

Infectious RNA transcribed from stably cloned full-length cDNA of dengue type 4 virus

(dengue virus/infectious transcripts/virus genetics)

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ABSTRACT Dengue virus is an enveloped positive-strand RNA virus with a genome \approx 11 kilobases in length. The four serotypes of dengue virus are currently the most important members of the flavivirus family in terms of geographical distribution and the incidence of infection in humans. In this communication we describe successful cloning of a stable full-length cDNA copy of dengue type 4 virus that can be used as the template for *in vitro* transcription of infectious RNA. Evidence is presented that dengue virus recovered from permissive cells transfected with the *in vitro* RNA transcripts retained a mutation that was engineered into full-length cDNA. The properties of the virus produced by cells transfected with infectious RNA transcripts of dengue cDNA resembled those of the virus from which the cDNA clone was derived. The dengue virus recombinant DNA system should prove helpful in gaining a better understanding of the molecular biology of dengue viruses and should facilitate the development of a safe and effective live vaccine for use in humans.

The family *Flaviviridae* contains some 68 viruses most of which are arthropod-borne and cause disease of varying severity in humans or animals. The four dengue virus serotypes (types 1–4) of the flavivirus family form a distinct antigenic subgroup known as the dengue complex. In terms of geographic distribution and incidence of infection, dengue viruses rank highest among flaviviruses (1). Although typical dengue illness is moderately severe, mortality is usually low. However, a life-threatening form of dengue disease with hemorrhagic fever or shock syndrome can occur under certain circumstances in young children. Because an effective vaccine against dengue is still not available, the World Health Organization has designated the dengue viruses a priority area for accelerated vaccine development. Dengue viruses, like other flaviviruses, contain a positive-strand RNA genome and three virus-coded structural proteins: the capsid (C) protein, the membrane (M) protein, and the envelope (E) glycoprotein. Virion RNA is capped at the 5' end but a poly(A) sequence is not present at the 3' end (2, 3). Virion RNA is infectious when inoculated into experimental animals or into cells in culture (4–6). Sequence information obtained from molecular cloning of nearly all major strains of flaviviruses has contributed much to our current understanding of flavivirus gene organization, expression, and replication (7). Sequence analysis showed that the dengue type 4 virus genome contains 10,646 nucleotides (nt); the first 101 nt at the 5' end and the last 384 nt at the 3' end are noncoding and the remaining sequences code for a 3386-amino acid polyprotein in the order of C-preM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (where the NS proteins are nonstructural proteins) (8, 9). Several strategies of proteolytic cleavage are employed to process the polyprotein into individual

proteins. For example, cleavage between C and preM, preM and E, or E and NS1 is mediated by a host-cell signal peptidase ("signalase") (10, 11). Cleavage between NS1 and NS2A is effected by NS2A and requires an 8-amino acid sequence at the carboxyl terminus of NS1 (12, 13). It appears that NS3 is a viral protease; however, it was recently observed that both NS2B and NS3 are required for the proteolytic processing of most of the remaining nonstructural proteins (14–16).

Genetic analysis of positive-strand RNA viruses has been greatly facilitated by the use of recombinant DNA techniques. It was shown that cloned full-length cDNA of poliovirus is infectious for susceptible cells in culture (17). Subsequent studies showed that RNA transcripts produced *in vitro* from a poliovirus cDNA template were more infectious than the cDNA itself (18). RNA transcripts produced by *in vitro* transcription of cloned full-length cDNA derived from several other positive-strand RNA viruses (19–21) and, more recently, from yellow fever virus (22) have also been demonstrated to be infectious. One of the objectives of our dengue research has been to construct cloned full-length dengue virus cDNA that could serve as a template for infectious RNA transcripts. This would allow us to introduce specific mutations into the dengue virus genome at the cDNA level and isolate dengue virus mutants for biological studies and potential use in a live virus vaccine for humans. In this study we describe the construction and cloning of a stable full-length dengue cDNA copy in a strain of *Escherichia coli* using the pBR322 plasmid vector. RNA molecules produced by *in vitro* transcription of the full-length cloned DNA template were infectious, and progeny virus recovered from transfected cells was indistinguishable from the parental virus from which the cDNA clone was derived.

