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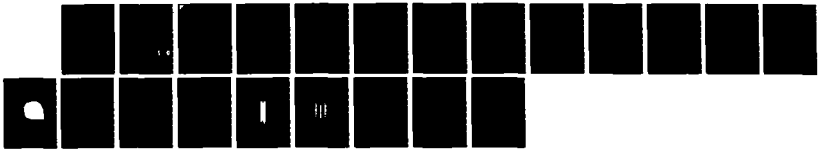

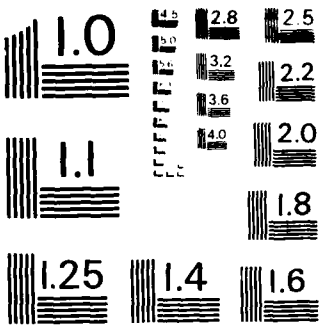

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"Structure and Functional Studies of DEN-2 Virus Genome"

Progress Report

by

Radha Krishnan Padmanabhan, Ph.D.

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Complementary DNA synthesis of Dengue-2 RNA by avian reverse transcriptase in vitro. The size of the DNA copy of Dengue RNA is in the range of 800 to >5000 nucleotides.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Dengue-2 RNA was extracted from the virus particles. Poly A tail was added to the 3' end of DEN-2 RNA using E. coli poly A polymerase and ATP as well as T_4 RNA ligase and Oligo (A). The poly (A) tailed DEN-2 RNA was used as a template for complementary DNA synthesis using oligo(dT) as primer and reverse transcriptase. The size of the cDNA synthesized was determined by alkaline agarose gel electrophoresis using standard DNA marker fragments. The size is heterogeneous as expected and it falls in the range of 800 to >5000 nucleotides.

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Abstract

1. Dengue-2 RNA (DEN-2 RNA) was extracted from the virus particles by treatment with sodium dodecyl sulfate and protease-K, followed by phenol extraction and ethanol precipitation.

2. Small amounts of DEN-2 RNA were used for E. coli poly A polymerase reaction and for T_4 RNA ligase reaction. The former enzyme catalyzes the polymerization of AMP residues from the precursor ATP and the latter enzyme catalyzes the ligation of oligo A residues, and both reactions occur at the 3' end of RNA. We demonstrate that both enzymes catalyze the addition of poly A tract efficiently to the 3' end of DEN-2 RNA.

3. Poly A (or oligo A in the case of RNA ligase reaction) -tailed DEN-2 RNA was used as a template for the reverse transcriptase reaction for cDNA synthesis in the presence of 3 unlabeled and one labeled deoxynucleoside triphosphates. Irrespective of the method used to add the poly A tail, cDNA of DEN-2 RNA was synthesized by reverse transcriptase. We are currently working on the synthesis of the second strand and cloning of the dC-tailed double-stranded cDNA into dG-tailed pBR322 DNA.

4. For structural analysis near the 3' end of DEN-2 RNA, we labeled the 3' end by using RNA ligase and ^{32}pCp. The labeled DEN-2 RNA was analyzed by HCHO-agarose denaturation gel electrophoresis. We found by autoradiography that the labeled RNA was intact and behaved similarly to Sindbis RNA in this gel system. We are currently determining the nucleotide sequence near the 3' end using well-established RNA sequencing procedures.

5. We have developed a computer program to analyze a nucleotide sequence of any length up to 10,000 bases for the occurrence of restriction sites or other unique nucleotide sequences. A second program was developed which is analogous to the above program, except it is designed to search for and print out fragment sizes predicted from digests with two different restriction enzymes. A listing is printed for each of the enzymes individually and then a composite listing is printed for the double digestion fragments. This is useful for preparing DNA standards for restriction site mapping using DNA of known nucleotide sequence as a standard. A third program was designed to assist in the restriction enzyme mapping experiments. If the migration distances of DNA fragments of unknown sizes are compared to those of standard DNA fragments in this program, the sizes of the unknown DNA fragments can be calculated.

