Immunochemical and Oligonucleotide Fingerprint Analyses of Venezuelan Equine Encephalomyelitis Complex Viruses

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SUMMARY

RNA oligonucleotide fingerprint analyses indicate that the genome RNA obtained from Trinidad donkey (TRD) Venezuelan equine encephalomyelitis (VEE) virus serotype I A, its vaccine strain derivative TC-83, and the VEE I B virus isolate PTF-39, have almost identical patterns of characteristic ribonuclease T₁ resistant oligonucleotides. The TC-83 strain and the I B isolate can, on the basis of these analyses, be considered as variants of the TRD virus and categorized as I AB serotypes. Comparisons made by single and co-electrophoreses of the ribonuclease T₁ digests of the RNA species of TC-83 and a VEE I C isolate P676 indicate that 16 of 37 large oligonucleotides of the TC-83 virus co-migrate with the oligonucleotides obtained from the I C isolate. Similar single and co-electrophoreses of ribonuclease T₁ digests of the RNA species of TC-83 and a VEE I D isolate 3880 indicate that 18 of 41 TC-83 large oligonucleotides co-migrate with the oligonucleotides obtained from the I D virus isolate. At least nine of the TC-83 large oligonucleotides appear on the basis of these analyses, to be present in the digests of the genome RNA obtained from these selected I B, I C and I D virus isolates. The ribonuclease T₁ digests of three I E virus isolates (Mena II, 63U2 and 71U388) give oligonucleotide fingerprints which, although comparable to each other, are more distinct from the I A and I B RNA fingerprints than are those of the I C and I D RNA species. The ribonuclease T₁ resistant oligonucleotide fingerprints of VEE virus isolates belonging to serotypes (VEE subtypes) II, III and IV show little similarity to each other or to those of the serotype I virus isolates we have studied. The results obtained here agree with the reported close antigenic relationships of VEE, I A, I B, I C and I D virus isolates, and our studies suggest that these viruses have conserved nucleotide sequences. The I E virus isolates appear to have more distinct nucleotide sequences than do the other serotype I viruses. The results also agree with the serological differentiation of VEE, I, II, III and IV subtypes in that the oligonucleotide fingerprints of subtypes II to IV are different from each other and from those of the different serotype I virus isolates. On the basis of antigenic and genome relationships, VEE isolates can be classified as serotypes I to IV with serotype I viruses differentiated into the categories I AB, I C, I D and I E.

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Antigenic similarities established by haemagglutination inhibition (HI), complement-fixation (CF) and neutralization of infectivity tests have led to the establishment of the alphavirus genus of the family Togaviridae (Casals, 1957, 1963; Fenner, 1976). By serological analyses, 16 of the 20 registered alphaviruses have been placed in three groups or serocomplexes: the Venezuelan equine encephalomyelitis (VEE) complex, the western equine encephalomyelitis (WEE) complex and the Semliki Forest virus complex (Berge, 1975; Karabatsos, 1975; Chanas et al. 1976). The VEE complex includes, in addition to the type VEE virus, three other serologically distinguishable registered alphaviruses: the VEE subtype II Everglades FE3-7C isolate (EVE), the VEE subtype III Mucambo Be An8 isolate (MUC), and the VEE subtype III Pixuna Be Ar35645 isolate (PIX). Although these viruses are related to subtype IA Trinidad donkey 1943 virus isolate (TRD), they can be serologically differentiated (Shope et al. 1964; Young & Johnson, 1969a; Johnson & Martin, 1974; Scherer & Pancake, 1977; France et al. 1979). Young & Johnson (1969a), using antisera produced in the spiny rat (Proechimys semispinosus), examined a large number of VEE virus strains from all areas of the Americas by short-incubation HI testing. They thereby categorized the VEE complex viruses into four subgroups (I to IV), and within subtype I they recognized five antigenic variants (IA to IE), with subtype IA including the prototype VEE isolate (Kubes & Rios, 1939) and the TRD isolate. Johnson & Martin (1974) suggested that the epizootic variants IA, IB and IC were antigenically very similar and that viruses should be grouped into a single category designated IABC. Antisera prepared in rhesus monkeys (Monath et al. 1974) or chickens (Scherer & Pancake, 1977) have not been serologically specific for VEE virus subtype I viruses, although with most VEE virus antisera the subtype I, II, III and IV viruses could be easily distinguished. Recently France et al. (1979), using rabbit antisera raised against the largest envelope glycoprotein (gp-57) of VEE virus in the HI test, were able to separate the VEE complex viruses into the four subtypes (and the antigenic varieties of subtype I) described by Young & Johnson (1969a).

