A-5	E2 <sup>e</sup> P676	-	-	2	-	4	2
1A4D-1	E2 <sup>f</sup> TRD	-	-	3	3	4	4
1A3A-9	E2 <sup>g</sup> PTF-39	-	-	3	3	4	4
1A3B-7	E2 <sup>h</sup> PTF-39	-	-	3	1	4	4
3B2D-5	E1 <sup>a</sup> TC-83	-	-	-	-	3	3
3B2A-9	E1 <sup>b</sup> TC-83	-	-	3	3	3	3
5B6A-6	E1 <sup>c</sup> TC-83	-	-	-	-	3	1
3A5B-1	E1 <sup>d</sup> TC-83	-	-	3	3	4	4

\* Alphanumeric designations are anti-VEE virus monoclonal antibodies (mouse ascitic fluids). Eptiope specificity and virus used to elicit monoclonal antibody are shown. MHIAF = mouse hyperimmune ascitic fluid. All antibody preparation were tested at 1:300 dilution.

† Relative fluorescence as determined by two observers in at least two experiments.

- = negative  
4 = maximum

**Table 10. HI and ELISA serum titers in A/J mice immunized with TC-83 or recombinant vaccines.**

Vaccine	Log <sub>10</sub> Geometric Mean Titer (±S.E.) <sup>d</sup>										
	Prechallenge					Postchallenge					Ratio <sup>e</sup>
	Dose <sup>a</sup> (PFU)	Challenge <sup>b</sup>	Survival <sup>c</sup>	HI	ELISA	HI	ELISA	HI	ELISA	HI	
TC-83	10 <sup>4</sup>	TRD	8/8	2.47 (0.09)	≥5.01	3.03 (0.14)	N.D.	4	N.D. <sup>f</sup>		
VACC/TC-5A	10 <sup>7</sup>	-	8/8	1.64 (0.13)	3.17 (0.13)	1.94 (0.09)	3.24 (0.14)	2	1		
VACC/TC-5A	10 <sup>7</sup>	TRD	8/8	1.56 (0.09)	3.24 (0.14)	2.77 (0.17)	4.67 (0.22)	16	29		
VACC/TC-5A	10 <sup>6</sup>	TRD	7/8	1.43 (0.04)	3.03 (0.07)	2.72 (0.13)	4.75 (0.09)	19	53		
VACC/TC-5A	10 <sup>5</sup>	TRD	5/8	1.18 (0.03)	2.36 (0.06)	3.10 (0.16)	5.01 (0.10)	85	445		
VACC	10 <sup>8</sup>	TRD	0/8	<1.0	<1.0	-	-	-	-		
PBS	-	TRD	0/8	<1.0	<1.0	-	-	-	-		

<sup>a</sup> PFU determined in Vero cells. TC-83 and VACC viruses given by intraperitoneal injection or tail scarification, respectively.

<sup>b</sup> Challenge = 100 IPLD<sub>50</sub> (15 PFU) of TRD virus.

<sup>c</sup> Number of survivors/number in group.

<sup>d</sup> Determined from individual titers of five (10<sup>5</sup> PFU VACC/TC-5A), seven (10<sup>6</sup> PFU VACC/TC-5A), or eight (all others) mice per group.

<sup>e</sup> Postchallenge GMT/prechallenge GMT (GMT = geometric mean titer).

<sup>f</sup> N.D. = not done.

**Table 11.** VEE neutralizing antibody titers in A/J mice 3 weeks after immunization with VACC/TC-5A virus.

Mouse Number	Vaccine dose (PFU)		
	$10^7$	$10^6$	$10^5$
1	2,560 <sup>a</sup>	160	10
2	640	< 10 <sup>b</sup>	10
3	320	80	< 10 <sup>b</sup>
4	640	< 10	< 10 <sup>b</sup>
5	5,120	320	20
6	80	320	160
7	80	20	< 10 <sup>b</sup>
8	20	320	20

<sup>a</sup> Reciprocal of highest antiserum dilution that inhibited 70% of more of the VEE TRD virus (60 PFU) used in the test.

<sup>b</sup> Mouse died after challenge with 100 IPLD<sub>50</sub> of VEE TRD virus.

**Table 12. Duration of immunity in individual A/J mice immunized intradermally with  $10^7$  PFU of recombinant VACC/TC-5A virus.**

Mouse	Days Postimmunization		
	18	33	86
1	320 <sup>a</sup>	2,560	1,280
2	640	160	320
3	2,560	640	320
4	640	2,560	2,560
5	5,120	5,120	10,240
6	20	80	40

<sup>a</sup> Reciprocal of highest antiserum dilution that inhibited 70% or more of VEE TRD virus (60 to 80 PFU) used in neutralization test.

**Table 13. Cross-reactivities of sera pooled from A/J mice immunized with VEE TC-83 or recombinant VACC/TC-5A virus.**

Virus	Pooled Mouse Serum	
	VACC/TC-5A	TC-83
VACC	80 <sup>a</sup>	< 10
VACC/TC-5A	160	< 10
TRD (1AB) <sup>b</sup>	1,280	≥ 20,480
P676 (1C)	320	≥ 640
3880 (1D)	10	160
Mena II (1E)	10	80
EVE (2)	40	320
PIX (4)	< 10	< 10
WEE	< 10	< 10

<sup>a</sup> Endpoint = 70% neutralization of 60 to 90 PFU.

<sup>b</sup> VEE subtype.



**Table 14. VACC/TC-5A and TC-83 vaccine efficacy in protecting C3H or Swiss NIH mice from intraperitoneal challenge with four VEE virus strains.**

Challenge		Survival <sup>a</sup>			
Virus	Dose (PFU)	VACC/TC-5A		TC-83	
		C3H	Swiss	C3H	Swiss
TRD (1AB) <sup>b</sup>	6 X 10 <sup>1</sup>	6/6 <sup>c</sup>	6/6	6/6	6/6
	6 X 10 <sup>2</sup>	6/6	6/6	6/6	6/6
	6 X 10 <sup>3</sup>	6/6	5/6	6/6	6/6
	6 X 10 <sup>5</sup>	6/6	5/6	6/6	6/6
P676 (1C)	1.1 X 10 <sup>4</sup>	6/6	6/6	6/6	6/6
	1.1 X 10 <sup>6</sup>	6/6	5/6	6/6	6/6
3880 (1D)	1 X 10 <sup>1</sup>	6/6	6/6	6/6	6/6
	1 X 10 <sup>3</sup>	6/6	6/6	6/6	6/6
EVE (2)	1 X 10 <sup>8</sup>	8/8	8/8	8/8	8/8

<sup>a</sup> All mice in 17 PBS control groups (nine virus challenge doses, two mouse strains) succumbed to virus challenge. Only three of eight PBS control Swiss NIH mice died after EVE virus challenge.

<sup>b</sup> VEE subtype.

<sup>c</sup> Number of survivors/total.

**Table 15. Summary of survival of VACC/TC-5A- or TC-83-immunized mice challenged intraperitoneally or intranasally with virulent VEE TRD virus.**

Mouse Strain	Intraperitoneal challenge <sup>a</sup>		Intranasal challenge <sup>b</sup>	
	VACC/TC-5A	TC-83	VACC/TC-5A	TC-83
A/J	42/42	8/8	6/24	16/16
C3H	30/30	30/30	0/30	15/20
Swiss NIH	22/24	24/24	1/30	18/18

<sup>a</sup> Challenge dose = 15 PFU to  $6 \times 10^5$  PFU of TRD virus.

<sup>b</sup> Challenge dose = 3,300 (A/J) or 10,500 (C3H and Swiss NIH) PFU of TRD virus.

**Table 16. Phenotyping of nylon wool passaged T-cells from normal and alphavirus primed C3H mice after depletion with specific antisera.**

Treatment <sup>a/</sup>	% Cytotoxicity	T-cell depletion		
		Pre-proliferation	Post-proliferation	
		VEE TC-83 <sup>b/</sup>	Sindbis	VEE TC-83
Diluent	5	97,791 ± 13,626	34,054 ± 2,197	44,859
Anti-Thy-1	60	3,046 ± 3,118	No data	1,879
Anti-Lyt-1.1	49	3,250 ± 992	<1,000	7,366
Anti-Lyt-2.1	29	92,457 ± 5,098	20,132 ± 2,467	36,596

<sup>a/</sup> Plus complement.

<sup>b/</sup> Results shown are from stimulation of virus-primed T-cells with homologous virus only. Similar treatment of normal, unprimed T-cells gave negative lymphoproliferation values. Results are c.p.m. of <sup>3</sup>H-thymidine incorporation after complement treatment.

**Table 17. Phenotypic analysis of nylon wool packaged lymphocytes by flow cytometry.**

Antibodies <sup>a/</sup>	Cell Type	Percent Staining Lymphocytes	
		Normal <sup>b/</sup>	VEE TC-83 <sup>c/</sup>
Anti-Thy-1	All T-cells	65	74
Anti-L3T4	All Th-cells	43	50
Anti-Lyt-2	All Tc-cells	21	20
Anti-IgG	B-cells	No data	20

<sup>a/</sup> Thy-1, Lyt-2, and IgG antisera were conjugated to fluorescein isothiocyanate and L3T4 antisera to phycoerythrin.

<sup>b/</sup> Lymphocytes from uninoculated C3H mice were labeled and phenotyped following nylon wool chromatography.

<sup>c/</sup> Lymphocytes were processed from mice four weeks after inoculation with VEE TC-83 virus and incubated in vitro with homologous virus.

**Table 18. Stimulation of VEE TC-83 virus-primed nylon wool passaged T-cells with VEE subtype viruses.**

Virus <sup>a/</sup>	Subtype	Lymphoproliferation	%
TC-83	IAB	66,376 ± 3,349 <sup>b/</sup>	100
PTF-39	IAB	94,660 ± 5,359	142
P676	IC	45,344 ± 1,897	68
3880	ID	27,545 ± 2,411	41
Mena II	IE	36,862 ± 2,986	56
Everglades	II	71,327 ± 6,271	93
Mucambo	III	22,130 ± 1,209	33
Pixuna	IV	23,781 ± 3,274	36

<sup>a/</sup> Stimulator splenocytes not preinfected; purified virus (1 μg) added to wells at time of assay.

<sup>b/</sup> Values are c.p.m. of adjusted means ± associated S.E.

Table 19. Serological reactivities of horse vaccinees with TC-83 virus.

