Comparison of Dengue Viruses and Some Other Flaviviruses by cDNA–RNA Hybridization Analysis and Detection of a Close Relationship between Dengue Virus Serotype 2 and Edge Hill Virus

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SUMMARY
Variable amounts of cDNA were synthesized in vitro from RNA extracted from several flaviviruses, including the four prototype dengue (DEN) virus serotypes. The synthesis was carried out using an oligo(dT) primer, suggesting the presence of a short poly(A) region at or near the 3' end of some flavivirus genomes. The DEN-1 and DEN-2 prototype strains produced the largest amount of cDNA and were therefore used to investigate further the relatedness of flavivirus genomes by cDNA–RNA hybridization. The flaviviruses studied are related to each other to some extent since the hybrids formed exhibited about 30% nuclease resistance, but a closer relationship was detected between dengue viruses of serotype 1 and 4 and between dengue virus serotype 2 and Edge Hill virus. A monoclonal antibody to the envelope protein (V3) of dengue viruses reacted with Edge Hill virus, confirming the genetic relationship between the viruses.

INTRODUCTION
Flaviviruses consist of a group of about 50 serologically related viruses in the family Togaviridae which replicate in arthropod and in vertebrate cells. Some of these viruses cause major diseases in humans, such as yellow fever, dengue haemorrhagic fever and Japanese encephalitis. Flaviviruses consist of a single-stranded RNA genome, a capsid protein, a low mol. wt. membrane protein and an envelope glycoprotein (for reviews, see Schlesinger, 1977; Strauss & Strauss, 1977; Westaway, 1980). The molecular structure of the viral genome has not been studied extensively. The presence or absence of a poly(A) tail at the 3' end has not been proven but RNA from West Nile (WN) and Japanese encephalitis (JE) viruses does not bind to oligo(dT) or poly(U) columns (Wengler et al., 1978; Russell et al., 1980). Several groups have used ribonuclease T1 oligonucleotide fingerprinting to study RNA from dengue (DEN), Saint Louis encephalitis (SLE), Uganda S, WN and yellow fever viruses (Vezza et al., 1980; Walker et al., 1982; Trent et al., 1981, 1983; Wengler et al., 1978; Monath et al., 1983) but these studies have not detected poly(A) tracts. Neither method excludes the presence of very short regions of poly(A) residues, less than 10 nucleotides in length. This report describes a more sensitive method of detecting a poly(A) tract. It involves the synthesis in vitro of complementary DNA (cDNA) from flavivirus RNAs using an oligo(dT) primer which will bind to a poly(A) tract, and the enzyme reverse transcriptase. The cDNAs produced were used to examine the relationships of flavivirus RNA genomes by cDNA–RNA hybridization.

METHODS
Cells. Cells of the C6/36 clone from Aedes albopictus were grown in Eagle’s MEM with Earle’s salts (EMEM) containing 10% foetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 0.1 mM non-essential amino acids. Maintenance medium for infected cell cultures consisted of the appropriate growth medium.
containing 0.6% bovine plasma albumin instead of foetal bovine serum. The LLC-MK2 (monkey kidney) cell line was grown in Medium 199, supplemented with 20% foetal bovine serum, penicillin and streptomycin. Labelling medium for radiolabelling infected cell proteins consisted of EMEM deficient in methionine but supplemented with 0.6% bovine plasma albumin, penicillin and streptomycin.

**Viruses.** The flaviviruses used were the prototype strains of the four dengue virus serotypes: DEN-1 (Haw), DEN-2 (NGC), DEN-3 (H87) and DEN-4 (H241); two DEN-2 viruses isolated in Thailand in 1980 designated T80G and T80H (provided by Dr D. S. Burke, Armed Forces Research Institute of Medical Sciences, Bangkok); and three viruses isolated in Australia: Edge Hill (C281), Stratford (C338) and Kunjin (MRM16) (Doherty, 1974).

Viruses used to produce monoclonal antibodies were reference prototype DEN serotypes derived from suckling mouse brain virus pools routinely maintained at the Walter Reed Army Institute of Research. Each was passed four times in C6/36 cells. Kunjin and Edge Hill viruses were kindly provided as lyophilized stocks by Dr R. Shope, Yale Arbovirus Research Unit (YARU), New Haven, Conn., U.S.A. Additionally, the C281 strain of Edge Hill virus (VR-377) was obtained as lyophilized stock from the American Type Culture Collection (ATCC). Each of these viruses was passed once in C6/36 cells.