Biochemical studies of the structural proteins of the VEE complex viruses by gel electrophoresis have given five distinguishable polypeptide patterns with differences in the number and mol. wt. of the envelope proteins (Pederson et al. 1975; Pedersen & Eddy, 1975). These differences, however, do not correlate completely with either the antigenic or epidemiological classification of VEE strains (Young & Johnson, 1969a; Johnson & Martin, 1974; France et al. 1979). Although recent studies by France et al. (1979) indicate that SDS gel electrophoresis patterns cannot unequivocally be used to classify VEE virus strains, Jahrling & Eddy (1977), have recently shown that hydroxyapatite column chromatography of the polypeptides of VEE virus can be used to differentiate the various VEE subtypes, correlating well with the serological typing scheme of Young & Johnson (1969a).

In this study we used the technique of oligonucleotide fingerprinting of ribonuclease T1 resistant oligonucleotides to investigate the genome relationships of VEE complex viruses. This technique has been used to characterize the RNA species of several groups of single stranded RNA containing animal viruses, including the picornaviruses (Frisby et al. 1976; Harris et al. 1977), rhabdoviruses (Clewley et al. 1977b), alphaviruses (Wengler et al. 1977) and bunyaviruses (Clewley et al. 1977a). We report the results of analyses of 11 VEE viruses of subtypes I, II, III and IV isolated from various parts of the Americas. The evidence presented indicates that VEE IA and IB isolates can be considered as virus variants, while the isolates representing the IC, ID and to a lesser extent IE viruses, are more distantly related to the IA and IB viruses. The oligonucleotide fingerprints of the subtype II (EVE), III (MUC)
### Table 1. Strains of Venezuelan equine encephalomyelitis virus studied

<table>
<thead>
<tr>
<th>Virus strain*</th>
<th>HI subtype†</th>
<th>Original host</th>
<th>Geographic origin</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trinidad donkey 1 (TRD)</td>
<td>IAB</td>
<td>Donkey</td>
<td>Trinidad</td>
<td>1943</td>
</tr>
<tr>
<td>TC-83</td>
<td>IAB</td>
<td>Vaccine derivative of Trinidad donkey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTF-39</td>
<td>IAB</td>
<td>Human</td>
<td>Guatemala</td>
<td>1969</td>
</tr>
<tr>
<td>P676</td>
<td>IC</td>
<td>Anopheles triannulus</td>
<td>Venezuela</td>
<td>1963</td>
</tr>
<tr>
<td>3880</td>
<td>I D</td>
<td>Human</td>
<td>Panama</td>
<td>1961</td>
</tr>
<tr>
<td>Mena II</td>
<td>IE</td>
<td>Human</td>
<td>Panama</td>
<td>1961</td>
</tr>
<tr>
<td>63U2</td>
<td>IE</td>
<td>Sentinel hamster</td>
<td>Mexico</td>
<td>1963</td>
</tr>
<tr>
<td>7U388</td>
<td>IE</td>
<td>Sentinel hamster</td>
<td>Guatemala</td>
<td>1971</td>
</tr>
<tr>
<td>FE3-7c (EVE)</td>
<td>II</td>
<td>Mosquito pool (Culex)</td>
<td>U.S.A.</td>
<td>1963</td>
</tr>
<tr>
<td>Be An8 (MUC)</td>
<td>III</td>
<td>Cebus apella</td>
<td>Brazil</td>
<td>1954</td>
</tr>
<tr>
<td>Be Ar35645 (PIX)</td>
<td>IV</td>
<td>Anopheles nimbus</td>
<td>Brazil</td>
<td>1961</td>
</tr>
</tbody>
</table>

* Virus abbreviations are EVE for Everglades, MUC for Mucambo and PIX for Pixuna.

† Designation of subtypes (serotypes) based on short-incubation HI results using antisera raised against the VEE virus envelope glycoprotein gp-57 (France et al. 1978).

and IV (PIX) viruses are unique and quite distinct from those of the subtype I viruses. The results confirm the currently accepted classification of VEE complex viruses with the exception that VEE IA and IB isolates appear to be variants of each other.

### METHODS

**Viruses.** The VEE virus strains used in these studies have been cloned in Vero cell monolayers and are designated by the subtype classification of Young & Johnson (1969a), isolation identification code, and geographic origin (Table 1).