MATERIALS AND METHODS

Cloning Subfragment and Full-Length cDNA of Dengue Virus. DNA segments spanning nearly the entire genome were initially cloned from a cDNA library of dengue type 4 virus strain 814669 (virus kindly supplied by W. Brandt, Walter Reed Army Institute of Research, Washington) (23). These cDNA clones, used to establish the dengue type 4 sequence, were joined to form full-length clone 1A by shared restriction enzyme sites: namely, pF19 *HindIII*⁴⁴-*Sst* I¹⁹³¹, pE19 *Sst* I¹⁹³¹-*Sst* I³⁰³³, pD20 *Sst* I³⁰³³-*Hae* II⁴¹⁸⁹, pC20 *Hae* II⁴¹⁸⁹-*Hae* II⁵⁸⁸¹, pA9 *Hae* II⁵⁸⁸¹-*Xba* I⁷⁷²⁵, pA28 *Xba* I⁷⁷²⁵-*HindIII*⁹⁸²⁹, and pB81 *HindIII*⁹⁸²⁹-*Hae* II¹⁰⁶⁰⁸. The 5' and 3' subfragments used for replacement in the full-length clone were constructed from other independently isolated cDNA clones: clone 2 of the 5' half-fragment was joined by pF34 *HindIII*⁴⁴-*Sst* I¹⁹³¹, pS15 *Sst* I¹⁹³¹-*Sst* I³⁰³³, pD16 *Sst* I³⁰³³-*Spe* I³³³⁸, and pD17 *Spe* I³³³⁸-*EcoRI*⁵¹²⁵; clone B of the 3' half-fragment was joined by pD13 *BstBI*⁵⁰⁶⁹-*Nco* I⁶³³², pX6

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Abbreviation: nt, nucleotide(s).

*Nco*I⁶³³²-*Xba*I⁷⁷²⁵, and pK2 *Xba*I⁷⁷²⁵-*Hae*II¹⁰⁶⁰⁸; and clone C of the 3' subfragment by pD13 *Bst*BI⁵⁰⁶⁹-*Nco*I⁶³³², pX10 *Nco*I⁶³³²-*Xba*I⁷⁷²⁵, and pK1 *Xba*I⁷⁷²⁵-*Hae*II¹⁰⁶⁰⁸.

The SP6 promoter sequence was positioned upstream of the 5' end of the dengue sequence as follows: the 287-base-pair (bp) *Sau*3A1 fragment of pGEM-3 (nt 2618-37) (Promega) containing the SP6 promoter sequence (nt 2809-2867) was inserted at the *Bgl*II site created from the *Pst*I site of pBR322. A dengue cDNA fragment (nt 1-88) flanked by the *Pst*I cleavage sequence at its 5' end and the *Bgl*II cleavage sequence at its 3' end was inserted between the *Pst*I and *Bgl*II sites downstream of the promoter sequence. Additional sequence between the SP6 transcription-initiating G residue and the first nucleotide of the dengue sequence was removed by oligonucleotide-directed mutagenesis in an M13 cloning vector. Also, in the pBR322 plasmid, the cleavage sequence GGTACC for *Asp*718 was engineered immediately following the dengue 3' end sequence. *E. coli* strain HB101 was used for cloning dengue sequences from the cDNA library and for cloning intermediate-length cDNA. Strains HB101 and DH5 α were employed in the initial attempt to clone full-length cDNA. Successful cloning of full-length dengue cDNA was accomplished with *E. coli* strain BD1528 (*thy*A, *met*⁻, *nad*BF, *ung*⁻, *gal*⁻, *sup*E, *sup*F, *nsd*R⁻, *hsd*M⁺; originally from B. Duncan, obtained through D. Nathans, Johns Hopkins University, Baltimore).