Horse	Immunogen	Test	Serum Sample <sup>a/</sup>		
			1 <sup>0</sup>	2 <sup>0</sup>	Post-Challenge
70	TC-83	EIA	25600	n.a. <sup>b/</sup>	32000
		N	≥ 1024	n.a.	128
182	TC-83	EIA	25600	n.a.	12800
		N	≥ 1024	n.a.	128
14	TC-5A	EIA	100	3200	25600
		N	8	32	128
77	TC-5A	EIA	≤ 100	3200	6400
		N	16	128	256
134	TC-5A	EIA	100	3200	12800
		N	8	32	64
160	TC-5A	EIA	100	25600	12800
		N	8	128	256
84	Vacinnia	EIA	≤ 100	≤ 100	1600
		N	< 4	≤ 2	≤ 2
86	Vaccinia	EIA	≤ 100	≤ 100	1600
		N	< 4	≤ 2	≤ 2

<sup>a/</sup> Highest observed titers.

<sup>b/</sup> Not applicable.

**Table 20. Neutralization reactivities of horse vaccinees with equine virulent VEE virus 71-180 and vaccinia viruses.**

Horse	Immunogen	Test	Serum Sample <sup>a/</sup>		
			1 <sup>0</sup>	2 <sup>0</sup>	Post-Challenge
70	TC-83	71-180	≥ 256	n.a. <sup>b/</sup>	256
		Vaccinia	< 2	n.a.	≤ 2
182	TC-83	71-180	≥ 1024	n.a.	256
		Vaccinia	< 2	n.a.	≤ 2
14	TC-5A	71-180	< 2	32	256
		Vaccinia	64	64	64
77	TC-5A	71-180	< 2	32	64
		Vaccinia	64	128	128
134	TC-5A	71-180	< 2	32	128
		Vaccinia	≥ 128	128	256
160	TC-5A	71-180	< 2	32	128
		Vaccinia	≥ 128	128	512
84	Vaccinia	71-180	< 2	≤ 2	1600
		Vaccinia	≥ 128	64	128
86	Vaccinia	71-180	< 2	≤ 2	1600
		Vaccinia	≥ 128	128	128

<sup>a/</sup> Highest observed titers.

<sup>b/</sup> Not applicable.

Table 21. ELISA cross-reactivity of sera from immunized equines.

Horse	Immunogen	Time <sup>a/</sup>	Antigens									
			IAB	IC	ID	IE	II	III	IV	WEE	EEE	
70	TC-83	Pre	-	-	-	-	-	-	-	-	-	-
		Post	+	+	+	+	+	+	+	+	-	+
182	TC-83	Pre	-	-	-	-	-	-	-	-	+	+
		Post	+	+	+	+	+	+	+	+	+	+
14	TC-5A	Pre	-	-	-	-	-	-	-	-	-	-
		Post	+	+	+	+	+	+	+	+	-	-
77	TC-5A	Pre	-	-	-	-	-	-	-	-	-	-
		Post	+	+	+	+	+	+	+	+	-	-
134	TC-5A	Pre	-	-	-	-	-	-	-	-	+	-
		Post	+	+	+	+	+	+	+	+	+	+
160	TC-5A	Pre	-	-	-	-	-	-	-	-	-	-
		Post	+	+	+	+	+	+	+	+	-	-

<sup>a/</sup> Time pre- or post-primary (TC-83) or secondary (TC-5A) immunization.



**Table 22. Equine Th-cell stimulation index to various VEE virus vaccines and heterologous alphaviruses.**

Horse	Immunogen	Time <sup>b/</sup>	Antigen <sup>a/</sup>				
			TC-83	71-180	WEE	EEE	
70	TC-83	1 <sup>0</sup>	4.5				
		C	4.4	3.5	1.0	1.0	
		182	1 <sup>0</sup>	10.0			
		C	3.0	2.5	2.7	1.0	
14	TC-5A	1 <sup>0</sup>	1.0				
		2 <sup>0</sup>	2.4	3.2			
		C	13.0	16.0	1.0	1.0	
77		1 <sup>0</sup>	2.8				
		2 <sup>0</sup>	2.8	4.4			
		C	3.7	3.1	5.8	1.0	
134		1 <sup>0</sup>	4.5				
		2 <sup>0</sup>	2.6	2.3			
		C	7.8	6.6	1.0	1.0	
160		1 <sup>0</sup>	1.0				
		2 <sup>0</sup>	1.0	1.0			
		C	2.2	2.2	1.0	1.0	
84	Vaccinia	1 <sup>0</sup>	1.0				
		2 <sup>0</sup>	1.0	1.0			
		C	1.9	2.9			
86		1 <sup>0</sup>	1.0				
		2 <sup>0</sup>	1.0	1.0			
		C	2.2	3.3			

<sup>a/</sup> Stimulation index is  $\frac{\text{C.P.M. test (Lymphocytes + Virus)}}{\text{C.P.M. Control (Lymphocytes + Diluent)}}$

A value of 1 indicates no stimulation.

<sup>b/</sup> Results are from the day with the highest values, usually 2-3 weeks post-primary inoculation (1<sup>0</sup>), 4 days post-boost (2<sup>0</sup>), and 14 days post-challenge (C).

Table 23. Equine Clinical Course -- VEE IB

Immunization & Challenge	Animal I.D.	VEE IB -- Post-Challenge Clinical Course
Vaccinia TC-5A	77	No Clinical Abnormalities Noted
(I.D.)	160	No Clinical Abnormalities Noted
	14	No Clinical Abnormalities Noted
VEE IB (SC)	134	No Clinical Abnormalities Noted
Vaccinia (I.D.)	84	<ol style="list-style-type: none"> <li>*1. Temperature elevated = D-1 (am) - D-2 (pm) Biphasic fever as normal D-3 (am) then increased D-3 (pm) - D-6 (pm)</li> <li>2. Depressed mental status = D-1 (pm) -- D-6 (pm)</li> <li>3. Anorexia = Incomplete progressing to complete by Day 4 - Day 6.</li> <li>4. Oliguria = anuria D-1 -- D-6 (Progressive)</li> </ol>
VEE-IB (SC)	86	<ol style="list-style-type: none"> <li>*5. Anemia + decreased platelet counts + hemoconcentration (not thrombocytopenic)</li> <li>6. Ataxia with wide based stance.</li> <li>7. Aberrant mental status (teeth grinding, constant chewing motion).</li> <li>8. Terminally somnolent - very reluctant to ambulate.</li> </ol>
TC-83 (SC)	70	No Clinical Abnormalities Noted
VEE-IB (SC)	182	No Clinical Abnormalities Noted
	Synopsis	<p>Animals became depressed and off feed and water initially beginning on Day 1 Post-Challenge (pm). This trend continued but on Day 3 the fever subsided, the animals appeared more responsive, and they ate/drank that morning. By pm of Day 3 - the fever returned, as did the depression. By Day 4, the animals were eating, drinking little or nothing, and the depressed mental status returned. The animals became progressively reluctant to move, became ataxic, and stopped eating/drinking or moving altogether - until euthanized.</p>

**Table 24. Summary of histopathologic findings in the CNS of horses immunized with recombinant vaccine TC-5A, TC-83 or wild-type vaccinia virus and challenged with virulent VEE virus.**

Mare	Vaccine	Histopathologic Findings in CNS
70	TC-83	Focal perivascular lymphoid infiltration - mild; cerebellum, hippocampus, thalamus.
182	TC-83	Meningeal lymphoid infiltration - mild.
14	TC-5A	Focal perivascular lymphoid infiltration - mild; pons chronic focal perivascular fibrosis - mild; cerebrum, chorioid plexus, pons (probably incidental finding).
77	TC-5A	Focal perivascular lymphoid infiltration - mild; cerebrum.
134	TC-5A	Meningeal lymphoid infiltration - mild.
160	TC-5A	Meningeal lymphoid infiltration - mild; focal perivascular lymphoid infiltration - mild; cerebrum, hippocampus, pons, cerebellum.
84	Vaccinia	Non-suppurative meningoencephalitis - severe.
86	Vaccinia	Non-suppurative meningoencephalitis - severe, with thrombosis.

FIGURE 1  
Page 1 of 6

- nsP1

MEK V H V D I E E D S P F L R A L O R T F P Q F E NS1 26

AUGGGCGGCGCAUGAGAGAAGCCAGACCACAUUACCUACCCAAAUGGAGAAAAGUUCACGUUGACAUGGAGGAGACAGCCCAUUCUUCAGAGCUUUGCAGCGGACGUUCCCGCAGUUG 120

[A] > pTRD-45,71,TC-19,46

V E A K Q V T D N D H A N A R A F S H L A S K L I E T E V D P S D T . L D I G S NS1 66

AGGUAGAAGCCCAAGCAGGUCACUGAUAAUAGCCAUUGCUAAUGCCAGAGCGUUUCGCAUCUGGCUUCAAAAUGAUCGAAAACGGAGGUGGACCCAUCCGACACCGAUCCUUGACAUUGGAA 240

A P A R R M Y S K H K Y N C I C P M R C A E D P D R L Y K Y A T K L K K N C K E NS1 106

GUGCGCCCGCCCGCAGAAUGUAUUCUUAAGCACAAGUAUCAUUGUAUCUGUCGCAUGAGAGUGUGCGGAAGAUCCGGACAGAUUGUAUUAAGUAUGCAACUAAGCUGAAGAAAAACUGUAAGG 360

I T D K E L D K K M K E L A A V M S D P D L E T E T M C L H D D E S C R Y E G Q NS1 146

AAUUAACUGAUUAGGAUUGGACAAGAAAUGAAGGAGCUCGCCGCCGUCUGAGCGACCCUGACCUUGAAAACUGAGACUUAUGGCCUCCACGACGCGAGUCGUGUGGCUACGAAGGGC 480

V A V Y Q D V Y A V D G P T S L Y H Q A N K G V R V A Y W I G F D T T P F M F K NS1 186

AAGUCGCUUUUACCGAGGAUGUAUACCGGUGAGCGGACCGACAAGUCUCUACCAAGCCAAUUAAGGGAGUUAAGUCGCUACUGGAAUGGCUUUGACACCCCCUUUUUUGUUUA 600

M L A G A Y P S Y S T N W A D E T V L T A R N I G L C S S D V M E R S R R G M S NS1 226

AGAACUUGGCGUGGAGCAUUAUCCAUCAUUCUACCAACUGGGCCGACGAAACCGUGUUAACGGCUCGUAACAUAGGCCUUAUGCAGCUCUGACCUUAUGGAGCGGUCACGUAGAGGGAUUG 720