**Infection of cells and virus purification.** Confluent monolayers of CER cells (Wiktor et al. 1977) were infected with stock virus at a multiplicity of 0.001 p.f.u./cell and overlaid with 30 ml of Eagle's minimal essential medium supplemented with 2% (v/v) foetal calf serum. The infected cells were incubated at 37 °C for 3 h, at which time 100 μCi/ml of carrier-free 32P-orthophosphate (ICN, Irvine, Calif.) was added and the cultures were incubated at 37 °C for an additional 36 h. The supernatant fluids were recovered and the cell debris removed by centrifugation at 4 °C for 30 min at 10000 g in a Sorvall RC-2B centrifuge. Virus was purified from these fluids by polyethylene glycol precipitation followed by sedimentation first in a combination gradient of potassium tartrate and glycerol, and then on a sucrose gradient (Obijeski et al. 1974). The virus band recovered from the sucrose gradient was diluted fourfold with the buffer TNE (0.01 M-tris-HCl, pH 7.8, 0.15 M-NaCl and 0.002 M-EDTA) and pelleted through a cushion of 35% (w/v) sucrose in TNE buffer by centrifugation at 200000 g for 180 min at 4 °C in a Beckman SW 40 rotor. Virus pellets were resuspended in TNE and stored at −70 °C until used.

**Extraction of RNA from virus particles.** Suspensions of VEE virus were treated with Proteinase (Beckman Instruments Inc., Palo Alto, Calif.) solubilized with SDS and the RNA extracted with a 25:24:1:0.01 (w/w) mixture of phenol:chloroform:isoamylalcohol:8-hydroxyquinoline. After extraction, the RNA was precipitated with 2 vol. of ethanol for 18 h at −20 °C (Trent et al. 1979). The RNA was reprecipitated once with ethanol and resuspended in TE buffer (0.02 M-tris-HCl, pH 7.4 and 0.002 M-EDTA) and stored at −70 °C.

**Digestion of virus RNA species with ribonuclease T1; two-dimensional polyacrylamide gel electrophoresis of ribonuclease T1 resistant oligonucleotides.** Virus RNA species labelled with 32P were digested for 30 min at 37 °C with 10 units of ribonuclease T1 and the resulting oligonucleotides separated by two-dimensional gel electrophoresis (de Wachter & Fiers,
Radioimmune precipitation of VEE viruses by subunit antisera. $^3$H-leucine labelled 200 ng quantities of VEE subtype IA, Trinidad donkey (TRD) virus (●), subtype II, FE3-7C virus (▲), subtype III, Mucambo Be An8 virus (◇), or subtype IV, Pixuna Be Ar35645 virus (○), were treated with (a) TRD gp-57 subunit antisera, or (b) TRD gp-50 subunit antisera, as described in the text. Each point represents an average of triplicate samples.

Analyses of selected oligonucleotides recovered from the two dimensional polyacrylamide gels. Radioactive oligonucleotides were eluted from the two-dimensional polyacrylamide gels, digested with ribonuclease A, and the products resolved by two-dimensional chromatography using polyethyleneimine thin layer plates (Volckaert & Fiers, 1977).

Preparation of antisera and serological tests. Antisera to VEE virus envelope glycoproteins were prepared in rabbits as described previously (France et al. 1979).

The radioimmune precipitation (RIP) test for assaying alphavirus antibodies has been described (Trent et al. 1979). Briefly, 250 ng of purified $^3$H-leucine labelled VEE virus and 0.1 ml of diluted antiserum were incubated for 1 h at 37 °C. Formaldehyde heat-fixed protein A-bearing *Staphylococcus aureus* cells were then added as an immunoadsorbent,
Table 2. Radioimmune precipitation tests using antisera to gp-57 envelope glycoproteins of VEE virus strains