In Vitro Transcription. Plasmid containing full-length dengue cDNA was linearized by cleavage with *Asp*718 (Boehringer Mannheim) followed by phenol extraction and ethanol precipitation. The linearized DNA template (2 μ g) was added to a transcription reaction mixture (50 μ l) containing 40 mM Tris (pH 7.5), 6 mM MgCl₂, 10 mM NaCl, 10 mM dithiothreitol, 50 units of RNasin, and 0.5 mM each NTP (Promega). To incorporate a cap structure at the 5' end of RNA transcripts, m⁷G(5')ppp(5')G (0.5 mM) was also added to the reaction mixture, which was incubated at 37°C for 1 hr (24). The RNA product was analyzed by agarose gel electrophoresis in Tris/borate buffer containing 0.1% SDS. RNA transcripts were visualized and quantitated by ethidium bromide staining. The transcription product was also treated with DNase or RNase to confirm that the infectious moiety of the transcription product was RNA.

RNA Transfection. Simian LLCMK₂ cells were grown in medium 199 supplemented with 10% fetal bovine serum. For transfection, subconfluent LLCMK₂ cells in a 24-well plate were rinsed once with serum-free medium 199 and then covered with 0.3 ml of serum-free medium per well. The transfection mixture containing DNase-treated *in vitro* RNA transcripts (0.1-1 μ g) or virion RNA (0.01-0.1 μ g) in 17 μ l of 0.02 mM Hepes buffer (pH 7.05) and 6 μ l of Lipofectin (Bethesda Research Laboratories) was mixed thoroughly and added to the medium. After incubation at 37°C for 12-18 hr, medium 199 plus 10% fetal bovine serum was added. Eight days after transfection, cells were trypsinized and transferred to a 6-well plate or to a chamber slide for an additional 2 days of incubation in the growth medium. Cells in the chamber slide were tested by immunofluorescence to detect the presence of dengue antigens. Infected cells in the culture plate were collected together with the fluid medium, lysed by freezing and thawing, and used as the source of recovered dengue virus.

Dengue Virus Characterization. Parental and recovered dengue viruses were characterized for plaque phenotype on LLCMK₂ cells (25). To analyze dengue virus proteins, confluent LLCMK₂ cells in a 6-well plate were infected with parental or recovered virus at 0.2 plaque-forming unit per cell in medium 199 containing 2% fetal bovine serum. Six days after infection cells were metabolically labeled with [³⁵S]methionine (50 μ Ci per well; specific activity, 600 Ci/mmol; 1 Ci = 37 GBq) in methionine-free medium for 2 hr and the lysate

was prepared in RIPA buffer for radioimmunoprecipitation using dengue virus hyperimmune mouse ascitic fluid (12). The immunoprecipitate was analyzed by SDS/12% PAGE (9).

RESULTS

Initial Attempt to Clone Full-Length Dengue DNA in Plasmid pBR322 Containing the SP6 Promoter. A series of dengue cDNA inserts had been cloned at the *Pst*I site of pBR322 that spanned the entire length of the dengue type 4 virus genome (8, 9). These cDNA inserts were joined at shared restriction enzyme sites to form a full-length dengue DNA copy (1A) by using the same *Pst*I cloning site of pBR322. For *in vitro* transcription, the SP6 polymerase promoter sequence was placed at the 5' end preceding the dengue sequence. The DNA structure for the predicted 5' sequence of RNA transcripts is shown in Fig. 1. The A residue of the first dengue nucleotide was positioned immediately following the normal SP6 polymerase transcription-initiating G residue. The m⁷G cap structure that is present at the 5' end of the virion RNA was provided by incorporation of m⁷G(5')pppG(5') as a cap analog in the transcription reaction. Thus, the DNA template would yield an m⁷G-capped dengue RNA transcript containing an additional G residue at the 5' terminus. To produce run-off transcripts, the template was linearized at the unique *Asp*718 cleavage site immediately following the 3' end of the dengue sequence. As shown in Fig. 1, five additional nucleotides are present in the template strand preceding the *Asp*718 cleavage site. If transcription proceeded to the last nucleotide, the RNA transcripts would contain these five additional residues at the 3' terminus.