OBJECTIVES: The overall objective of this project is to analyze the structure of Dengue-2 virus RNA using recombinant DNA technology in order to understand the biological role of the specific antigens coded by the RNA genome after infection of the host cell. The project will be carried out in the following steps: (1) complementary DNA copy of the viral RNA will be synthesized using the enzyme reverse transcriptase; (2) complementary DNA will be cloned in *E. coli* host/vector system in order to get large amounts of cDNA; (3) a physical map of the cDNA clone will be constructed using several restriction endonucleases; (4) location of the genes for the various viral antigens on the cDNA will be identified; (5) DNA sequence analysis of cDNA coding for specific viral genes will then be carried out. Finally, the gene for V3 will be expressed in *E. coli*.

INTRODUCTION

Dengue-2 belongs to the group of Flaviviruses which are the most abundant of Togaviruses. The genome is a non-segmented single-stranded RNA that has a sedimentation coefficient in the range of 38-45S and a molecular weight of 3-4 x 10^6 (~12,000 nucleotides in length; see Russell et al., 1980). The RNA lacks a poly(A) tract at its 3' end. Virion structural polypeptides first identified for DEN-2 virus (Stollar et al., 1969) and KUN virus (Westaway and Reedman, 1969) have been shown to be similar for eleven flaviviruses. In all instances, mature virions have a nucleocapsid or core protein V2 (M.W. 13,000 daltons), a large envelope glycoprotein V3 (M.W. 59,000 for DEN-2 protein) and a small non-glycosylated envelope protein V1 (M.W. 7000-8000 daltons).

In addition to the three virion (capsid) proteins, there are a number of non-virion proteins presumably coded by the viral genome. The size of the flavivirus genome (M.W. 4 x 10^6 daltons) is sufficient to code for all the virus-specified protein, their total molecular weight being about 370,000 daltons. Available evidence published elsewhere suggests that the synthesis of virus-specific protein does not go through an intermediate polyprotein which is processed further. This aspect of control of translation seemed to be different from those of other positive-strand RNA containing animal viruses in which the initiation of translation and termination occurs in each case at only a single site on mRNA.

The elucidation of the physical order of the coding sequences of the various virus-specific proteins on the viral RNA from 5' → 3' was attempted by observing the sequence of termination of incorporation of amino acid into translation products in infected HeLa cells treated with pactamycin or with hypertonic salt. Both treatments allow ribosomes to elongate nascent polypeptides but block reinitiation. In preliminary experiments with KUN-virus infected cells, Westaway observed that, subsequent to synchronous reinitiation, the total translation time is less than that of the much smaller poliovirus genome. This observation could be explained by a single initiation resulting in a polyprotein which then undergoes proteolytic cleavages to give rise to all the virus-specific proteins. Westaway interpreted these data to mean that there are multiple and independent internal initiations of translation occurring on the flavivirus genome.

Wengler et al. (1979) and Svitkin et al. (1981) used an in vitro translation system in order to investigate the problem of initiation of translation of flavivirus genome. The analysis of the polypeptide translated from the 42S in a reticulocyte lysate in vitro indicates that a series of polypeptides of increasing size, all starting probably at the same initiation sequence on the mRNA are synthesized. From the in vitro experiments using West Nile system, the following order of coding sequence, 5'-V2'V3 (V1, p_i20; p_i27; p_i37; p_i71; p_i100)-3' terminus, was deduced. The relative order of proteins within brackets is unknown (Wengler et al., 1979). Svitkin et al. (1981) used an in vitro translation system prepared from Krebs-2 cells and 42S RNA from tick-borne encephalitis virus (TBEV). In this system, polypeptides with size ranging from 13,000 to>160,000 were produced. Two of these polypeptides, V2 and V3, have been identified as virion core polypeptides. The amino acid sequences of these two polypeptides were also shown to be present in a larger polypeptide with the size of 118,000 daltons.

These data do not support the hypothesis proposed by Westaway (1977) for the occurrence of multiple initiation sites for the various virus-specific proteins.

RATIONALE

In order to understand the mechanism of initiation of translation of flavivirus genomes, its primary structure needs to be determined. This approach has been recently accomplished in the case of the poliovirus genome. From the nucleotide sequence, it was possible to locate the initiation site of translation for the polyprotein and its predicted amino acid sequence.