I L R K K Y L K P S N M V L F S V G S T I Y H E K R D L L R S W H L P S V F H L NS1 266

CCAUUCUUAAGAAAGUAUUGAAACCAUCCAAACAUGUUCUUAUCUGUGGCUCCGACCAUCCACGAGAAGAGGGACUUAUGAGGAGCUGGCACCUGCCGUCUGUAUUCACU 840

R G K Q N Y T C R C E T I V S C D G Y V V K R I A I S P G L Y G K P S G Y A A T NS1 306

UACGUGGCAAGCAAAUUAACAUUGCGGUGAGACUUAUAGUAGUUGCGAGGGUACGUCGUUAAAAGAAUAGCUUUCAGUCCAGGCCUGUAUGGGAAGCCUUCAGGCCUUGGUGCUA 960

M H R E G F L C C K V T D T L N G E R V S F P V C T Y V P A T L C D Q M T G I L NS1 346

CGAUGCACCGGAGGGAUUCUUGUGUGCAAAGUGACAGACACAUUGAACGGGGAGAGGGUCUUUUCGUGGACGUAUUGGCCAGCUACAUUGUGUGACCAAUGACUGGCAUAC 1080

A T D V S A D D A Q K L L V G L N Q R I V V M G R T Q R N T N T M K N Y L L P V NS1 386

UGGCAACAGAUUGCAGUGGCGGACGCGCAAAAACUGCUGGUUGGGCUCAACCAGCGUAUAGUCGUAACGGUGCGCACCCAGAGAACAACCAUUAUCCAUUAAUACCUUUGCCCC 1200

V A Q A F A R W A K E Y K E D O E D E R P L G L R D R Q L V M G C C W A F R R H NS1 426

UAGUGGCCCAGGCAUUGCUUAGGUGGGCAAAGGAUUAUAGGAAGAUCAAGAAGAUAAAGGCCACUAGGACUACGAGAUAGACAGUUAUGCAUGGGGUGUUGGGCUUUUUAAGGC 1320

K I T S I Y K R P D T Q T I I K V N S D F H S F V L P R I G S N T L E I G L R T NS1 466

ACAAGAUAAACUUAUUUAUAGCCCGGUAUCCAAACCAUCAUCAAAGUGAACAGCGAUUCCACUUAUCUGUGGCCAGGAUAGGCAUAACACAUUGGAGUAGCGGGCUGAGAA 1440

> pTRD-30

R I R K M L E E H K E P S P L I T A E D V Q E A K C A A D E R K E V R E A E E L NS1 506

CAAGAAUCAGGAAAUGUUAAGAGGAGCACAGGAGCCGUCACCCUCUUAUACCGCGAGGACGUACAAGAAAGCUAAGUGGGCAGCCGAUGAGGCUAAGGAGGUGCGUGAAGCCGAGGAGU 1560

- nsP2

R A A L P P L A A D V E E P T L E A D V D L M L O E A G A G S V E T P R G L I K NS2 11

UGCGCGCAGCUCUACCACCUUUGGACGUGAUGUUGAGGAGCCACUCUGGAAAGCCGAUGUCGACUUGAUGUUAACAAGAGGCGUGGGCCGGCUCAGUGGAGACACCCUGGCGUUGAUAA 1680

V T S Y D G E D K I G S Y A V L S P Q A V L K S E K L S C I H P L A E Q V I V I NS2 51

AGGUUACCAGCUACCGUGGCGAGGACAAGAUCCGGUCUUAUCGUGGCUUUCGCGAGGCGUACUACAAGAGUAAAUAUUCUGCAUCCACCCUCUGGUGAAACAGUCUUAUGUGA 1800

pTC-46 < pTRD-71 <

T H S G R K G R Y A V E P Y H G K V V V P E G H A I P V Q D F Q A L S E S A T I NS2 91

UAACACACUCUGGCCGAAAAGGGCGGUUAUGCCGUGGAACCAUUAUCCAUUGUAAAGUAGUGGUGCCAGAGGGAUGCAUACCCGUCCAGGACUUAAGCUCUGAGUGAAAUGGCCACCA 1920

[T]=Leu (TRD error)

V Y N E R E F V N R Y L H H I A T H G G A L N T D E E Y Y K T V K P S E H D G E NS2 131

UUGUGUACAACGAACGUGAGUUCGUAAAACAGGUACCUGCACCAUUAUUGCCACAUUGGAGGAGCGGUGAACACUGAUGAAGAAUUAUUAACAAACUGUCUAGCCGAGGACGACGCGG 2040

Y L Y D I D R K Q C V K K E L V T G L G L T G E L V D P P F H E F A Y E S L R T NS2 171

AAUACCUGUACGACAUUGCAGGAAACAGUGCGUCAAAGAAAGAACUAGUCACUGGGCUAGGGUCACAGGGGAGCUGGUGAUCCUCCUCCAUUGAUAUUGCCUACGAGAGUCUGAGAA 2160

R P A A P Y Q V P T I G V Y G V P G S G K S G I I K S A V T K K D L V V S A K K NS2 211

CAGCAGCGCCUCUUAACCAAGUACCAACCAUAGGGUGUAUGGCGUGCCAGGAUCAGGCAAGUCUGGCAUUAUAAAGCGGAGUACCAAAAAGAUUAUUGGUGAGCGCCCAAGA 2280

FIGURE 1  
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E N C A E I I R D V K K M K G L D V N A R T V D S V L L N G C K H P V E T L Y I NS2 251  
 AAGAAAACUGUGCAGAAAUAAAGGGACGUAAGAAAUGAAGGGCUGGACGUAAGCCAGAACUGUGGACUCAGUGUCUUGAAUGGAAUGCAAACACCCCGUAGAGACCCUGUAUA  
 2400  
 D E A F A C H A G T L R A L I A I I R P K K A V L C G D P K Q C G F F N M H C L NS2 291  
 UUGACGAAGCUUUUGCUUGCAUGCAGGUACUCUCAGAGCGCUCUAGCCAUUAUAAGACCUAAAAAGGCAGUGUCUCUGGGGACCCCAAACAGUGCGGUUUUUUUAACAUUGUGCC  
 2520  
 K V N F N H E I C T Q V F H K S I S R R C T K S V T S V V S T L F Y D K K M R T NS2 331  
 UGAAAGUCAUUUUAACACGAGAUUGCACAAGUCUCCACAAAAGCAUCUCUGCCGUGGACUAUAUCUGUGACUUCGGUCGUCUACCUUGUUUUUACGACAAAAAUGAGAA  
 2640  
 T N P K E T K I V I D T T G S T K P K Q D D L I L T C F R G W V K Q L Q I D Y K NS2 371  
 CGACGAUCCGAAAGAGACUAAGAUUGAUUGACACUACCCGGCAGUACCAAACCUAAGCAGGACGAUCUUCUCACUUGUUCAGAGGGUGGGUAGGACAGUUGCAAUAGAUUA  
 2760  
 G N E I M T A A A S Q G L T R K G V Y A V R Y K V N E N P L Y A P T S E H V N V NS2 411  
 AAGGCAACGAAAUAAUGACGGCAGCUCUCUAGGGCUGACCCGUAAAGGUGUGUAGCCGUGCGUUAACAGGUGAAUUGAAAUCCUUGUAGCCACCCACCUAGAACAUUGAAGC  
 2880  
 L L T R T E D R I V W K T L A G D P W I K T L T A K Y P G N F T A T I E E W Q A NS2 451  
 UCCUACUGACCCGACGGAGGACCGCAUCGUGUGGAAACACUAGCCGGCAGCCAUUGGAAUAAAAACUCAGUCGCCAAGUACCCUGGGAAUUUCACUGCCAAGUAGAGGAGUGGCAAG  
 3000  
 E H D A I M R H I L E R P D P T D V F O N K A N V C W A K A L V P V L K T A G I NS2 491  
 CAGAGCAUGAUGCCAUCAUGAGGCACAUUUGGAGAGACCGGACCCUACCGACGUCUCCAGAAUAAAGGCAACGUGUGUUGGGCAAGGCUUUGUGCCGGUGCUGAAGACCCGUGG  
 3120  
 D M T T E Q W N T V D Y F E T D K A H S A E I V L N Q L C V R F F G L D L D S G NS2 531  
 UAGCAUGACCAUCUGAACAAUGAACACUGUGGAAUUAUUUGAAAGGCAAAAGCUCACUCAGCAGAGAUAGUAGAACCAACUAGCCGUGAGGUUCUUGGACUUGAUCUGGACUCCG  
 3240  
 L F S A P T V P L S I R N N H M D N S P S P N M Y G L N K E V V R Q L S R R Y P NS2 571  
 GUCUUAUUUCUGCACCACUGUCCGUUAUCCAUUAGGAUUAUCACUGGGAAUACUCCCGUCGCCUAAACUAGUACGGGCUAAUAAAGAGUGGUCGUCAGCUCUCUGCAGGUACC  
 3360  
 Q L P R A V A T G R V Y D M N T G T L R N Y D P R I N L V P V N R R L P H A L V NS2 611  
 CACAACUGCCCGGGCAGUUGCCACUGGAAGAGUCUAGACAUAGAACUCUGGUACACUGCCGAAUUAUGAUCGGCGCAUAAACCUAGUACCUUAACAGAAAGACUGCCUUCUUGU  
 3480  
 pTC-19 ◀  
 L H H N E H P Q S D F S S F V S K L K G R T V L V V G E K L S V P G K M V D W L NS2 651  
 UCCUCCACCAUUAUAGAACACCCACAGAGUCACUUCUUCUUCUUCGACGACAAUUAAGGGCAGAACUCUGUCUGGUGUGCGGGGAAAGUUGUCCGUCACAGGCAAAUUGUAGCUGU  
 3600  
 S D R P E A T F R A R L D L G I P G D V P K Y D I I F V N V R T P Y K Y H N Y Q NS2 691  
 UGUCAGACCGGGCUGAGGCUACCUUCAGAGCUCGGCUGGAAUUGGCAUCCAGGUGAUGUGCCAAAUUAUGACAAUUAUUUGUUAUUGGAGGACCCAUUAUAAUACCAUCUUC  
 3720  
 ▶ pTC-9 (Sst I #1)  
 Q C E D H A I K L S M L T K K A C L H L N P G G T C V S I G Y G Y A D R A S E S NS2 731  
 AGCAGUUGAAGACCAUGCCAUUAAGCUUAGUUGUUGALCAAGAAAGCCUGUUGUUGAUCUGAAUCCCGGGAACCUUGUCAGCAUAGGUUAUGGUUACCGCUGACAGGGCCAGGAAA  
 3840  
 ▶ pTRD-26  
 I I G A I A R O F K F S R V C K P K S S L E E T E V L F V F I G Y D R K A R T H NS2 771  
 GCAUCAUUGGUGCUAUGCCGGCAGUUCAGUUUUCGGGUUAGCAAACCGAAAUCCUACUUGAAGAGACGGAAGUUCUGUUUGUUAUUGGUAUGGUAUGGCAAGGCCCGUACCG  
 3960  
 pTRD-30  
 ~ nsP3  
 N P Y K L S S T L T N I Y T G S R L H E A G C A P S Y H V V R G D I A T A T E G NS3 17  
 ACAAUCCUUAACAGCUUUAUUAACCUAGACCAAUUAUUAACAGGUCCAGACUCCACGAAGCCGGAUGGACCCUUAUUAUUGGUGGAGGGGAAUUGCCACGGCCACCGAAG  
 4080  
 V I I N A A N S K G Q P G G G V C G A L Y K K F P E S F D L Q P I E V G K A R L NS3 57  
 GAGUGAUUAUUAUUGCUGUAACAGCAAAGGACAACUGCCGGAGGGGUGUGCGGAGCGCUGAUAAGAAAUUCCCGAAAGCUUGCAUUAUUAACGCCAUUCGAAUGGAAAGGCGGAC  
 4200  
 V K G A A K H I I H A V G P N F N K V S E V E G D K Q L A E A Y E S I A K I V N NS3 97  
 UGGUCAAAGGUGCAGCUAAACAUUAUUAUUGCCGUGAGGACAAACUUAACAAGAUUUGGAGGUGAAGGUGACAAACAGUUGGCAAGGCUUUGAUGUCCAUUGCUAAGAUUGUA  
 4320  
 D N M Y K S V A I P L L S T G I F S G N K D R L T Q S L N H L L T A L D T T D A NS3 137  
 ACGAAUACAUAUUAAGUCAGUAGGCAUUCACUGUUGUCCACCGGCAUCUUUUCGGGAAACAAGAUUGGACUAAACCAUUAUUAUUGGACCAUUGGUGACAGCUUUAAGACCAACUGAUG  
 4440  
 D V A I Y C R D K K W E M T L K E A V A R R E A V E E I C I S D D S S V T E P D NS3 177  
 CAGUAGUAGCCAUUAUUCUGCAGGACAGAAUUGGAAUUGACUCUACAGGAGCAGUGGCUAGGAGAGAAGCAGUGGAGGAGAUUGCAUUAUUGGACGACUUCAGUGACAGAACUG  
 4560  
 A E L V R V H P K S S L A G R K G Y S T S D G K T F S Y L E G T K F H Q A A K D NS3 217  
 AUGCAGAGCUGGUGAGGGUGCAUCCGAAGAUUCUUGGGUGGAAAGGAGGCUACAGCACAAGCGAUGGCAAAACUUUUCUUAUUAUUGGAAAGGACCAAGUUCACCGCCGGCCAAAGG  
 4680