In the earlier experiments in which *E. coli* HB101 was employed as the host for transformation, we observed that full-length dengue cDNA was often unstable in the plasmid. Thus, dengue cDNA underwent rearrangement and many colonies had to be screened to isolate a clone of DNA with the predicted restriction enzyme digestion pattern. In an attempt to solve this problem, we examined stability of clone 1A plasmid produced in other strains of *E. coli*. The highly transformation-competent strain DH5 α and strain BD1528 used in bisulfite-induced mutagenesis were compared with

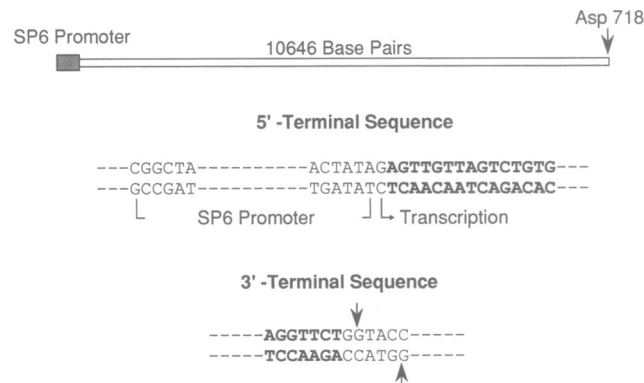


FIG. 1. Terminal sequences of cloned full-length dengue virus cDNA used for transcription of infectious RNA. The full-length dengue virus cDNA (10,646 nt long) was cloned with the adjacent SP6 promoter sequence (filled box) at the 5' end and the unique *Asp*718 cleavage sequence (arrow) at the *Pst*I site of pBR322 plasmid. Note that there is one additional C residue at the position between 10,448 and 10,449 and one additional G residue between 10,470 and 10,471, both of which were missing in the published dengue type 4 sequence (8). The template sequences for the predicted 5' terminus and 3' terminus of the RNA transcript are also shown. The SP6 polymerase transcription-initiating G is retained and is located preceding the 5' dengue sequence shown in bold letters. The *Asp*718 cleavage sequence GGTACC is positioned immediately following the 3' dengue sequence also shown in bold letters.

strain HB101. We found that strain DB1528 produced transformants exhibiting a colony size 3–4 times larger than HB101 transformants. More important was the observation that transformants of DB1528 generally yielded a plasmid with the predicted restriction enzyme pattern, suggesting that *E. coli* DB1528 was the strain of choice to produce stable clones of full-length dengue DNA. However, *in vitro* RNA transcripts made from such a full-length dengue DNA clone (1A) were not infectious when tested by transfection of permissive cells in culture, whereas dengue virion RNA used as the positive control at a 100-fold lower concentration yielded infectious dengue virus as detected by indirect immunofluorescence assay.