**Growth of flaviviruses.** Virus growth was monitored by assay of viral haemagglutinin (Clarke & Casals, 1958). The dengue viruses did not produce a cytopathic effect. When maximum haemagglutinin titre was reached, the tissue culture fluid was centrifuged to remove cells and debris, and the virus precipitated by addition of polyethylene glycol 6000 (PEG) to 6% (w/v). The viruses were recovered by centrifugation of the mixtures at 7500 g for 30 min.

**Synthesis of cDNA probes.** RNA was extracted from virus pellets with phenol/chloroform and precipitated three times with cold ethanol before use in cDNA synthesis. RNA (2 μg) and the primer p(dT)20 (P-L Biochemicals) were heated to 90 °C for 1 min, quickly chilled in ice and used as a template in cDNA synthesis. This cDNA synthesis was carried out (essentially as described by Gough et al., 1980) in a 20 μl vol. of 50 mm-Tris–HCl pH 8.3, 4 mm-dithiothreitol (DTT), 65 mm-magnesium acetate buffer containing 1 mm-dCTP, 1 mm-dTTP, 1 mm-dGTP, 0.05 mm-dATP, 1 μCi/μl [32P]dATP (sp. act. 2000 to 3000 Ci/mmol, Amersham) and 16 units of avian myeloblastosis virus reverse transcriptase (Life Sciences). The reaction mix was incubated at 37 °C for 1 h, for 30 min. followed by the addition of 100 μM-deoxyribonucleotides and a further incubation of 30 min at 37 °C.

**Purification of cDNA probes.** In order to obtain a pure single-stranded cDNA probe, the RNA template was hydrolysed with 0.5 μl NaOH/0.2% SDS at 37 °C for 2 h. The cDNA was then electrophoresed through a 1.5 mm-thick 3% polyacrylamide gel containing 7 M-urea and Tris/borate/EDTA buffer (Peacock & Dingman, 1968) for 3 h. The radioactive band representing the largest mol. wt. cDNA was cut out, electroeluted from the gel, extracted with phenol and precipitated with cold ethanol twice before being used in hybridization experiments.

**cDNA–RNA hybridization assays.** cDNA–RNA hybrids were formed in capillaries at 65 °C for 20 h in 10 mm-Tris–HCl pH 7, 1 mm-EDTA, 0.18 m-NaCl, 0.05% SDS as described by Gould & Symons (1977). They were assayed at 45 °C for 30 min with 100 units/ml S1 nuclease (Miles Laboratories) in buffer containing 30 mm-sodium acetate pH 4.6, 50 mm-NaCl, 1 mm-ZnSO4, 5% glycerol, 40 μg/ml denatured carrier DNA. The 32P counts were precipitated with 10% TCA prior to collection onto GF/A filters (Whatman) and liquid scintillation counting. The formula used to calculate the percentage S1 nuclease resistance of the homologous and heterologous hybrids was: Corrected percentage S1 nuclease resistance = 100 × [S1 nuclease resistance of hybrid (%) – S1 nuclease resistance of cDNA (%)]/100 – S1 nuclease resistance of cDNA (%). This percentage S1 nuclease resistance reflects the sequence homology of the cDNA–RNA hybrids and its validity has been discussed elsewhere (Gonda & Symons, 1978).

**Antibody preparations.** The preparation and characterization of ascitic fluids containing monoclonal antibodies directed against DEN antigens has been described previously (Gentry et al., 1982; Henchal et al., 1982). The following type-specific monoclonal antibodies were used: 15F3 (DEN-1), 3H5 (DEN-2), 5D4 (DEN-3) and 1H10 (DEN-4). DEN complex-specific monoclonal antibodies included 1B7 and 2H12. Flavivirus group-reactive monoclonal antibodies, 4G2, and reference hyperimmune mouse ascitic fluid (HMAF) prepared against DEN-2 virus have been shown to cross-react with most flaviviruses.

**Indirect immunofluorescence assay.** Details of the indirect immunofluorescence assay have been described previously (Henchal et al., 1982). LLC-MK2 cells were infected with each of the viruses. After 3 to 5 days when cytopathic effects were seen, virus-infected and uninfected cells were spotted and fixed with cold acetone onto glass slides. Normal mouse ascitic fluid and DEN-2 HMAF were used at a 1:100 dilution. Each of the monoclonal antibody preparations was diluted 1:10 and then serially twofold.