<table>
<thead>
<tr>
<th>VEE virus subtype</th>
<th>TRD (IA)</th>
<th>PTF-39 (IB)</th>
<th>P676 (IC)</th>
<th>388o (D)</th>
<th>Mena (IE)</th>
<th>EVE (II)</th>
<th>MUC (III)</th>
<th>PIX (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRD (IA)</td>
<td>25,600†</td>
<td>800</td>
<td>1,600</td>
<td>800</td>
<td>≤ 100</td>
<td>400</td>
<td>1,600</td>
<td></td>
</tr>
<tr>
<td>PTF-39 (IB)</td>
<td>25,600</td>
<td>400</td>
<td>6,400</td>
<td>3,200</td>
<td>≤ 100</td>
<td>800</td>
<td>1,600</td>
<td></td>
</tr>
<tr>
<td>P676 (IC)</td>
<td>25,600</td>
<td>3,200</td>
<td>12,800</td>
<td>6,400</td>
<td>1,600</td>
<td>400</td>
<td>≤ 100</td>
<td></td>
</tr>
<tr>
<td>388o (D)</td>
<td>12,800</td>
<td>800</td>
<td>3,200</td>
<td>3,200</td>
<td>800</td>
<td>1,600</td>
<td>200</td>
<td>3,200</td>
</tr>
<tr>
<td>Mena II (IE)</td>
<td>12,800</td>
<td>1,600</td>
<td>3,200</td>
<td>100</td>
<td>800</td>
<td>200</td>
<td>1,600</td>
<td>800</td>
</tr>
<tr>
<td>EVE (II)</td>
<td>3,200</td>
<td>1,600</td>
<td>200</td>
<td>400</td>
<td>≤ 100</td>
<td>12,800</td>
<td>≤ 100</td>
<td>400</td>
</tr>
<tr>
<td>MUC (III)</td>
<td>100</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>400</td>
<td>≤ 100</td>
<td>≤ 100</td>
</tr>
<tr>
<td>PIX (IV)</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td>400</td>
<td>≤ 100</td>
<td>25,600</td>
</tr>
</tbody>
</table>

* Abbreviations: TRD, Trinidad donkey; EVE, Everglades FE3-7C; MUC, Mucambo, Be An8; PIX, Pixuna, Be Ar35645.
† Results expressed as the serum dilution 50% precipitation endpoint.

and the mixture was incubated for 30 min at 25 °C (Trent et al. 1979). The immunoglobulin-coated cells were collected by centrifugation and the bound radioactivity was measured. Endpoints are expressed as the serum dilution which precipitated 50% of the radioactive virus in the mixture.

RESULTS

The serological relationships of the VEE complex viruses used in these studies

Rabbit antisera raised against the VEE glycoprotein gp-57 purified by isoelectric focusing as a pI 9.0 fraction, have been shown to react very specifically in the short incubation HI test (France et al. 1979). With such sera it has been possible using radioimmune precipitation tests (RIP), to confirm the serological groupings of the VEE complex viruses obtained by Young & Johnson (1969a), i.e. subtype I viruses can be categorized into the antigenic varieties IA, IB, IC, ID and IE and also differentiated from the subtype II, III, and IV VEE viruses. Homologous and heterologous RIP reactions to VEE complex viruses with antisera raised against their respective gp-57 glycoprotein preparations are shown in Fig. 1a and Table 2. With regard to the serological cross-reactivities of the TRD (IA), EVE (II), MUC (III) and PIX (IV) viruses, some one-way cross-reactivity was detected between the IA virus antiserum and the VEE subtype II virus, suggesting that they share certain antigenic determinants. Little cross-reactivity was detected between the subtype II, III and IV viruses. Between the other subtype I members and the subtype II, III and IV viruses, different amounts of low cross-reactivities (in most cases) were found (see Table 2).

In the serological tests involving particular subtype I viruses, although the homologous test results were not always the highest, extensive reactivities were found (Table 2). The results indicate that VEE virus haemagglutinins on the gp-57 envelope glycoprotein of subtypes IA, IB, IC, ID and IE are antigenically related (Young & Johnson, 1969a; Johnson & Martin, 1975; Scherer & Pancake, 1977; France et al. 1979). Likewise results indicate that as far as the haemagglutinin antigenic determinants are concerned, the subtype I, II, III and IV viruses are antigenically quite distinct.

RIP tests have also been performed with antisera raised against the purified gp-50 envelope proteins of TRD, EVE and PIX viruses. As seen in Fig. 1(b) and Table 3, antisera to the gp-50 of TRD virus reacted at high titre with its homologous virus, at much lower titre with EVE and MUC viruses and not at all with PIX virus. The cross reactions
Table 3. Radioimmune precipitation tests using antisera to the gp-50 envelope glycoproteins of prototype VEE virus strains

<table>
<thead>
<tr>
<th>VEE virus strain</th>
<th>Antisera to gp-50 glycoprotein of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRD (IA)</td>
</tr>
<tr>
<td>TRD (IA)</td>
<td>25600†</td>
</tr>
<tr>
<td>EVE (II)</td>
<td>200</td>
</tr>
<tr>
<td>MUC (III)</td>
<td>100</td>
</tr>
<tr>
<td>PIX (IV)</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