Replacement of Dengue DNA Segments in the Full-Length Construct with Independently Derived DNA Clones. We reasoned that the failure to produce an infectious RNA transcript from the cloned dengue virus cDNA template might be due to the presence of one or more deleterious mutations in the full-length cDNA clone 1A. Such mutations could presumably arise from cloning of a defective genomic RNA in the virus stock or from a copying error during cloning and/or propagation of the plasmid. In an attempt to correct the defect in cDNA clone 1A, we replaced dengue DNA segments that might contain such deleterious mutations with the corresponding segments from independently cloned dengue cDNA. We adopted the strategy of cloning the 5' and 3' halves of the dengue cDNA sequence separately. The unique *Bst*BI site at nt 5069 was used to divide the full-length dengue sequence into two fragments, each representing $\approx 50\%$ of the genome. A unique *Asp*718 site was introduced at the *Pst*I site of pBR322 downstream of the *Bst*BI site of the dengue sequence. For convenience, the 5' fragment of the first full-length clone containing the SP6 promoter was designated 5'-1 and the remaining 3' sequence between the *Bst*BI and *Asp*718 sites was designated 3'-A. A plasmid containing the second 5' fragment, 5'-2, was constructed from a second set of dengue cDNA inserts. This plasmid was also suitable for use as a cloning vector for insertion of a 3' fragment to yield a full-length DNA construct. Two additional 3' fragments flanked by *Bst*BI and *Asp*718 sites, 3'-B and 3'-C, were also constructed from an independent series of dengue cDNA inserts (Fig. 2). Replacement of the 3' fragment in the first full-length clone 1A with 3'-B or 3'-C fragments yielded two other full-length clones, 1B and 1C. Similarly, substitution of the 5'-2 fragment into three other full-length DNA constructs yielded full-length DNA clones 2A, 2B, and 2C. In this manner, six full-length cDNA combinations were available for analysis.

Initial Evidence for Infectivity of RNA Transcripts Produced *in Vitro*. RNA transcripts produced from the six cloned full-length dengue DNA templates were tested for infectivity

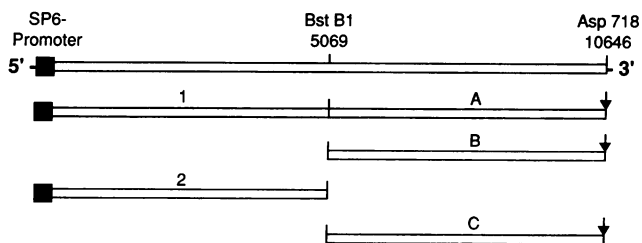


FIG. 2. Full-length dengue cDNA clones. The diagram depicts the full-length dengue cDNA clone containing the SP6 promoter sequence at the 5' end and an *Asp*718 cleavage site at the 3' end as shown in Fig. 1. The *Bst*BI cleavage site at nt 5069 separates the dengue genome into the 5' and 3' half-fragments. Replacement of these fragments in full-length clone 1A with the corresponding 5'-2, 3'-B, or 3'-C fragments that were constructed from an independent set of cDNA clones gives rise to five other full-length combinations as shown.

by transfection of simian LLCMK₂ cells. Dengue virus-infected cells were readily observed by an indirect immunofluorescence assay 10 days following transfection with 2A RNA. In contrast, RNA transcripts from the five other full-length DNA clones were negative by this assay, indicating that these cDNA clones contained one or more lethal mutations that were not detected by restriction enzyme analysis. Confirmation of the infectivity of the RNA transcripts of clone 2A was provided by recovery of dengue virus from the medium or the cell lysate of 2A RNA-transfected cells. The titer of dengue virus present in the transfected cell lysate was 10^5 plaque-forming units/ml. Treatment of 2A RNA transcripts with DNase I did not affect infectivity, whereas RNase treatment completely abolished infectivity (data not shown). In these experiments the infectivity of RNA transcripts was not analyzed by plaque assay. Instead, the production of infectious virus in cell culture was sought after an extended incubation that permitted virus amplification. When assayed in this manner by indirect immunofluorescence the minimum concentration required for infectivity was 1 ng for virion RNA and 10 ng for 2A RNA transcripts. Thus, specific infectivity was estimated to be 100 infectious units/ μ g of 2A RNA or 1000 infectious units/ μ g of virion RNA. These values were 10–100 times less than those observed for yellow fever virus (22), suggesting that dengue RNA transfection is relatively less efficient or that one or more attenuating but nonlethal mutations are present in clone 2A. In any event, these analyses indicated that the RNA transcripts made from clone 2A were infectious following transfection of permissive cells in culture.