Our present understanding of the control of the synthesis of virus-specific polypeptides coded by the flavivirus genome is incomplete. The controversy whether the genome is translated from a single initiation site producing a precursor polypeptide which is subsequently cleaved or from multiple internal initiation sites is far from settled. Moreover, for the development of new vaccines, one approach is the construction of attenuated viral strains incapable of reverting to a virulent strain. A second approach is to make E. coli express cloned segments of the viral genome coding for specific antigens (like V3) that can synthesize neutralizing antibodies in man. In order to achieve these goals, the genomes of the flaviviruses need to be investigated.

PROGRESS REPORT (March 1, 1982-September 1, 1982)

The contract from USAMRDC was awarded to support this project officially on March 15, 1982, at The University of Kansas Medical Center. Dr. Masatoshi Nohara, Research Associate, joined my laboratory on May 7, 1982, and Dr. Narender Kalyan, Research Associate, joined on July 7, 1982.

1. Extraction of DEN-2 RNA from the virions. The virions were purified by sucrose density gradient and supplied to me by Dr. Walter E. Brandt. The virus particles were disrupted by treatment with sodium dodecyl sulfate (2%) and protease K (50 µg/ml) at 37°C for 1 hr. The solution was extracted with phenol three times. The resultant aqueous layer was extracted with ether three times and precipitated with ethanol (2 volumes) in the presence of 0.3 M sodium acetate. The ethanol precipitated RNA was centrifuged at 8000 rpm using SS34 rotor in a Sorvall RC-5B centrifuge for 30 min. The supernatant was removed and the precipitate, after drying in the dessicator, was resuspended in 100 µl of TE buffer (10 mM Tris and 1 mM EDTA) and divided into four aliquots. Each was frozen at -70°C until further use.

2. Estimation of the amount of the viral RNA present. An estimation of the amount of RNA in a solution can be determined by the measurement of the absorbance of a solution of RNA at 260 nm. A solution of 1.0 A_{260} contains approximately 40 µg/ml. We used an alternate method which is extremely sensitive to estimate the amount of RNA in a solution. The principle of this method is that ethidium bromide binds to RNA and DNA to give rise to fluorescent complexes which can be photographed under ultraviolet light. This method is extremely sensitive and can detect and quantitate an unknown

RNA when compared to the fluorescence of standard RNA solution. We used E. coli tRNA of known concentration to titrate the amount of DEN-2 RNA. Fig. 1 shows the ethidium bromide fluorescence of DEN-2 RNA and E. coli tRNA standard. The amount of RNA extracted from the virions was estimated from the intensity of fluorescence at 125 µg/ml. Hence, the yield of DEN-2 RNA from the virions (which were originally isolated from the infected cells grown in ten 32 ounce bottles) is 12.5 µg.

3. Addition of poly A tract to the 3' end of DEN-2 RNA. DEN-2 RNA has no poly A tract at its 3' end. In order to synthesize the cDNA of an RNA molecule, one approach involves the use of oligo(dT) as primer for reverse transcriptase. This requires the presence of poly A (or oligo A) at the 3' end of the RNA. I proposed two approaches for the addition of poly A to the 3' end: use of E. coli poly A polymerase which catalyzes the polymerization of AMP residues (from the precursor ATP) to the 3' end of an RNA; and use of T4 RNA ligase which can join short oligo(A) to the 3'-OH of DEN-2 RNA.

a) Use of E. coli poly A polymerase

Poly A polymerase from E. coli readily polymerizes the AMP of ATP onto the free, 3' terminal hydroxyl group of RNA. The length of poly A synthesized varies from 300 to several thousand residues depending on the time of incubation. We used this enzyme to add poly A tract to the 3' end of DEN-2 RNA in a reaction containing about 3 µg of RNA, 50 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.25 M NaCl, 250 µM ATP-^{3}H (8.7 mCi/µmole) and one unit of poly A polymerase. The incubation was carried out at 37°C for 60 min. The reaction was stopped by extracting with $CHCl_3$:isoamyl alcohol (24:1) mixture and ether extracting the aqueous layer to remove $CHCl_3$. The aqueous layer was used for cDNA synthesis by reverse transcriptase.