* Abbreviations: TRD, Trinidad donkey; EVE, FE3-7C; MUC, Mucambo, Be An8; PIX, Pixuna, Be Ar35645.
† Results expressed as the serum dilution 50% precipitation endpoint.

observed with the TRD gp-50 antiserum are comparable to the RIP cross reactions obtained with antibodies raised against TRD gp-57 (Table 2). The antiserum to the gp-50 of the subtype II virus reacted (1) at high titre with its homologous virus, (2) at a significant but lower serum dilution with TRD virus, and (3) not at all with the subtype II and IV viruses. The PIX gp-50 antiserum is type specific, as shown in Table 2. These results with the gp-50
Fig. 3. The oligonucleotide fingerprints of ribonuclease T<sub>1</sub> digests of <sup>32</sup>P-labelled RNA species of VEE subtype I viruses. (a) IA TRD; (b) IA TC-83; (c) IB PTF139; (d) IC P676; (e) ID 3880; (f) IE Mena II. For technical details see text and the legend to Fig. 2. In (a) and (c) the oligonucleotides that are missing (*), or new (arrows) when compared to the fingerprint of (b) are indicated.
It

e% d ~ °oee
• o
o O~ e
• o

t)

Fig. 4. Co-electrophoreses of the ribonuclease T₁ digests of ³²P-labelled VEE subtype I viruses. (a) IA TC-83 and ID 388o virus RNA digests; (c) IA TC-83 and IC P676 RNA digests; schematic representations of these are shown in (b) and (d) respectively, with the TC-83 oligonucleotides represented by closed circles, those of the other virus by open circles, and shared oligonucleotides by half-filled circles. For discussion of the cross hatched areas, see text.

antisera demonstrate the serological differentiation of VEE subtype I, II, III and IV viruses, particularly with regard to the distant antigenic relationships of MUC and PIX viruses with the other VEE subtypes.

Oligonucleotide fingerprint analyses of subtype I VEE virus RNA species

Two procedures have been used to compare the ribonuclease T₁ resistant oligonucleotide fingerprints of the genome RNA species of different complex viruses: (1) direct visual comparison of the maps of individual VEE viruses using the internal dye markers as reference points; and (2) comparison of co-electrophoreses of the digests of two VEE RNA species with those of the single VEE RNA digests.

We have used the reference strain Trinidad donkey (TRD) and its TC-83 vaccine derivative as a basis for comparing the oligonucleotide fingerprints of the various VEE complex viruses. The oligonucleotide fingerprint obtained for TRD is shown in Fig. 2(a), with an
oligonucleotide spot schematic given in Fig. 2(b). The fingerprint of TC-83 is given in Fig. 3(b). The final positions of the two reference dye markers, bromophenol blue (x, top centre) and xylene cyanol FF (x, bottom left) are indicated in Fig. 2(a, b). Not shown in the print is the polyadenosine tract which appeared in the lower left portion of the electropherogram. For different digests of TRD RNA or the RNA species of other VEE viruses, the pattern obtained is highly reproducible with the exception of one spot (No. o). We have observed that either spot No. o is present (see Fig. 3b for TC-83) or, when absent, an alternative spot sometimes appears just above the spots No. 12 and 13 (see Fig. 8a also for TC-83). The alternative spot is often large, not as well defined and much fainter than the other spots in the electropherogram. When it occurs, it is indicated in the schematics by a cross-hatched region (see Fig. 4). In view of these observations, an analysis of the composition of oligonucleotide No. o obtained from a TC-83 RNA digest has been undertaken. Pancreatic ribonuclease digestion of oligonucleotide No. o has given a compositional analysis of 1 AAAAU:5 to 6 AU:35 U:7 C:1 G. No analyses have been undertaken on the alternative spot due to the difficulty in obtaining enough of it, so that we have not been able to prove that it is a derivative of spot No. o. It is conceivable that due to its high content of uridylic residues, spot No. o is more susceptible than are the other oligonucleotides to digestion by trace amounts of pyrimidine specific nucleases contaminating the ribonuclease T1 preparations.