Additional Evidence for Infectivity of RNA Transcripts. To provide additional evidence that infectious dengue virus was produced by cells transfected with clone 2A RNA transcripts, we introduced two silent mutations into full-length dengue clone 2A DNA (G3473 \rightarrow T and C3476 \rightarrow A) that created a new *Pst*I site at nt 3476. These mutations created a reporter sequence but did not alter the amino acid sequence. RNA transcripts were then prepared from reengineered 2A(P) DNA, containing the new *Pst*I cleavage site, and complete removal of the DNA template was effected by exhaustive digestion with DNase I. These transcripts were then used for transfection of cells. The transfected cells produced infectious dengue virus designated 2A(P). Genomic RNA extracted from progeny virus derived from 2A or 2A(P) was reverse-transcribed using appropriate primers and the cDNA product was employed as a template for a polymerase chain reaction (PCR) to generate a DNA fragment between nt 3193 and 4536. A DNA band was observed at a gel position predicted for the 1343-bp fragment produced by PCR (Fig. 3). *Pst*I digestion of the PCR DNA product from 2A(P) virus yielded two fragments that were 280 and 1063 bp long as predicted by the presence of the *Pst*I cleavage sequence. The control PCR DNA product of virus recovered from 2A RNA was insensitive to *Pst*I digestion. This observation indicated that progeny virus 2A(P) was derived from the RNA transcripts of mutant 2A(P) DNA containing the engineered *Pst*I site.

Dengue Virus Recovered from Infectious RNA Transcribed *in Vitro*. The progeny dengue virus recovered from the lysate of cells transfected with RNA transcripts produced from clone 2A or clone 2A(P) was compared with parental wild-type virus for ability to produce plaques on LLCMK₂ cell monolayers. Six days after infection, both progeny virus and parental virus produced characteristic dengue plaques. Although the parental virus previously subjected to passage in mosquito cells produced both small and large plaques, both progeny viruses recovered from LLCMK₂ cells yielded mostly large plaques. It is not known whether the mixed plaque morphology of the wild-type virus on LLCMK₂ cells was the result of passage in mosquito cells, leading to

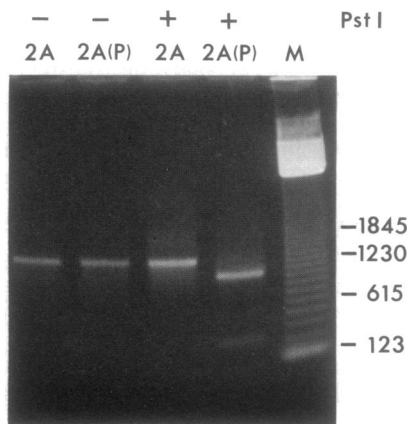


FIG. 3. Dengue virus recovered from infectious RNA contains the genomic sequence of the DNA template. Progeny dengue virus was recovered from LLCMK₂ cells transfected with DNase-treated RNA transcribed from clone 2A DNA (wild-type control) or from clone 2A (P) DNA containing an engineered *Pst* I site at nt 3476 of the dengue sequence. The recovered dengue virus was then grown in C6/36 mosquito cells. Medium from infected C6/36 cells was collected and virion RNA was extracted from the pelleted virus. Virion RNA was used for reverse transcription and the cDNA product was used as template to produce the 1343-bp dengue virus cDNA fragment (nt 3193–4536) by PCR using the appropriate primers. *Pst* I digestion of the PCR product is shown. Lane M shows the 123-bp DNA ladder as size markers.

emergence of plaque morphology mutants. The more uniform plaque morphology of the viruses recovered from cells transfected with full-length RNA transcripts probably reflects the clonal origin of the clone 2A- or 2A(P)-derived virus. Dengue-specific proteins produced by progeny virus-infected cells and by parental virus-infected cells were also compared. Fig. 4 shows the profile of protein bands including dengue virus preM, E, NS1, NS3, and other, unassigned, dengue-specific