**Determination of specificity of monoclonal antibodies.** The reaction of the monoclonal antibodies with viral proteins was determined by immune precipitation of lysates from virus-infected LLC-MK2 cells radiolabelled with [35S]methionine or DEN-2 virions with mouse ascitic fluids (Henchal et al., to be published). Each ascitic fluid was reacted with DEN antigens overnight at 4°C. Immune complexes were collected using Sepharose 4B-Protein A and analysed by electrophoresis in polyacrylamide gels.
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Fig. 1. Autoradiograph exposed for 16 h at room temperature of $^{32}$P-labelled cDNA synthesized from flavivirus RNAs electrophoresed for 3 h at 35 mA on a 0.4 mm-thick 3% polyacrylamide gel containing 7 M-urea and Tris/borate/EDTA buffer. (a) Influenza RNA (A/Mem/102/72); (b) DEN-2 RNA; (c) DEN-1 (Haw) cDNA; (d) DEN-2 (NGC) cDNA; (e) DEN-2 (T80H) cDNA; (f) DEN-3 (H87) cDNA; (g) DEN-4 (H241) cDNA; (h) Edge Hill cDNA. (a) and (b) were stained with ethidium bromide.

RESULTS

Synthesis of cDNA to flavivirus genomes

The dengue virus RNA used in cDNA synthesis was extracted from PEG-precipitated virus. It was considered free from host cell nucleic acid on the basis of the following experiments. (i) Dengue virus-infected and uninfected C6/36 cells were labelled in vivo with $[^{32}P]$ orthophosphate. Another batch of dengue virus-infected and uninfected C6/36 cells were not labelled with $^{32}$P. For the virus purification procedure, tissue culture fluid from the $^{32}$P-labelled uninfected cells was combined with unlabelled dengue virus-infected cells (A), while $^{32}$P-labelled dengue-infected culture fluid was combined with unlabelled infected cell fluid (B). RNA extracted from both of the resulting PEG viral pellets was used in ribonuclease T1 oligonucleotide fingerprinting (as described by Walker et al., 1982). No radioactive T1 oligonucleotides were detected for (A) but a characteristic fingerprint was obtained for (B). (ii) The HaeIII digest pattern of cDNA synthesized from RNA extracted from the PEG viral pellet was compared with that extracted from sucrose gradient-purified virus. The one-dimensional HaeIII patterns were identical.
cDNA to the flavivirus genomes was synthesized using reverse transcriptase in the presence of an oligo(dT) primer. The cDNAs produced were separated on a denaturing polyacrylamide gel (Fig. 1). The prototype strains for DEN-1 and DEN-2 produced more cDNA than the other flaviviruses even though the same concentration of RNA was used for each virus isolate. The level of cDNA produced by Kunjin and Stratford RNA (data not shown) was similar to that of Edge Hill which was about 19 times less than that of DEN-1 and DEN-2 (NGC) according to laser densitometer tracings. This ability of all of the flavivirus RNAs tested to produce a certain amount of cDNA was not due to self-priming since no distinct cDNA bands were produced in the absence of p(dT)12-18 primer.

The major cDNA molecule synthesized was similar in electrophoretic mobility to the RNA from which it was produced, indicating that it was a near full-length cDNA copy (Fig. 1). The smaller cDNA bands are most likely degradation products but could also represent partial or incomplete cDNA synthesis products. The oligo(dT)-primed cDNA from DEN-1 RNA was also compared with cDNA produced by random priming this RNA (Fig. 2). The electrophoretic mobility of the oligo(dT)-primed cDNA was less than that of the randomly primed cDNA suggesting that oligo(dT) primes predominantly at or near the 3' end. Both of these cDNA molecules were then digested for 16 h with the restriction enzyme HaeIII (about 100 units/μg cDNA in buffer containing 6.6 mM-Tris-Cl pH 7.4, 6.6 mM-MgCl2, 10 mM-DTT, 50 mM-NaCl), and the products were separated on a denaturing polyacrylamide gel (Fig. 2). The resulting HaeIII digest patterns of cDNA obtained by oligo(dT) or random priming were similar, indicating that the oligo(dT)-primed cDNA represents predominantly a single polyadenylated RNA species and that there is little or no contamination of the RNA with non-polyadenylated RNA.