FIGURE 1  
Page 3 of 6

I A E I N A M W P V A T E A N E Q V C M Y I L G E S M S S I R S K C P V E E S E NS3 257  
 AUAAAGCAGAAAUAUAGCCAUUGGCCUUGCAACCGGAGGCAUAGAGCAGGUAUGCAUUAUUCUCGGAGAAAGCAUGAGCAGUAUAGGUCGAAAUGCCCGUCGAAAGUCJCGG  
 4800  
 A [A]=Thr (TRD error)

A S S P P S T L P C L C I H A M T P E R V Q R L K A S R P E D I T V C S S F P L NS3 297  
 AAGCCUCUCACCACCUAGCAGCUGCCUUGCUUGUGCAUCCAUGCCAUGACUCCAGAAAGAGUACAGCGCCUAAAAGCCUCACGUCAGAAACAAUUAUCUGUGUCUACUUCUUCU  
 4920  
 [A]=Thr

P K Y R I T G V Q K I Q C S Q P I L F S P K V P A Y I H P R K Y L V E T P P V D NS3 337  
 UGCCGAAGUAUAGAAUACUCUGGUGUGCAGAAUCCAAGUCUCCAGCCUUAUUGUUCUACCCGAAAGUCCUGCGUAUUAUCCAAGGAAGUAUCUGGUGAAACACCACCGGUAG  
 5040

E T P E P S A E N Q S T E G T P E Q P P L I T E D E T R T R T P E P I I I E E E NS3 377  
 ACGAGACUCGGGAGCCAUCCGGCAGAGAACCAUCCACAGAGGGGACCCUGAACACCACCAUUAUACCGGAGGAGAGACCAGGACUAGAACGCCUGAGCCGAUCAUCAUGAAGAGG  
 5160

E E D S I S L L S D G P T H Q V L Q V E A D I H G P P S V S S S S W S I P H A S NS3 417  
 AAGAAAGAGUAUAGCAUAGUUGCUGKAGAUUGGCCGCCACCCAGGUGCUGCAAGUCGAGGAGACAUUCACGGGCGCCUCUGUAUCUAGCUCAUCUGGUCUACUUCUUGCAU  
 5280

D F D V D S L S I L D T L E G A S V T S G A T S A E T N S Y F A K S M E F L A R NS3 457  
 CCGACUUGAUUGGACAGUUAUCCAUUCUAGACACCCUGGAGGGAGUAGCGUGACCAGCGGGCAACGUCAGCCGAGACUAAUCUUAUCUUGCAAAGUAUGGAGUUCUGGGCC  
 5400

Repeat 1a Repeat 1b Repeat 2a  
 P V P A P R T V F R N P P H P A P R T R T P S L A P S R A C S R T S L V S T P P NS3 497  
 GACCGGUGCCUGGGCCUCGAACAGUAUUCAGGAACCCUCCACAUCCCGCUCGGCCACAAGAACCCGUCACUUGCACCCAGCAGGGCCUGCUGGAGAACAGCCUAGUUCACCCCGC  
 5520

Repeat 2b  
 G V N R V I T R E E L E A L T P S R T P S R S V S R T S L V S N P P G V N R V I NS3 537  
 CAGCGGUAUAGGUGUAUCUAGAGAGAGGAGUCGAGGGCCUACCCCGUACCGCACUCCUAGCAGGUCGGUCUCGAGAACCCAGCCUGGUCUCCAACCCGCCAGGGCUAAUAGGGUGA  
 5640  
 pTC-9 (Sst I #1) ← pTC-2 (Sst I #2)

T R E E F E A F V A Q Q Q \* R F D A G A Y I F S S D T G Q G H L Q Q K S V R Q T NS4 20  
 UUAACAAGAGAGGAGUUGAGGGCUGCUAGCACAACAACAAGACGGUUGUUGCGGGUGCAUACAUUUUCUCCGACACCGGUAAGGGCAUUAACAACAAAAUACAGUAGGCAAA  
 5760

V L S E V V L E R T E L E I S Y A P R L D Q E K E E L L R K K L Q L N P T P A M NS4 60  
 CGGUGCUAUCGGAAGUGGUGUUGGAGAGGACCGAAUUGGAGAUUUCUUGCCCGCCGUCGACCAAGAAAAAGAAUUAUACGCAAGAAAUUACAGUAAAUCCACACCUUGUA  
 5880  
 [A]=  
 Thr (TC-83 error)

R S R Y Q S R K V E N M K A I T A R R I L Q G L G H Y L K A E G K V E C Y R T L NS4 100  
 ACAGAAGCAGAUACAGUCCAGGAPGGUGGAGAACAUAGAAAGCAUACAGCUAGACGUUUUCUGCAAGGCCUAGGGCAUUAUUGAAGGCAGAAAGGAAAAGUGGAGUGCUACCGAACCC  
 6000

H P V P L Y S S S V N R A F S S P K V A V E A C N A M L K E N F P T V A S Y C I NS4 140  
 UGCAUCCUGUUCUUGUAUUAUCUAGUGGUAACCGUGCCUUAUCAAAGCCCAAGGUCGACUGGAGGACCGUUAACGCCAUGUUAAGAGAAACUUCGACUGGGUUCUUCUAGUA  
 6120

I P E Y D A Y L D M V D G A S C C L D T A S F C P A K L R S F P K K H S Y L E P NS4 180  
 UUAUUCAGAGUACGAUGCCUUAUUGGACAUGGUJGACGGAGCUUAGCUGCUGUAGACACUGCCAGUUAUUGCCUGCAAAGCUGCGCAGCUUCCAAAGAAACACUCCUUAUUGGAA  
 6240

T I R S A V P S A I Q N T L Q N V L A A A T K R N C N V T Q M R E L P V L D S A NS4 220  
 CCACAUACGAUCCGAGUGCCUUCAGCGAUCCAGAACACCGCUCAGAACCGUCCAGGAGCUGCCACAAAAGAAUUGCAAUGUCACGAAAUGAGAGAAUUGCCCGUAUUGGAAUCGG  
 6360

A F N V E C F K K Y A C H N E Y W E T F K E N P I R L T E E M V V N Y I T K L K NS4 260  
 CGGCCUUAUUGGAAUGCUCAAGAAUUAUGCGUUAUUAUGAAUUAUGGGAACGUUAUAAAGAAAACCCCAUAGGCUUAUJGAAGAAAACGUGGUAUUAUUAUUAUACCAAUUA  
 6480

G P K A A A L F A K T H N L N M L Q D I P M D R F V M D L K R D V K V T P G T K NS4 300  
 AAGGACCAAAAGCUGCUGCUUUAUUGGAGACACAUUAUUAUUAUUGUAGGAGCAUACCAUUGGACAGGUUUGUAUUGGACUUAUUAAGAGAGACGUGAAAGUGACUCCAGGAACA  
 6600

H T E E R P K V Q V I Q A A D P L A T A Y L C G I H R E L V R R L N A V L L P N NS4 340  
 AACAUUCUAGAAACGGCCCAAGGUACAGGUGAUCCAGGCGCCGUAUCCGUAACAGCGUAUCUGUCGGAAUCCACCGAGAGCUGGUUAGGAGAUAAAUGCGGUCUGCUCCGA  
 6720