A 1 µl sample from a total of 50 µl at 0 min and 60 min after incubation was tested for trichloracetic acid insoluble radioactivity. The following was observed:

	(^{3}H) radioactivity (cpm)
0 min	227
60 min	2292

b) Addition of poly A tract to the 3' end of DEN-2 RNA using T4 RNA ligase

T_4 RNA ligase catalyzes the formation of an internucleotide bond between a 5' terminal phosphate and a 3' terminal hydroxyl of oligo- or polyribonucleotides with the accompanying hydrolysis of ATP (England and Uhlenbeck, 1978). RNA ligase was used to add 5' ^{32}P labeled oligo(A) to the 3' end of DEN-2 RNA. OligoA from P.L. Biochemicals was phosphorylated in a reaction mixture containing 262 pmoles of oligo A, 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 10 mM dithiothreitol and 20 units of polynucleotide kinase. The 5' ^{32}P labeled oligo(A) was fractionated from unutilized (γ-^{32}P)ATP by Sephadex G-50 column chromatography.

The labeled oligo(A) peak was concentrated by evaporation and taken up in 10 µl of 10 mM Tris HCl containing 1 mM EDTA.

The RNA ligase reaction contained 1-3 µg DEN-2 RNA, 225 pmoles of ^{32}P labeled oligoA, 5 µM ATP, 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 10% dimethyl sulfoxide and 6 units of RNA ligase. The incubation was carried out for six hours at 4°C. The product was analyzed by formaldehyde-agarose gel electrophoresis (Figure 2) (see Lehrach et al., 1977). RNA was denatured by heating in a mixture of formaldehyde and formamide and applied to an HCHO-agarose gel (1.5%) and electrophoresed at 25 mA overnight. The gel was dried under vacuum and autoradiographed. A labeled band that migrated very little from the origin was obtained. Sindbis RNA whose size is close to that of DEN-2 RNA was reported to have a similar electrophoretic mobility in this system (Lehrach et al., 1977). From these results, it is evident that at least a portion of DEN-2 RNA remained intact in this reaction after the addition of poly A tract.

DEN-2 RNA containing a poly A tract at its 3' end, added in a reaction catalyzed by either E. coli poly A polymerase or RNA ligase, was used for cDNA synthesis, catalyzed by reverse transcriptase.

4. cDNA synthesis. The reaction mixture contained 1-3 µg of poly A-tailed DEN-2 RNA, 53 pmole of oligo(dT), 209 µM of dCTP-^3H, (or α-^{32}PdCTP), 270 µM of three unlabeled dNTPs, 60 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 120 mM NaCl, 5 mM 2-mercaptoethanol, 25 units of human placental RNAse inhibitor and 10 units of reverse transcriptase. The incubation was carried out at 41°C for 1 hr. The reaction was stopped with a solution (25 µl) containing 0.02% bromophenol blue, 50% glycerol, 25 mM EDTA and 0.1% SDS. The cDNA synthesized was fractionated by gel filtration on Sephadex G-75 chromatography (Fig. 3 a & b). In Fig. 3a, the cDNA of DEN-2 RNA has dCMP-^3H incorporated in it and in Fig. 3b, the cDNA contains dCMP-(α-^{32}P) incorporated in it. In addition, in Fig. 3a, the peak which was excluded in the void volume contained cDNA:DEN-2 RNA hybrid. The peaks that contained ^{32}P radioactivity in this profile are presumably due to DEN-2 RNA labeled at the 3' end due to ligation of ^{32}P-oligo A, and the peaks which eluted subsequently are presumably due to oligomers of ^{32}P-oligo(A). In Fig. 3b, the reverse transcriptase reaction mixture was incubated with 0.4 N NaOH for 4 hours to digest the RNA moiety of cDNA:RNA hybrid, prior to G-75 column chromatography. Hence, a single peak of cDNA of DEN-2 RNA was obtained.