Comparison of the individual oligonucleotide fingerprints of VEE virus isolates I A TRD (Fig. 3a), I A TC-83 (Fig. 3b), I B PTF-39 (Fig. 3c), I C P676 (Fig. 3d), I D 3880 (Fig. 3e), and I E Mena II (Fig. 3f), indicates that the subtype I A and I B viruses are more closely related to each other than to the other subtype I viruses. The oligonucleotides absent in TRD and PTF-39 but present in TC-83 are indicated by asterisks (*), while those that are present in TRD or PTF-39, but are absent in TC-83 are indicated by arrows. Of the 70 oligonucleotides identified for TRD (other than No. o see Fig. 2), three are absent in TC-83 (No. 17, 48 and 32), and TC-83 has three new oligonucleotides. For two of the missing oligonucleotides, the new spots are to the left of the missing oligonucleotides while in the third case (No. 48) the new spot is to the right of the missing oligonucleotide. Sequence analyses will be needed to determine whether single C to U or U to C base changes have been involved in the apparent transpositions of these oligonucleotides. The RNA fingerprint of the VEE I B isolate also very closely resembles that of TRD. It lacks oligonucleotide No. 32 (and possibly No. 48) and has a new oligonucleotide (which is shared by TC-83) to the left of No. 48.

By determining the total radioactivity present in the 70 oligonucleotides identified in the fingerprint of TRD RNA and relating this to the total amount of 32P-labelled RNA applied to the gel, we have calculated that the 70 large oligonucleotides represent approx. 12% of the VEE genome. Preliminary studies indicate that approximately half of the 70 large oligonucleotides are present in the fingerprints of TC-83 and PTF-39 26S RNA (data not shown). The fingerprints of the 26S RNA of TC-83 and PTF-39 differ by only two oligonucleotides, indicating that the mRNA of these viruses are very similar and therefore specify structural proteins which are antigenically indistinguishable (France et al. 1979). In view of this, it is reasonable to suggest that the VEE viruses designated I A and I B be considered as variants of one another, or of a common progenitor.

Although certain similarities are apparent in the VEE I A, I C, I D and perhaps I E, RNA fingerprints (Fig. 3), we have resorted to co-electrophoreses of the digests of VEE I A TC-83 and the I D isolate (Fig. 4a, b), TC-83 and the I C isolate (Fig. 4c, d), and TC-83 and the I E isolate (Fig. 5a, b) to determine the T1 RNase resistant oligonucleotides in the genomes of these viruses which co-migrate.
Fig. 5. Co-electrophoresis of the ribonuclease T₁ digests of ³²P-labelled RNA species of VEE IA and IE viruses. The autoradiogram of the VEE IA TC-83 and IE Mena II RNA digest is shown in (a) with a schematic representation given in (b). In the latter, TC-83 oligonucleotides are indicated by filled circles, Mena II oligonucleotides by open circles and shared oligonucleotides by half-filled circles.

Fig. 6. Oligonucleotide fingerprints of the ribonuclease T₁ digests of ³²P-labelled RNA species of subtype IE VEE viruses: (a) 63U2, (b) 71U388.
For those regions of the electropherogram for which conclusions could be drawn concerning the origin of the oligonucleotides (see schematics diagram in Fig. 4b, 4d and 5b), as indicated by the half-filled circles, 18 out of 41 of the TC-83 large oligonucleotides co-migrated with those of the ID isolate (TRD No. 7, 10, 12, 13, 14, 15, 16, 19, 20, 24, 40, 50, 51, 52, 53, 54 and 55, plus one of the oligonucleotides found in the TC-83 fingerprints near No 51 but not present in the fingerprint of TRD). Similarly, 16 out of 37 of the TC-83 large oligonucleotides co-migrated with those of the IC isolate (TRD No. 1, 7, 10, 13, 14, 15, 16, 19, 21, 24, 28, 34, 40, 46, 47 and 50). These results suggest that a substantial number of the
oligonucleotides derived from the RNA species of these three VEE viruses (IA, IC and ID) may be homologous. Sequence analyses of the particular putative homologous oligonucleotides will be needed to provide conclusive evidence for their identity or relationship.

In contrast to the results obtained with the serotype IA, IB, IC and ID viruses, the co-electrophoresis of TC-83 (IA) with Mena II (a VEE I E serotype virus) suggested that few of the large oligonucleotides of these two viruses co-electrophoresed (Fig. 5). At best seven out of 49 TC-83 large oligonucleotides co-electrophoresed with those of the I E isolate (see Fig. 5b, TRD No. 18, 29, 34, 39, 40, 54 plus one of the TC-83 oligonucleotides, just above the TRD No. 18 which is not present in TRD).