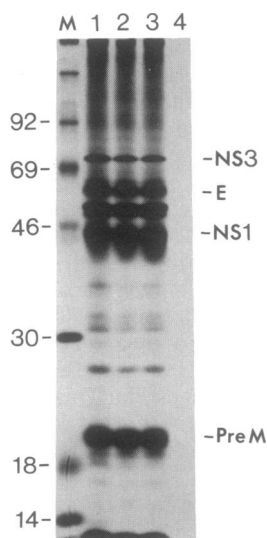


FIG. 4. Comparison of dengue virus proteins from LLCMK₂ cells infected with recovered virus or parental virus. [³⁵S]Methionine-labeled cell lysates were prepared for immunoprecipitation with dengue virus hyperimmune mouse ascitic fluid. The immunoprecipitates were separated by electrophoresis in SDS/12% polyacrylamide gel (acrylamide/*N,N'*-methylenebisacrylamide weight ratio, 60:1.6). Lanes: 1, cells infected with parental dengue virus; 2, cells infected with dengue virus recovered from clone 2A DNA; 3, cells infected with dengue virus recovered from clone 2A(P) DNA; 4, mock-infected cells; M, protein size markers (sizes in kilodaltons at left). Dengue virus proteins preM, E, NS1, and NS3 are indicated.

bands that were precipitated by dengue virus hyperimmune ascitic fluid. The size and relative intensity of each dengue protein appeared to be similar for progeny viruses and parental virus. Taken together, these results indicate that the recovered dengue viruses were similar to the virus from which the cDNA clone was derived.

DISCUSSION

We constructed stable full-length dengue type 4 virus cDNA clones that were used as templates for production of RNA transcripts that were evaluated for infectivity in cell culture. Evidence is presented that dengue virus was recovered upon transfection of permissive cultured cells with the *in vitro* RNA transcripts of one of six full-length DNA clones tested. To our knowledge, this represents the first demonstration that full-length flavivirus cDNA can be stably cloned and propagated in a cloning vector. In the case of yellow fever virus, the full-length cDNA used for successful production of infectious RNA transcripts was obtained by *in vitro* ligation of two separately cloned DNA segments (22). Instability of plasmid full-length dengue type 4 virus cDNA was initially observed when *E. coli* HB101 was used. Subsequent evaluation of plasmid dengue cDNA stability in other strains of *E. coli* showed that full-length dengue cDNA could be stably amplified in *E. coli* strain BD1528.

The full-length cDNA clone 1A, constructed from DNA segments that were used to establish the dengue type 4 virus sequence, did not yield infectious RNA transcripts. Replacement of the DNA segment in the region between the *Hind*III and *Bst*BI sites (nt 44–5069) with a corresponding DNA segment obtained from another set of independent clones from the same cDNA library corrected the defect, as infectious RNA was transcribed from the resulting full-length clone 2A. The failure of 1A DNA to serve as a template for infectious RNA transcripts was probably a manifestation of one or more deleterious mutations that were not detected by restriction enzyme analysis. Such mutations did not apparently affect expression of the encoded polypeptide, since recombinant vaccinia viruses containing sequences for one or more proteins from this region each produced properly processed and functional dengue proteins (15, 23, 26). It should be possible to further localize the site of lethal mutation(s) by using smaller DNA segments for substitution in a manner analogous to the marker rescue study originally performed to map the mutation sites of temperature-sensitive mutants of simian virus 40. (27). Comparison of the sequence in the functionally active segment with the established dengue type 4 sequence should clarify the nature of the deleterious mutation(s). One or more deleterious mutations also occurred in the 3' region between *Bst*BI and *Asp*718 sites (nt 5069–10,646), as two other separately cloned DNA segments in this 3' region failed to produce infectious RNA when used together with the functional 5' sequences to construct a full-length DNA clone. A possibly deleterious mutation in the 3' sequence of cloned yellow fever DNA was reported (22). This change apparently did not occur in dengue virus in the 3' region of 2A cDNA, which was stably cloned.