cDNA–RNA hybridization assays

Since DEN-1 and DEN-2 (NGC) RNA produced a substantial amount of cDNA, they were used to investigate further the relationship of some flavivirus RNAs by cDNA–RNA hybridization. In order to find the concentration of RNA suitable for the formation of hybrids with 5 pg cDNA, R₀t (RNA concentration × hybridization time, in mol·s/l) curves were determined for both DEN-1 and DEN-2 cDNAs. The curves were very similar and a log₁₀R₀t value of 1 was used in further experiments. The hybrids formed between homologous cDNAs and RNAs were 90 to 110% S₁ nuclease-resistant. These homologous hybrids were adjusted to 100% and all of the heterologous hybrids were then adjusted accordingly. The results of the heterologous hybridization experiments (Table 1) reveal that, firstly, the cDNA–RNA hybridization method cannot distinguish between genomes of viruses of the same serotype since the DEN-2 (NGC) probe hybridized to the same extent with the strains T80G and T80H of serotype 2 as it did with its homologous RNA. The RNA from these three isolates of serotype 2 exhibit different ribonuclease T₁ fingerprints (Walker et al., 1982). Secondly, the background of non-homologous RNA (in this case yeast RNA) was between 10 and 17%, while the minimum sequence homology observed for flavivirus RNA was about 25%. Thirdly, most of the viral RNAs showed between 25 and 40% sequence homology with DEN-1 and DEN-2 cDNAs while there were two pairs which exhibited a much greater sequence homology. These were DEN-1 and DEN-4, and DEN-2 (NGC) and Edge Hill. Edge Hill virus RNA protected the DEN-2 cDNA probe from nuclease digestion to a large extent (71%) and the nuclease resistance of this hybrid was confirmed by electrophoresis of S₁ nuclease-treated hybrids on a polyacrylamide gel followed by autoradiography (Fig. 3). The single-stranded DEN-2 cDNA probe was degraded to molecules ranging from about 500 to 4000 bases during the hybridization procedure, but only DEN-2 RNA (NGC and T80G) protected the probe completely; Edge Hill RNA protected the probe to a large extent, while RNA of other flaviviruses failed to protect the probe from S₁ nuclease cleavage.

All of the hybridization experiments were carried out under stringent conditions (0.18 M-NaCl during the hybridization and 0.08 M-NaCl during the S₁ nuclease assay). The S₁ nuclease resistance of the cDNA probe increased twofold when hybridization reactions were carried out at 0.56 M-NaCl and fourfold when the S₁ nuclease assay NaCl concentration was raised to
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Fig. 2. Autoradiograph exposed for 16 h at -70 °C of 32P-labelled cDNA synthesized from DEN-1 RNA and HaeIII-digested cDNA, electrophoresed on a 5% polyacrylamide gel containing 7 M-urea and Tris/borate/EDTA buffer. (a) Randomly primed cDNA; (b) oligo(dT)-primed cDNA; (c) HaeIII-digested randomly primed cDNA; (d) HaeIII-digested oligo(dT)-primed cDNA. The dye markers xylene cyanol and bromophenol blue are indicated by X and B respectively.

Fig. 3. Autoradiograph of S1 nuclease-treated 32P-labelled cDNA–RNA hybrids electrophoresed on a 3% polyacrylamide gel containing 7 M-urea and Tris/borate/EDTA buffer. The gel was exposed to an X-ray film at -70 °C for 2 weeks. (a to g) Hybrids formed with the DEN-2 (NGC) cDNA probe and RNA from (a) no RNA, (b) DEN-2 (NGC), (c) DEN-1, (d) DEN-2 (T80G), (e) DEN-3, (f) DEN-4 or (g) Edge Hill. The sample that contained no RNA was not treated with S1 nuclease and shows some degradation of the cDNA probe during the hybridization incubation. The dye markers xylene cyanol and bromophenol blue are indicated by X and B respectively.

0.56 M. The percentage S1 nuclease resistance obtained for the cDNA–RNA hybrids in Table 1 therefore represents the lowest percentage of sequence homology for the hybrids formed, since increasing the NaCl concentration would allow more mismatching of basepairing regions to occur in these hybrids.
Table 1. Estimated percentage sequence homology among flavivirus RNAs*

<table>
<thead>
<tr>
<th>RNA used for cDNA synthesis</th>
<th>RNA used in hybridization reaction</th>
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<tr>
<td></td>
<td>DEN-1 (Haw)</td>
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<td>DEN-1 (Haw)</td>
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</tr>
<tr>
<td>DEN-2 (NGC)</td>
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* cDNA–RNA hybrids were formed and assayed with S1 nuclease and the estimated percentage sequence homology was calculated as described in the text.
† Each percentage represents an average of six experiments.