To determine whether the Mena II isolate was representative of the I E serotype isolates, two additional virus isolates have been fingerprinted, 63U2 and 71U388 (Fig. 6a, b). The oligonucleotide fingerprints of both of these viruses are unique, and quite comparable to each other. Co-electrophoresis of the ribonuclease T1 digests of the virus RNA species of the 63U2 and 71U388 (Fig. 7c, d) agrees with this conclusion. The fingerprints of both 63U2 and 71U388 are evidently more similar to each other than to that of Mena II, although

Fig. 8. Oligonucleotide fingerprints of the ribonuclease T1 digests of VEE subtype I II, III and IV virus RNA species. (a) VEE IA TC-83, (b) VEE II EVE, FE3-7C, (c) VEE III MUC, Be An8 and (d) VEE IV PIX, Be Ar35645.
it is apparent that there are many oligonucleotides shared by all three viruses. Unfortunately, no co-electrophoreses have been performed between the RNA digests of TC-83 and either 63U2 or 71U388 to determine whether either of the latter two viruses is more closely related to TC-83 than is Mena II.

**Oligonucleotide fingerprint analyses of VEE subtype I, II, III and IV virus RNA species**

In order to investigate the interrelationships of subtypes I, II, III and IV viruses at the genome level, representative viruses have been analysed by oligonucleotide fingerprinting using either single or mixed virus RNA digests. Shown in Fig. 8 are the oligonucleotide fingerprints of 1 A TC-83 (Fig. 8a), VEE II, EVE FF3–7C (Fig. 8b) VEE III MUC Be An8 (Fig. 8c) and VEE IV PIX Be Arg35645 (Fig. 8d). From the single digests, each virus appears to have a unique fingerprint with little evidence of large T1 resistant oligonucleotides which have similar electrophoretic mobilities. The fingerprint obtained from the mixed virus RNA
Fig. 10. Co-electrophoresis of the ribonuclease T₁ digests of VEE virus RNA species. (a) VEE subtype I A TC-83 and VEE subtype IV Pixuna RNA species; (b) a schematic drawing of the same in which the TC-83 oligonucleotides are indicated by filled circles, the Pixuna oligonucleotides by open circles and shared oligonucleotides by half-filled circles; (c) co-electrophoresis of the ribonuclease T₁ digests of VEE subtype II (EVE) and subtype III (MUC) RNA species, and (d) a schematic drawing of the same showing the EVE oligonucleotides as filled circles, the MUC oligonucleotides as open circles, and shared oligonucleotides by half-filled circles.

digest of the TRD subtype I A and the subtype II virus is given in Fig. 9(a, b), while that of the TRD subtype I A and the subtype III virus is shown in Fig. 9(c, d). From those regions of the electropherograms from which assignments could be made, no more than five out of 32 of the TRD large oligonucleotides (No. 8, 12, 13, 19 and 24) appeared to co-electrophorese with those of subtype II virus, and no more than four out of 30 of the TRD large oligonucleotides (No. 4, 20, 38 and 50) appear to be present in the subtype III RNA digest. As discussed previously, sequence analyses will be needed to confirm whether these putative
homologous oligonucleotides are identical or indeed bear any relationship to each other.

The fingerprint obtained from the TC-83 VEE IA and subtype IV virus is given in Fig 10(a, b). Of 46 TC-83 large oligonucleotides (not counting No. 0), no more than five (TRD No. 18, 21, 24, 38 and 46) appear to co-electrophorese with those of the subtype IV virus. Co-electrophoresis of the RNA digests of the subtype II and subtype III viruses (Fig. 10 c, d) revealed that very few of their large oligonucleotides migrated in coincidence whereas co-electrophoresis of the RNA digest of VEE subtype IE Mena II isolate with that of the subtype III virus also gave little evidence of coincident electrophoresis of the large T1 RNase resistant oligonucleotides (Fig. 7a b).

In summary, the oligonucleotide analyses comparing subtype I, II, III and IV virus RNA species provide little evidence for coincident migration of the large T1 RNase resistant oligonucleotides among these serologically distinct VEE viruses.

DISCUSSION

Members of the VEE serocomplex of alphaviruses have been shown to be antigenically related to each other by HI, CF and neutralization of infectivity tests (Shope et al. 1964; Young & Johnson, 1969a; Johnson & Martin, 1974; Scherer & Pancake, 1977; France et al. 1979). All the VEE viruses analysed in our laboratory have three similar structural proteins, a nucleocapsid protein and two envelope glycoproteins gp-57 and gp-50, with the exception of Pixuna which has a third glycoprotein, gp-62, of uncertain origin (France et al. 1979).