5. 3' end group labeling of DEN-2 RNA with ^{32}pCp catalyzed by RNA ligase. In order to elucidate the sequence analysis of DEN-2 RNA near the 3' terminus, we used RNA ligase to label the 3' terminus with ^{32}pCp under conditions described by England and Uhlenbeck (1978). The reaction mixture (30 µl) contained 0.62 µg of DEN-2 RNA, 35 pmoles of ^{32}pCp, 50 µM ATP, 50 mM HEPES, 10 mM, 3.3 mM dithiothreitol, 10% dimethyl sulfoxide and 6 units of RNA ligase. The reaction was carried out at 4°C for 6 hours. The total radioactivity incorporated into DEN-2 RNA was determined by trichloracetic-acid-precipitable counts. A total of 1.1×10^6 cpm was incorporated per 0.65 µg of RNA. A small aliquot was applied to formaldehyde-agarose gel (1.5%) and electrophoresed at 25 mA for 16 hours. The gel was dried and autoradiographed. The results show that at least a portion of DEN-2 RNA was intact during this labeling reaction (Fig. 4). The labeled RNA of the size of Sindbis virus did not migrate from the origin under the

conditions used in this experiment (Lehrach et al., 1977). The labeled RNA obtained by this method is currently being analyzed for its 3' terminal sequence. We also determined the nearest neighbor analysis of the 3' labeled DEN-2 RNA by complete hydrolysis of DEN-2 RNA into mononucleotides by RNAse T2 and fractionation by high voltage electrophoresis of these mononucleotides, one of which would be expected to be labeled due to nearest neighbor transfer of ^{32}P from ^{32}pCp to the 3' terminal nucleotide of DEN-2 RNA. The 3' terminal nucleotide was found to be A by this analysis.

6. Size of the cDNA of DEN-2 RNA synthesized in the reverse transcriptase reaction. The size of the cDNA of DEN-2 RNA was determined by alkaline agarose gel electrophoresis using standard 5' ^{32}P-labeled λ DNA fragments generated by a double digestion of EcoRI and HindIII. In this digest, standard DNA fragments ranging in size from 21 kb to 0.8 kb in length are present. A small aliquot of cDNA from the peak eluted at V_0 in Fig. 3b (12,000 cpm) was applied on an alkaline agarose gel. The electrophoresis was carried out at 25 mA for about 16 hours. The gel was neutralized in a buffer containing 100 mM Tris-HCl, pH 7.6, 100 mM NaCl and 80% ethanol. The gel was then dried under vacuum and autoradiographed for 4 hours using intensifying screen at -70°C. The autoradiograph is shown in Fig. 5. The size of cDNA is heterogeneous as expected and within a range of 800 to >5000 nucleotides in length. The percentage of cDNA molecules with a size >5000 nucleotides is around 5%.

7. Development of computer programs for nucleic acid structural analysis.
Program Summaries: These programs were written in BASIC for the Heath H-89 microcomputer with 56K bytes of random access memory, a 5 1/4 inch disk drive, and a line printer.

a) RESTRICT.MSB
This program will analyze a nucleotide sequence of any length up to 10,000 bases for the occurrence of restriction sites or other unique nucleotide sequences. The program begins with a query for the name of the DNA fragment to be examined. The name is the data file name of the nucleotide sequence stored in ASCII code on a 5¼ inch disk. These sequences are always stored in a 5'-3' orientation as a single strand beginning at the left hand terminus of linear DNA or at the origin (or other specific locus) of circular DNA. The data analyst has the option of listing the nucleotide sequence for proofreading purposes.

After the sequence is read into memory from disk, the data analyst has another choice between searching for specific restriction enzyme recognition sequences or searching for unique sequences of interest. If the restriction site option is selected, one can either get a listing of sites for all restriction enzymes (93 unique recognition sequences) or get a listing of sites for individual enzymes.

If the unique sequence option is selected, one can enter the nucleotide sequence of interest in a 5'-3' direction. If the same sequence on the complementary strand is desired then the complement in the 3'-5' direction is entered on a second search cycle.