FIGURE 1  
Page 4 of 6

I N T L F D M S A E D F D A I I A E H F Q P G D C V L E T D I A S F D K S E D D NS4 380  
 ACAUUCAUACACUGUUUGAUAUUGUCGGCUGAAGACUUGACGCUAUUAUAGCCGAGCACUCCAGCCUGGGGAUUGUUCUGGAAACUGACAUCGCUCUUGAUAAAAGUGAGGACG  
 6840  
 A M A L T A L M I L E D L G V D A E L L T L I E A A F G E I S S I H L P T K T K NS4 420  
 ACCCAUGGGCUCUGACCCGCUAAUUGAUUCUGGAAGACUUAAGGUGUGGACGCAGAGCUGUUGACCGCUGAUGAGGGCGCUUUCGGCGAAAUUUCAUCAAUAUCAUUGCCCAUAAAACUA  
 6960  
 F K F G A M M K S G M F L T L F V N T V I N I V I A S R V L R E R L T G S P C A NS4 460  
 AAUUAAAUUCGGAGCCAUUGAUAUUGGAAUUGUUCUACACUUGUUGAACAACAGUCAUUAACAUGUAUUCGCAAGCAGAGUGUUGAGAGAACGGCUAACCGGAUCACCAUGUG  
 7080  
 A F I G D D N I V K G V K S D K L H A D R C A T W L N M E V K I I D A V V G E K NS4 500  
 CAGCAUUCUUGGAGAUACAUAUUCGUGAAGGAGUCAAAUUCGGACAAAUUAUUGGCAGACAGGUGCGCCACCUGGUGAUAUUGGAAGUCAAGAUUAUAGAUUGCUGUGGUGGGCGAGA  
 7200  
 A P Y F C G G F I L C D S V T G T A C R V A D P L K R L F K L G K P L A A D D E NS4 540  
 AAGCGCCUUAUUCUGUGGAGGGUUAUUAUUGUGUGACUCCGUGACCGGCACAGCGUGCCGUGUGGCAGACCCCUAAAAAGGCUUUAAGCUGUGGCAAAACCUUGGCAGCAGACGAUG  
 7320

[C]=Pro  
(TC-83 error)

H D D D R R R A L H E E S T R W N R V G I L S E L C K A V E S R Y E T V G T S I NS4 580  
 AACAUUGAUGACAGGAGAAGGGCAUUGCAUGAAGAGUCAACACCGUGAACCGAGUGGGUUAUUCUUCAGAGCUGUGCAAGGCAGUAGAUAUCAAGGUUGAAACCGUAGGAACUCCA  
 7440  
 > pTC-5

- 26S

I V M A M T T L A S S V K S F S Y L R G A P I T L Y G  
 UCAUAGUUAUUGGCCAUGACUACUUCUAGCUAGCAGUGUAAAUAUUCAGCUACCUAGAGAGGGGCCCCUUAUAUCUCUUCAGGGCUAACCUGAUUGGACUACGACAUAGUCUAGUCGCCCAA  
 7560  
 [A]=(TRD error)

> pTRD-1

- C  
 H F P F Q P M Y P M Q P M P Y R M P F A A P R R P W F P R T D P F L A M Q V Q E C 40  
 GAUGUUCGCCUUCAGCCAUAUGUUCGGAUGCAGCCAAUUGCCUUAUGCAACCCGUGCGGGCCCCGGCGAGGCCUUGUUCGCCAGAACCCUUCUUGCCGAUGCAGGUGCAGGA  
 7680  
 L T R S M A N L T F K Q R R D A P P E G P S A K K P K K E A S Q K Q K G G G Q G C 80  
 AUUAACCCGUCGUAUGGCUAACCUAGCUGUACAAGCAAACCGCGGGACCGCCACCUGAGGGGCCAUCCGCUAAGAAAACCGAAGAAAGGAGGCCUCCGCAAAAACAGAAAAGGGGGAGCCAAAG  
 7800

pTRD-26 <  
 K K K K N Q G K K K A K T G P P N P K A Q N G M K K K T N K K P G K R Q R M V M C 120  
 GAAAGAAGAAAGAACCAAGGAAGAAGAGGCUAAGACAGGGCCGCCUUAUCCGAAGGCACAGAAUGGAAACAAGAAGAACCAACAAGAAACCGGCAAGAGACAGCGCAUGGUCU  
 7920

K L E S D K T F P I M L E G K I N G Y A C V V G G K L F R P M H V E G K I D N D C 160  
 GAAUUGGAAUUCGACAAAGACGUCCAAUUCAUUGUUGGAAAGGAUAAAACGGCUACCGUUGUGUGGUGCGGAGGGAAGUUAUUCAGGCCGAUGCAUGGGAAGGCAAGUCCGACAAACGA  
 8040

V L A A L K T K K A S K Y D L E Y A D V P Q N M R A D T F K Y T H E K P Q G Y Y C 200  
 CGUUCUGGCCCGCCUUAAGACGAAGAAAGCAUCCAAUACGAUCUUGAGUAGCAGAUUGGCCACAGAAUUCGCGGCGGUAUCAAUCAAUACCCCAUGAGAAAACCCCAAGGCUAUA  
 8160

S W H H G A V Q Y E N G R F T V P K G V G A K G D S G R P I L D N Q G R V A I C 240  
 CAGCUGGCAUUGGAGCAGUCCAAUUAUAAAUGGGCGUUCACGGUGCCGAAAGGAGUUGGGCCAAAGGAGACAGGGGACGACCCCAUUCUGGAUAACCGGACCGGUGGUCGCUAU  
 8280

- E3

V L G G V N E G S R T A L S V V M W M E K G V T V K Y T P E N C E Q W S L V T T E3 5  
 UGUGCUGGGAGGUGUAUGAAGGAUUCUAGGACAGCCUUCACUUCGUCUUGGGAACGAGAAGGGAGUACCGUGAAGUUAUUCUCCGGAGAACUGCGAGCAAUGGUCUAGUGACCAC  
 8400

M C L L A N V T F P C A Q P P I C Y D R K P A E T L A M L S V N V D N P G Y D E E3 45  
 CAUGUCUCUGCCCAUUGGAGGUCUCCAUUGGUCUAAACCAUUAUUGCUACGACAGAAAACCGAGAGACUUGGCCAUGCUCAGCGUUAACGUGAACAACCGGGCUACGAUGA  
 8520

- E2

L L E A A V K C P G R K R R S T E E L F K E Y K L T R P Y M A R C I R C A V G S E2 26  
 CGUCUGGAAGCAGCUGUAAGUGCCCCGGAAGGAAAAGGAGUCCACCAGGAGCUGUUAAGGAGUUAAGCUAACCGCCCUUAUUGGCCAGAUUGCAUCAGAUUGCAGUUGGGAG  
 8640

[T]=Asn

C H S P I A I E A V K S D G H D G Y V R L O T S S Q Y G L D S S G N L K G R T M E2 66  
 CUGCCAUUGUCCAAUAGCAAUCCAGGCAGUAAAGAGCGAGCGGGCAGCAGGUAUUGUUAAGACUUCAGACUUCUCCGCAUUAUGGCCUGGUAUUCUCCGGCAACUUAAGGGCAGGACCAU  
 8760

FIGURE 1  
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R Y D M H G T I K E I P L H Q V S L H T S R P C H I V D G H G Y F L L A R C P A E2 106  
 CGGGUAGCAUGCACGGGACCAUUAAGAGAUACCAUACAUCAGUGUCACUCUCAUCUCGCCGUGUCACAUUGUGGGAUGGGCACGGUUAUUCUGCUUGCCAGUGGCCCGGCGG  
 . . . . .  
 . . . . .  
 (T)=Tyr

G D S I T M E F K K D S V T H S C S V P Y E V K F N P V G R E L Y T M P P E H G E2 146  
 AGGGGACUCCAUCACCAUGBAUUAUUAAGAAUUCUGUCACACUCUCUGUCGCGUGGCCGUAUGAAGUGAAAUAUUAUCUGUAGGCAGAGAACUCUUAUACUCAUCCCCAGAACACGG  
 . . . . .  
 . . . . .  
 (G)=Arg

V E Q A C Q V Y A H D A Q N R G A Y V E M H L P G S E V D S S L V S L S G S S V E2 186  
 AGUAGAGCAAGCGUGCCAAGUCUACGCACAUGAUGCAAGAACAGAGGAGCUUAUGUCGAGAUACCCUCCGCGGUCAGAAUGGGACAGCAGUUGGUUUUCUUGAGCGGCGAGUACAGU  
 . . . . .  
 . . . . .  
 9120

T V T P P V G T S A L V E C E C G G T K I S E T I N K T K Q F S Q C T K K E Q C E2 226  
 CACCGUGACACCUCCUGUUGGGAUAGCGCCUGGUGGAUUGCGAGUGGGCGGCAAAAGAUCCCGAGACCAUACAAGAACAAAACAGUUCAGCCAGUCCAGUCACAAAGAAGGAGCAGUG  
 . . . . .  
 . . . . .  
 [A]=Asp [A]=Lys (TRD error)

R A Y R L Q N D K W V Y M S D K L P K A A G A T L K G K L H V P F L L A D G K C E2 266  
 CAGAGCAUUCGGGUCGAGAACGUAUAGUGGGUGUAUUAUUCGACAAACUGCCCAAAGCAGCGGGAGCCACCUAAAAAGAAAAUCGCAUGUCCCAUUCUUGCUGGGCAGACGGCAAAG  
 . . . . .  
 . . . . .  
 9360

T V P L A P E P M I T F G F R S V S L K L H P K N P T Y L T T R Q L A D E P H Y E2 306  
 CACCGUGCCUAGCACCAGAACCUAUGAUAAACCUUUGGUUUCAGAUCAUGUCACUGAAAUCGACCCUAAAGAUCCCAUAUUAUACCCCGCCAAUUGCUGAUGAGCCUCACUA  
 . . . . .  
 . . . . .  
 [C]=Phe [T]=Ile

T H E L I S E P A V R N F T V T E K G W E F V W G M H P P K R F W A Q E T A P G E2 346  
 CACGCACGAGCUCAUUCUGAACCCAGCUGUAGGAUUUUUACCGUACCCGAAAAAGGGUGGGAGUUUGUAGGGGAAACCCCGCCGAAAAGGUUUUGGGCACAGGAAACAGCACCCGG  
 . . . . .  
 . . . . .  
 9600

N P H G L P H E V I T H Y Y H R Y P M S T I L G L S I C A A I A T V S V A A S T E2 386  
 AAUCCACAUUGGGCUACCGCAGGAGGUAUACCUAUAUUAUACCAAGAUACCUAUUGUCCCAUUCUGGGUUGUCAUUUUGUGCCGCCAUUGCAACCGUUUCUGUCCAGGCGUCAC  
 . . . . .  
 . . . . .  
 9720