Table 2. Reactions of monoclonal antibodies to dengue viruses with selected flaviviruses in indirect immunofluorescence assays

<table>
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<tr>
<th>Flavivirus</th>
<th>DEN1</th>
<th>DEN2</th>
<th>DEN3</th>
<th>DEN4</th>
<th>KUN</th>
<th>YARU</th>
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<td>320</td>
<td>320</td>
</tr>
</tbody>
</table>

* Protein detected in immune precipitation reactions with monoclonal antibodies: NS, unidentified non-structural protein; V3, envelope glycoprotein; NV2, intracellular protein.
† Dilution of monoclonal antibodies that reacted with virus-infected cells.
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**Detection of antigenic determinants shared by Edge Hill and dengue serotype 2 viruses**

Monoclonal antibodies with unique specificities for distinct antigenic determinants of DEN proteins were used to probe for similar determinants on Edge Hill proteins. Serial dilutions of monoclonal antibodies were reacted with cells infected with each of the viruses. Edge Hill virus obtained from two different sources (YARU and ATCC) did not react with monoclonal antibodies that were specific for DEN serotypes (Table 2); however, both reacted with DEN complex-specific monoclonal antibodies 1B7. In radioimmune precipitation analyses 1B7 react with the envelope glycoprotein V3. Monoclonal antibodies 2H2 which precipitate the intracellular protein NV2 of DEN viruses failed to react with Edge Hill virus. Kunjin virus reacted with serological reagents group-reactive for flavivirus.

**DISCUSSION**

The ability to make cDNA in the presence of an oligo(dT) primer suggests that a poly(A) tract, however short, is at or near the 3' end of the viral RNA. This may be a general property of the flaviviruses since it has been reported that SLE virus RNA contains a small region of poly(A) residues as tested by ribonuclease resistance (Brawner et al., 1977). It is interesting to note that two strains of DEN-2 (T80G and T80H) made cDNA to an extent similar to the other flaviviruses (data not shown for T80G) and not to that of the prototype strain. The reason for the varying amounts of cDNA synthesized from these flavivirus RNAs may be due to the variable length of the poly(A) region, or due to a varying ratio of polyadenylated RNA to RNA without any poly(A) residues.

The cDNAs produced from DEN-1 and DEN-2 RNA were used to examine the genetic relationship of several dengue virus isolates as well as other flaviviruses by hybridization. The results showed that this technique cannot be used to study closely related viruses such as those belonging to the same serotype, but that a broad relationship of viral genomes can be detected.

The flaviviruses are subdivided by their reactions in serological tests. Each virus contains antigenic determinants common to the group, others which specify a complex of viruses, and those which determine serotype specificity (Trent, 1977). The four dengue virus serotypes form a discrete complex of flaviviruses (Porterfield, 1980) and there is some evidence of a subcomplex of dengue virus serotypes 1 and 3 (Russell & Nisalak, 1967; Henchal et al., 1982). The hybridization results suggest a closer relationship between dengue serotypes 1 and 4 (73% homology) and a relationship between dengue 1 and other dengue viruses only slightly greater than that between other flaviviruses tested. There need not be a correlation between serological tests and hybridization since the antigenic determinants are located on the major virus glycoprotein (V3) which is encoded in less than 15% of the virus genome. The closer genetic relationship of serotypes 1 and 4 implies that evolutionary patterns may not be common to all four serotypes.

The hybridization test revealed an unexpected genetic relationship between Edge Hill virus and dengue virus serotype 2. Indirect immunofluorescence assays showed that LLC-MK2 cells infected with Edge Hill virus reacted not only with flavivirus group-reactive serological reagents, but also with monoclonal antibodies, 1B7, which had been previously thought to be specific for dengue viruses. It was particularly interesting to observe that other DEN complex-specific monoclonal antibodies, 2H2, did not react with cells infected with Edge Hill virus. Monoclonal antibodies 1B7 react with the envelope glycoprotein whereas 2H2 react with a non-virion intracellular protein (NV2). These data suggest that Edge Hill and dengue viruses share complex-specific genetic information in the region coding for the envelope glycoprotein. However, Edge Hill has lost a DEN complex-specific antibody-binding site on the NV2 intracellular protein. Edge Hill virus forms part of an antigenic complex distinct from other flavivirus complexes (Porterfield, 1980) and has not previously been considered closely related to dengue viruses. There are also distinct ecological differences between the viruses. Dengue viruses infect man and are transmitted by *Aedes* spp. of the subgenus *Stegomyia* (Schlesinger, 1977). The vertebrate hosts for Edge Hill virus are marsupials (Doherty et al., 1964) and the virus has been isolated in eastern Australia from *Anopheles amictus*, *Culex annulirostris* and *Aedes* spp. of the subgenus *Ochlerotatus* (Doherty, 1974, 1977; Doherty et al., 1979).
Hybridization of flavivirus RNA with complementary DNA of dengue viruses has provided estimates of relationships between genomes instead of portions encoding antigenic determinants. The results suggest that such analyses would be valuable in the study of the evolutionary origins of flaviviruses.

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REFERENCES


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