The reduced specific infectivity of dengue virus RNA transcribed *in vitro* might be explained by nonviral 5' and 3' sequences if internal sequences were otherwise identical to the virion RNA. The predicted 5' terminus of RNA transcripts contains an additional G following the added m⁷G cap. This additional 5' sequence was also present in the yellow fever RNA transcripts, which exhibited 2–3% of the specific infectivity of virion RNA (22). Additional nucleotides are also present in the 5' sequence of infectious RNA transcribed *in vitro* from cDNA of other RNA viruses including poliovirus (18), hepatitis A virus (20), and Sindbis virus (21). In each case, the specific infectivity of the *in vitro* RNA transcripts

was also lower than that of virion RNA. The presence of additional nucleotides at the 3' terminus might also reduce the specific infectivity of *in vitro* transcribed dengue RNA. While it is possible to remove additional nucleotides at the 3' template by exonuclease digestion, as was carried out for the yellow fever cDNA template, we found that additional nucleotides present in the 3' template of dengue cDNA did not abolish the infectivity of the RNA product.

Recovered dengue virus showed a more uniform plaque morphology than did the parental dengue virus, which had been propagated several times in C6/36 mosquito cells. This is not surprising, because recovered virus probably represents a clonal population of the original virus stock. Nonetheless, analysis of dengue proteins produced by recovered virus and by parental virus showed an identical profile. The terminal sequences of virion RNA from recovered virus were not verified by direct analysis. Conceivably, specific sequence recognition and initiation of flavivirus RNA replication coupled with specific packaging of virion RNA could provide a mechanism for precise trimming of the additional nucleotides present in transfecting RNA. For these reasons, it is possible that virion RNA of recovered dengue virus contains authentic terminal sequences despite the fact that additional nucleotides are present in RNA transcripts.

The successful recovery of dengue virus from cloned cDNA-derived RNA transcripts has implications for molecular analysis of dengue virus as well as the development of new dengue vaccine strategies. It is now possible to introduce mutations into dengue cDNA by site-directed mutagenesis and recover dengue virus for biological studies such as identification of regulatory elements involved in transcription, replication, and packaging of dengue RNA, as well as elucidation of the mechanism of polyprotein processing. Molecular techniques now become available for analysis of the antigenic structure of dengue envelope glycoprotein and other protective antigens and for characterization of other viral structural and nonstructural proteins to better understand their functional role in viral replication and possible involvement in dengue immunopathogenesis.

One immediate application of our current findings is in the area of vaccine development. Restriction of dengue virus replication should cause attenuation. For example, it should be possible to produce a panel of dengue viruses that are restricted in viral replication to a varying degree as a consequence of suboptimal polyprotein processing. The polyprotein NS1-NS2A cleavage site has been chosen as the first target for constructing dengue virus mutants that are restricted because of inefficient cleavage (M. Pethel and C.-J.L., unpublished observations). Similarly, it may be possible to isolate dengue virus mutants containing deletions in the coding regions as well as in the 3' or 5' noncoding region that result in reduced replicative capacity. Deletion mutants would offer the theoretical advantage of being less subject to reversion of phenotype than amino acid substitution mutants. Dengue virus deletion mutants with altered properties such as temperature sensitivity or reduced virulence could be subjected to further evaluation in monkeys for evidence of attenuation prior to initiation of clinical trials in volunteers. Because of the concern about immune enhancement as the underlying cause for severe dengue (1), the current strategy for immunization against dengue favors the use of a vaccine preparation that contains all four dengue serotypes. In this regard, the full-length cDNA of dengue type 4 virus could be used as a vector for engineering chimeric dengue viruses that

contain the DNA sequence coding for the structural proteins of one of the three other serotypes that are substituted for the corresponding DNA sequence of dengue type 4 virus. Such chimeric dengue viruses prepared from the other serotypes would share a common sequence of type 4 nonstructural proteins and the 5' and 3' noncoding regions of type 4 if the appropriate mutations conferring satisfactory attenuation could be engineered in these type 4 regions. In this manner it may be possible to construct chimeric dengue viruses of type 1, type 2, and type 3 antigenic specificity that could be included in a live quadrivalent dengue vaccine.

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