W L F C R S R V A C L T P Y R L T P N A R I P F C L A V L C C A R T A R A E T T 6K 3  
 CUGGCUGUUUUGCAGAUUCAGAGUUGCGUCCUAACUCUUAACCGGCUAACCAUACCGCUAGGUAUACCAUUAUGUCUGGCUUGCUUGCUGCGCCCGCCAGCUGCCCGGGCCGAGACCAC  
 . . . . .  
 . . . . .  
 9840

W E S L D H L W N N M Q Q M F W I Q L L I P L A A L I V V T R L L R C V C C V V 6K 43  
 CUGGGAGUCUUGGAUCACCUAUGGAACAUAACCAACAGAUUUCUGGUAUUAUUGUCUAGCCUUGGCGCCUUGAUUCUAGUGACUGCCUGUCUAGGUGGGUGUGCUGUGUCGU  
 . . . . .  
 . . . . .  
 [A]=Leu TRD error

P F L V M A G A A G A G A Y E H A T T M P S Q A G I S Y N T I V N R A G Y A P L E1 27  
 GCCUUUUUAGUCAUGGGCCGGCCGAGGCGCCGCGCCUACGAGCAGCGGACCAGUAGCCGAGCAAGCGGAAUUCUGUAUUAACAUUAGUCAACAGAGAGGGCUAGCCACCCU  
 . . . . .  
 . . . . .  
 10080

P I S I T P T K I K L I P T V N L E Y V T C H Y K T G M D S P A I K C C G S Q E1 67  
 CCCUUCAGCAUAAACCAACAAGAUCAAGCUGAUACCUACAGUGAACUUGGAGUAGCUCACCGCCACUAACAAAACAGAAUGGAUACCAAGCCAUCAAUUGCUGGGUUCAGCA  
 . . . . .  
 . . . . .  
 10200

C T P T Y R P D E Q C K V F T G V Y P F M W G G A Y C F C D T E N T Q V S K A Y E1 107  
 AUGCACUCCAUCUACAGGCCUGAUGAACAGUGCAAGUUCUACAGGGGUUUAACCCGUCAUGUGGGUGUGCAUUAUGCUUUUGCCACUCUGAGAACCCCAAGUCAGCAAGGCCUUA  
 . . . . .  
 . . . . .  
 10320

V M K S D D C L A D H A E A Y K A H T A S V Q A F L N I T V G E H S I V T T V Y E1 147  
 CGUAAUGAAAUCUGAGCAGUCCUUGCGGUAUCUGCUGAAGCAUUAUAAAGCGCACAGCCUCAGUGCAGGGGUUCUCAAACAUACAGUGGGAGAACACUUAUUGGACUACCGUGUA  
 . . . . .  
 . . . . .  
 [T]=Val (TC-83 error)

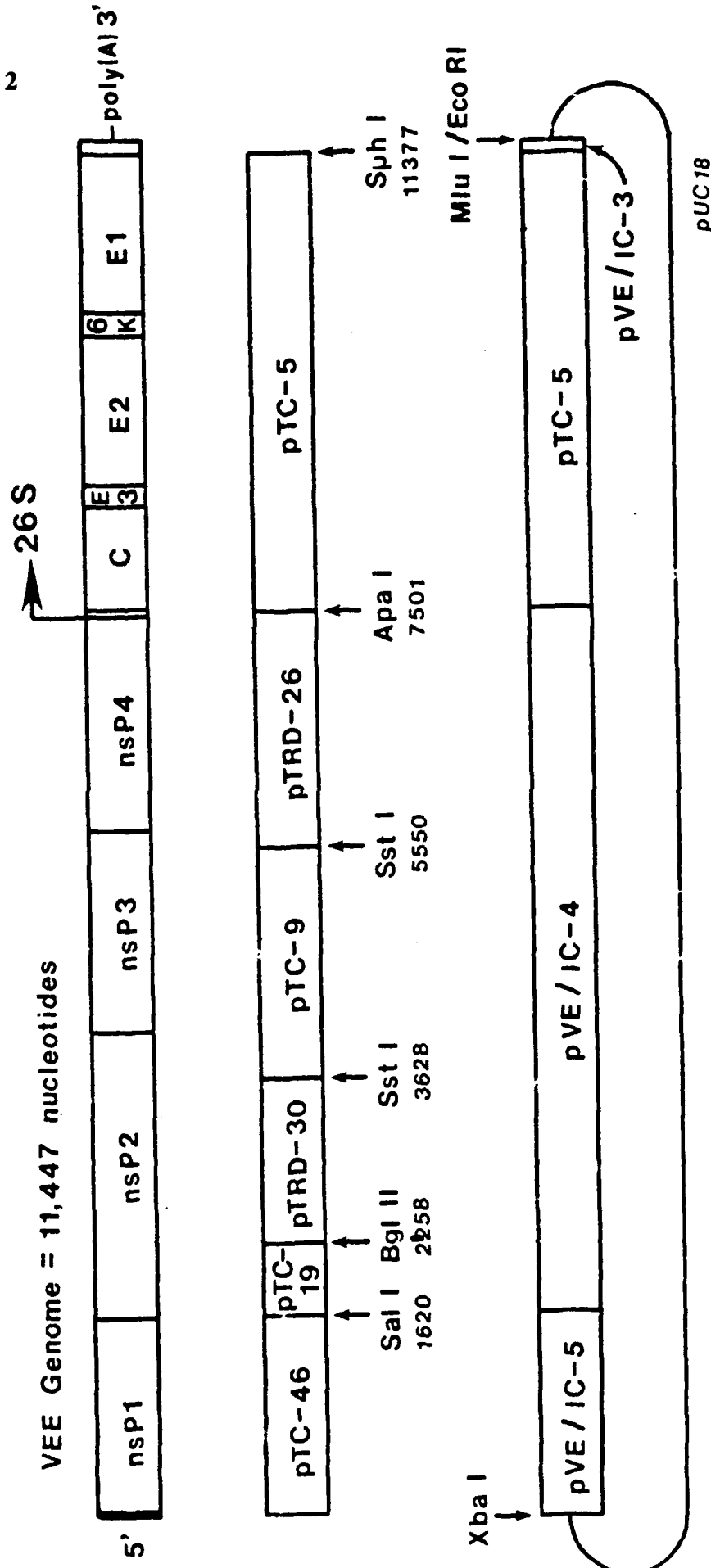
V N G E T P V N F N G V K L T A G P L S T A W T P F D R K I V Q Y A G E I Y N Y E1 187  
 UGUAAUGGAGAAAUCUCCUGGUAUUUUAUUGGGGUAUUUAUUAUCUGCAGGUCGGUUCACAGCUUGGACACCCUUGAUGCGAAAUCUGGUCAGUAGCCGGGGGAGUUAUUAUUA  
 . . . . .  
 . . . . .  
 [A]=Ile

D F P E Y G A G Q P G A F G D I Q S R T V S S S D L Y A N T N L V L Q R P K A G E1 227  
 UGAUUUUCUGAGUUGGGCAGGACAACAGGAGCAUUGGAGAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUAUUA  
 . . . . .  
 . . . . .  
 [T]=Ser