Serological and biological studies indicate that despite these similarities there is considerable divergence among the viruses of the VEE complex (Young & Johnson, 1969a, b; Zarate & Scherer, 1969; Jarhling & Scherer, 1973; Johnson & Martin, 1974; Pedersen & Eddy, 1975; Scherer & Chin, 1977; France et al. 1979). Our present investigation was undertaken to determine (1) whether the genome RNA oligonucleotide fingerprints of different VEE subtype viruses reflected this antigenic diversity, and (2) to what extent the oligonucleotide fingerprints of the subtype I viruses resembled each other.

The results of this study indicate that the ribonuclease T1 resistant oligonucleotides of subtype IA and IB virus RNA species are essentially identical so that these isolates should be considered as variants of each other, or as progeny of a common progenitor. The degree of identity of the oligonucleotide fingerprints of these viruses is similar to that reported previously for strains of certain other alphaviruses. For example, the oligonucleotide maps of the virus RNA species of Sindbis strains from Egypt (EgAr339) and South Africa (SAAR86) differ by only one large oligonucleotide, whereas certain Chikungunya (CHIK) virus strains from Africa differ by only two large oligonucleotides (Wengler et al. 1977). Similar results have been reported for vesicular stomatitis virus, Indiana or New Jersey serotype isolates (Clewley et al. 1977b).

The results obtained by fingerprinting the RNA species of different VEE subtype I viruses agree with the serological evidence for categorizing the IC, ID and IE viruses as varieties of subtype I virus, although the IE isolate, Mena II, appears to be less related to the subtype IA and IB viruses than are the IC and ID isolates. On the basis of the serological evidence and the results of RNA fingerprint analyses, we suggest that IAB should be used to designate those VEE virus strains previously designated as IA and IB by Young & Johnson (1969a).

Frisby et al. (1976), reported that no large oligonucleotides were shared between picornaviruses representing different serogroups. However, they found that those viruses within a serogroup which could be antigenically differentiated from other members of that serogroup, had fingerprints which were similar, but not identical to each other.

The oligonucleotide fingerprint analyses of the virus RNA species of VEE subtype II,
III and IV viruses indicate that these viruses are quite distinct from each other and from the subtype I viruses. Serological analyses by HI and RIP tests with subunit antisera indicate that subtype I, II, III and IV viruses are easily distinguished. Any cross-reactivity is usually of low titre (France et al. 1979). Our results support the differentiation of the four subtypes in the VEE complex of alphaviruses (Shope et al. 1964; Karabatsos, 1975; Chanas et al. 1976), with the subtype I viruses categorized into the varieties I AB, I C, I D and I E.

The origin of the VEE virus strains responsible for epidemic disease in man and animals is not known, although several authors have speculated about the maintenance and dispersal of epizootic virus strains and their possible derivation from, and relation to, epizootic virus strains (Young & Johnson, 1969a; Franck & Johnson, 1971; Young, 1972; Johnson & Martin 1974; Scherer et al. 1973). Our studies on the antigenic relationships of the envelope glycoproteins presented here and elsewhere (France et al. 1979) together with the oligonucleotide fingerprint analyses of the VEE virus genomes, can be considered in relation to the suggestion that the epizootic viruses I AB and I C may be related to or may result from mutation of enzootic subtype I virus strains (e.g. I D and, or I E). Both of the membrane glycoproteins of subtype I VEE viruses contain antigenic determinants common to other subtype I viruses but do not present as major antigens on subtype II, III, or IV viruses. Fingerprint analyses of the genomes of the I AB, I C and I D viruses show that each is unique, though all are quite similar. The results fit the hypothesis that there was probably a common progenitor for the I AB, I C and I D viruses, although whether the progenitor was epizootic and became enzootic, or was enzootic and shed epizootic varieties cannot be determined. The unique oligonucleotide patterns of the I E viruses and their more distant antigenic relationships with the other subtype I viruses, suggest that they are not as closely related to the epizootic I AB and I C viruses as are the current enzootic I D varieties.

Whether there was a more distant progenitor for all the subtype I viruses from which the I E varieties developed before it diversified into the I AB, I C, and I D strains or whether I E varieties have evolved faster than the other strains also cannot be determined at present.

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REFERENCES
VEE viruses


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