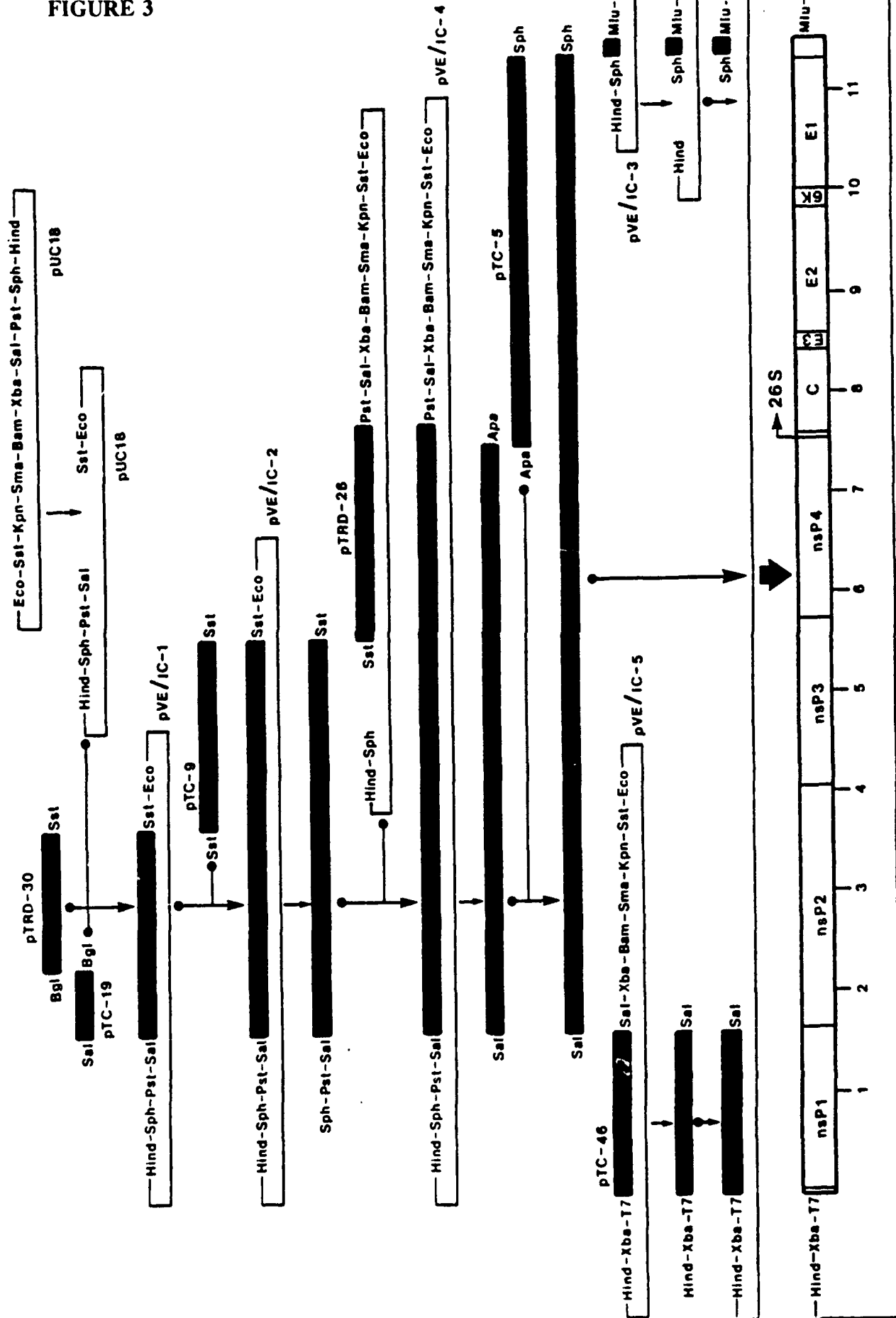


FIGURE 2



pVE / IC-92 | 11,447 bp + 2698 bp = 14,145 bp |

FIGURE 3



pVE/IC-92 | 11447 bp + 2698 bp = 14145 bp |

FIGURE 4

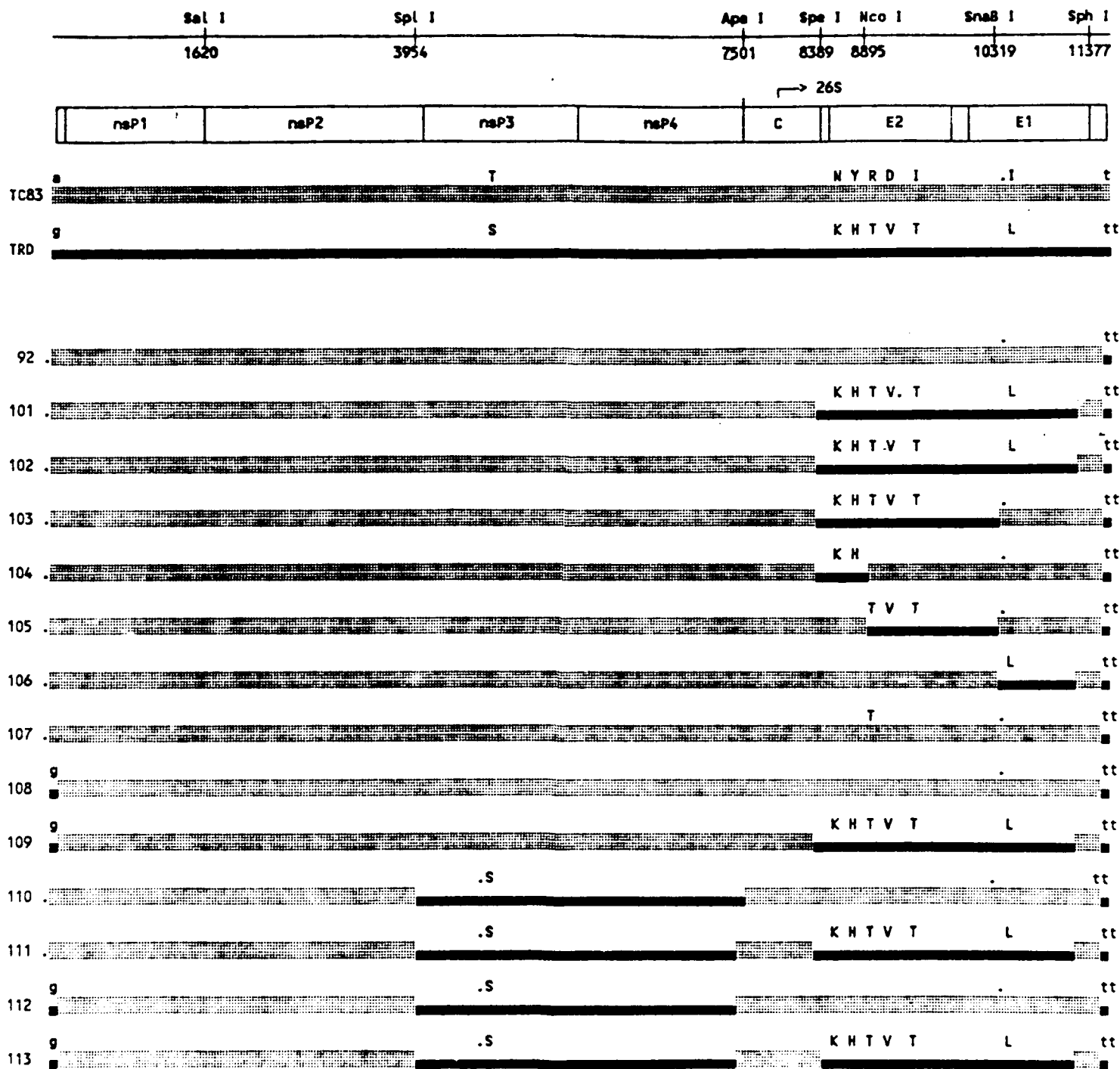


FIGURE 5

Translated 26S Polypeptide Precursor: Amino Acid Sequence

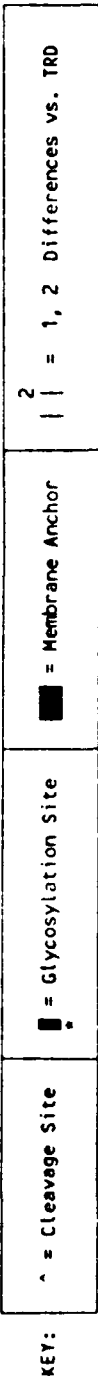
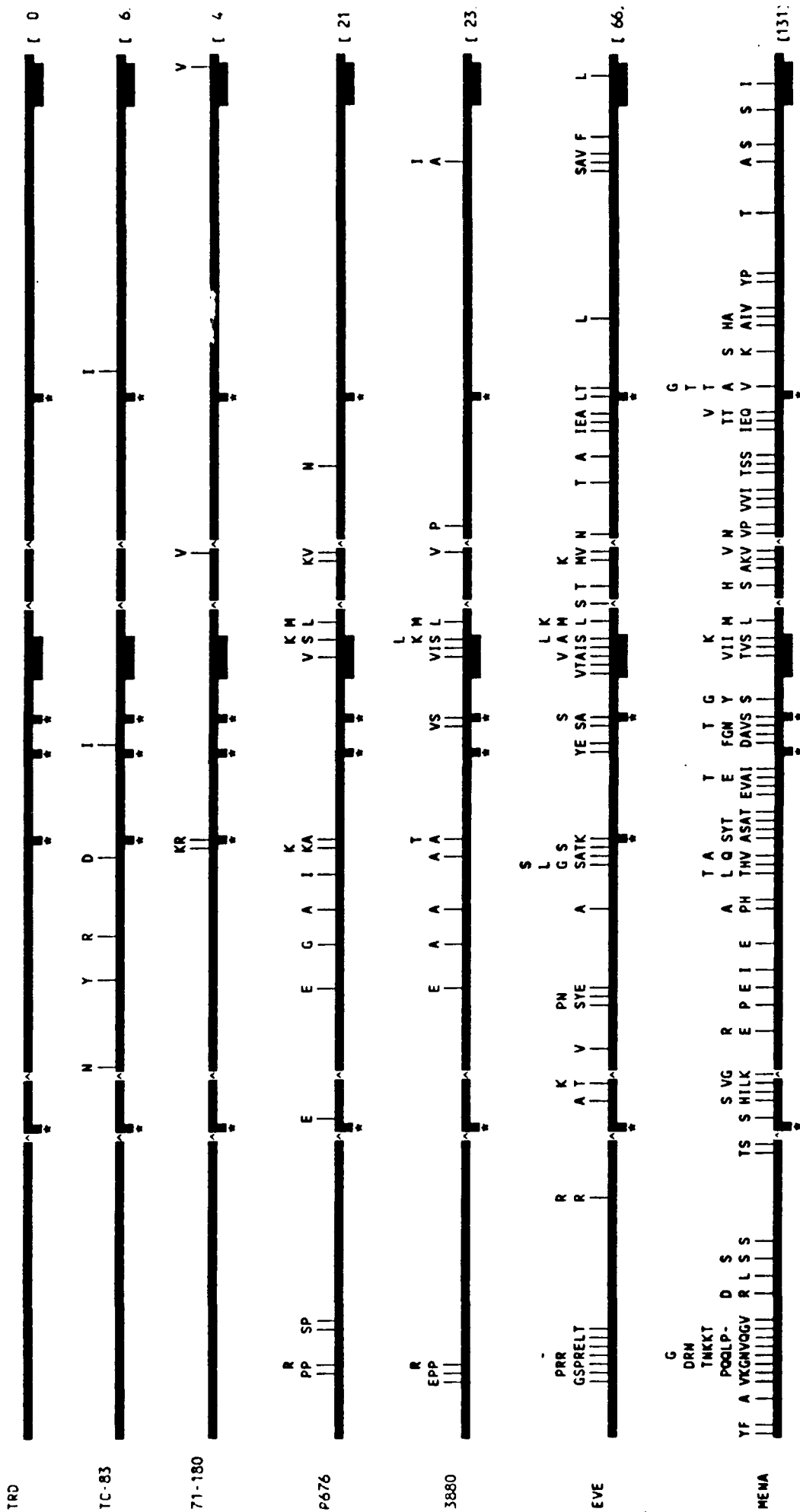


FIGURE 6

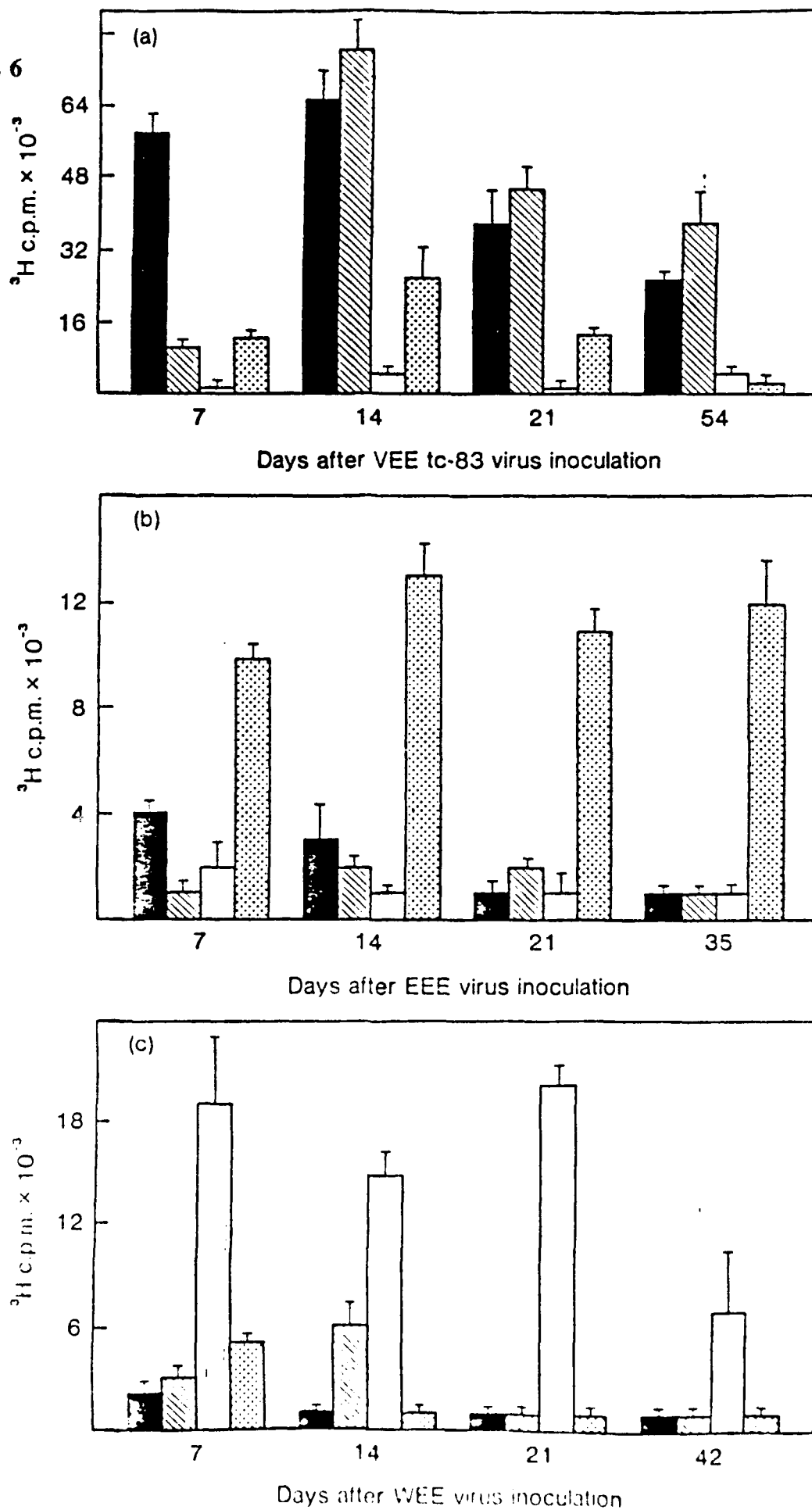
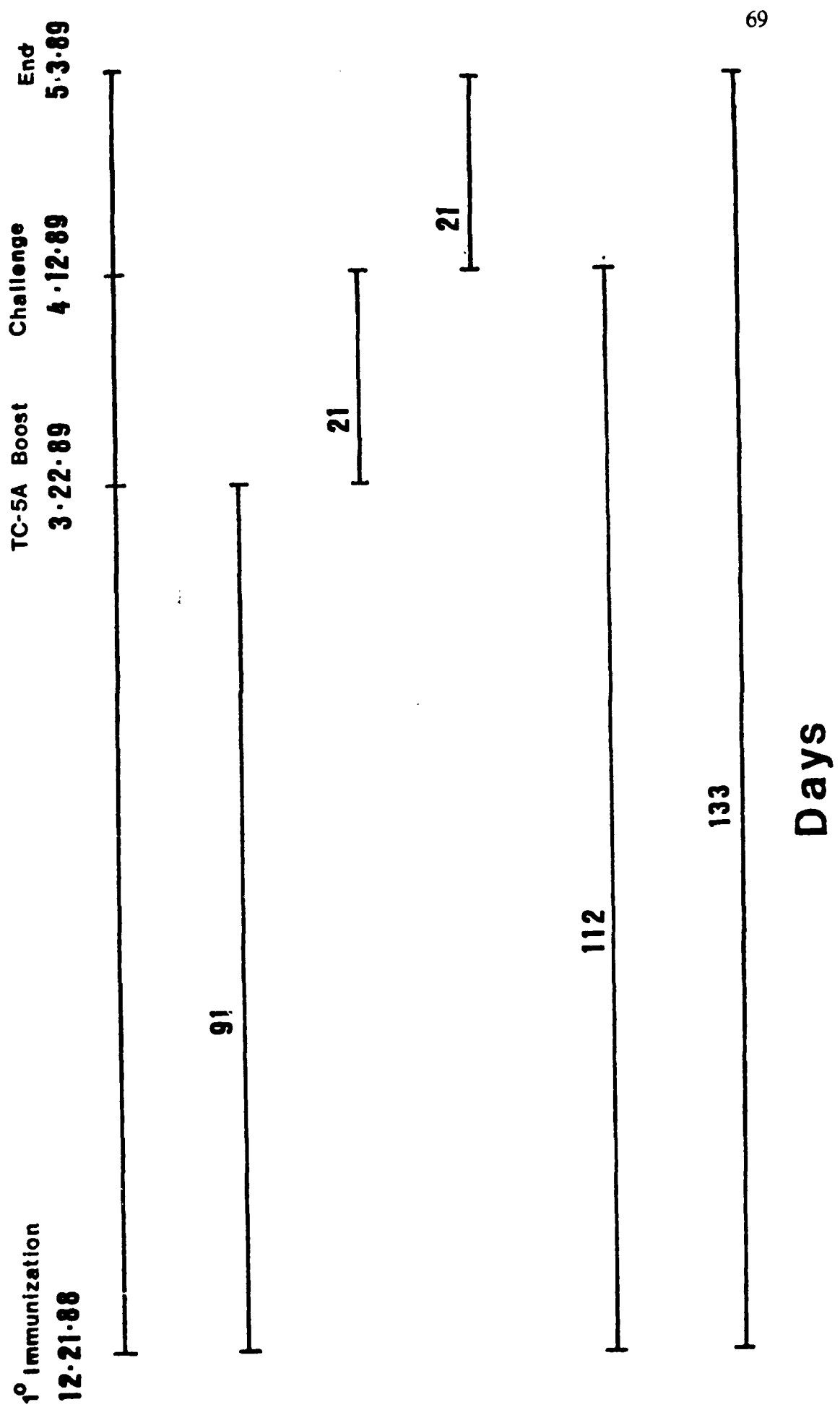


FIGURE 7



## FIGURE LEGENDS

**Figure 1:** Annotated full-length nucleotide and deduced amino acid sequences of the RNA genome of VEE TRD virus. The nucleotide sequence is numbered continuously from 1 through 11447. The amino acid sequences of the nonstructural and structural polypeptide regions are numbered using partitioned annotation. The single letter amino acid abbreviation is aligned with the first nucleotide of its codon.

The putative 26S mRNA start site and proteolytic cleavage sites of the translated nonstructural and structural polyproteins are indicated. The two peptide repeats in nsP3 and the transmembrane domains of the E1 and E2 envelope glycoproteins are indicated by overlying solid bars.

\* = Glycosylation site (N-X-S or T)

= Confirmed nucleotide sequence difference between TRD and TC-83 virus genomes

= Nonconfirmed nucleotide sequence differences ("artifacts," "errors," or variants) between TRD and TC-83 virus-specific cDNA clones

**Figure 2:** General construction strategy for pVE/IC-92. The cDNA regions and restriction enzyme splice sites used in the construction of the full-length cDNA clone of VEE TC-83 virus are shown. Genome nucleotide positions of the restriction enzyme sites used in the splicing are indicated.

**Figure 3:** Construction details for pVE/IC-92. cDNA regions that were used in the construction are shown as solid bars. The donor clones and restriction enzyme splice sites are indicated. The pUC18 plasmid backbone is indicated by open rectangles and available polylinker (MCS) restriction sites. Solid dots indicate ligated DNAs, arrows the ligated product. The organization of the full-length VEE TC-83 cDNA in pUC18, including kilobase length markers, is shown in the schematic diagram at the bottom of the figure. Restriction enzyme sites shown are Apa I, BamH I, Bgl II, EcoR I, Hind III, Kpn I, Mlu I, Pst I, Sma I, Sph I, and Xba I.



- Figure 4:** Summary schematic diagram of pVE/IC-92 and the recombinant full-length cDNA clones derived therefrom. cDNA splice sites and nucleotide positions, genome organization of VEE virus, TC-83 and TRD virus genomes annotated with amino acid (upper case, coding regions) and nucleotide (lower case, 5'- and 3'-noncoding regions), and annotated full-length cDNA constructs of TC-83 virus (pVE/IC-92), recombinant TC-83/TRD viruses (pVE/IC-100 - pVE/IC-102), putative wild-type TRD virus (pVE/IC-113) are shown. cDNA regions derived from pVE/IC-92 are indicated by wide, lightly stippled bars. Those regions resulting from insertion of appropriate domains TRD virus cDNA into the pVE/IC-92 backbone are indicated by narrow, solid bars. Single dots indicate positions of known cDNA "errors": (a) the extraneous G nucleotide at the 5'-end of the genome, (b) the pTC-5 error at genome position 10356 (C-to-T error; E1-119 Ala-to-Val error), and the pTRD-26 error at genome position 4698 (G-to-A error; nsP3-223 Ala-to-Thr error). All of the full-length cDNA clones have the TRD-sense 3'-noncoding region (TT versus TC-83 T at TRD positions 11408 - 11409).
- Figure 5:** Comparison of the amino acid sequences of structural genes comprising Venezuelan equine encephalitis viruses TRD (IAB), TC-83 (IAB), 71-180 (IAB), P676 (IC), 3880 (ID), Everglades (II), and Mena (IE) viruses.
- Figure 6:** Lymphoproliferative response of (a) VEE TC-83, (b) EEE, and (c) WEE virus-primed and pooled (three to six mice per virus group) NW-purified responder T cells. Stimulator cells were infected with purified viruses VEE TC-83 (solid columns), VEE Trinidad (hatched columns), WEE (blank columns) and EEE (dotted columns). Bars represent the adjusted mean counts per minute in three replicates with the standard error.
- Figure 7:** Time line indicating the days following immunization of horses with TC-83 (112 days), TC-5A (91 days after the primary immunization and 21 days following the second immunization) and wild-type vaccinia (112 days) prior to virulent virus challenge at 112 days. Control horses were euthanized on day six following challenge. The horses immunized with TC-83 and TC-5A were euthanized on day 21 after virulent virus challenge.