All test sequences are compared in a 5'-3' direction with the nucleotide sequence data. If a match is found, the location of the beginning of the site

is printed as the nucleotide number from the left hand terminus or point of origin. The percentage of genome size (or DNA fragment size) is printed in parentheses. For non-palindromic restriction enzyme recognition sequences, the complementary 3'-5' sequence is also searched. For restriction sites, the fragment sizes are ordered and printed in descending size with the sequence number of the first and last nucleotide indicated.

b) DOUBLE.MSB

This program is operationally similar to RESTRICT. MSB; however, it is designed to search for and to print out fragment sizes predicted from digests with two different restriction enzymes. A listing is printed for each of the enzymes individually and then a composite listing is printed for the double digest fragments. This is useful for preparing DNA standards for restriction site mapping using DNA of known nucleotide sequence as a standard.

c) DNASIZES.MSB

This program was designed to assist in the restriction enzyme mapping experiments. Restriction digests of test DNA were electrophoresed in horizontal submarine slab gels along with standard DNA fragments. The migration patterns were visualized and photographed by UV-induced fluorescence of ethidium bromide intercalated DNA. Migration distances were measured from photographs. These distances and the DNA fragment sizes for the standard DNA serve as input data for the computer program. The standard size and distance data are fitted by a polynomial regression algorithm to yield an equation into which the test DNA migration distances are entered. The resulting evaluation yields the observed fragment size.

d) CLONE.MSB

This program is designed to allow the generation of DNA sequence data files of cloned DNA. After using program RESTRICT.MSB to get the nucleotide numbers of restriction sites of interest, a cloned DNA fragment is generated by the computer simply by entering the file names of the DNA fragments to be used for cloning and the start and stop points for ligation of the fragments. The output is a sequence written on the disk and given a file name by the data analyst.

Experiments to be completed during the remainder of the contract period

The cDNA synthesized from polyA-tailed DEN-2 RNA template will be used for double-strand cDNA synthesis, following the procedure outlined in Fig. 4 of Renewal Contract Application. The ds-cDNA will be tailed with dC residues using calf thymus terminal nucleotidyl transferase. It will be annealed to pBR322, tailed with dG residues and the resultant recombinant plasmid will be used for transformation of E. coli HB101. The ampicillin-sensitive and tetracyclin resistant colonies will be isolated and screened for recombinant cDNA inserts. The size of the inserts and the restriction enzyme cleavage sites of the cDNAs will be determined. DNA sequence analysis of these cDNA molecules will also be started.

FIGURE LEGENDS

Fig. 1. Estimation of concentration of Dengue 2 RNA by ethidium bromide spot test analysis.

Standard tRNA solution of known concentration (determined by $O.D_{260}$ measurement) was mixed with ethidium bromide (1 µg/ml) solution. It was then diluted serially from a range of 2.5 µg/ml to 0.15 µg/ml. A 2 µl aliquot of unknown DEN-2 RNA solution was diluted serially in a similar manner. These diluted solutions were spotted on a sheet of Saran Wrap laid over a UV-transilluminator. The ethidium bromide fluorescence was photographed using Kodak Type 23A filters. The concentration of RNA was calculated by matching the intensity of fluorescence of DEN-2 RNA with that of standard tRNA.

Fig. 2. Formaldehyde-agarose gel electrophoresis of DEN-2 RNA tailed with oligo(A) in a reaction catalyzed by RNA ligase.

An aliquot (2 µl) of DEN-2 RNA labeled at the 3' end with ^{32}P-oligo A in a reaction catalyzed by RNA ligase was applied to a formaldehyde-agarose gel as described by Lehrach et al. (1977). The labeled DEN-2 RNA did not migrate from the origin of 1.5% agarose gel similar to the mobility of Sindbis RNA in this system.

Fig. 3. Complementary DNA synthesis.

The cDNA of DEN-2 RNA was synthesized as described in the text. The reverse transcriptase reaction contained dGTP, dTTP, dATP as non-radioactive triphosphates and dCTP-^{3}H as the labeled triphosphate in 3a and dCTP-^{32}P in 3b. In 3a, the reaction after 1 hr incubation was applied as such to Sephadex G-75 column, whereas in 3b, it was first treated with 0.4 N NaOH for 4 hours at room temperature before G-75 column chromatography. The ^{32}P radioactivity in 3a at V_o is due to DEN-2 RNA which was labeled by 5'-^{32}P oligo A in an RNA ligase-catalyzed reaction, and those ^{32}P peaks included in G-75 were presumably due to oligomers of oligo A.

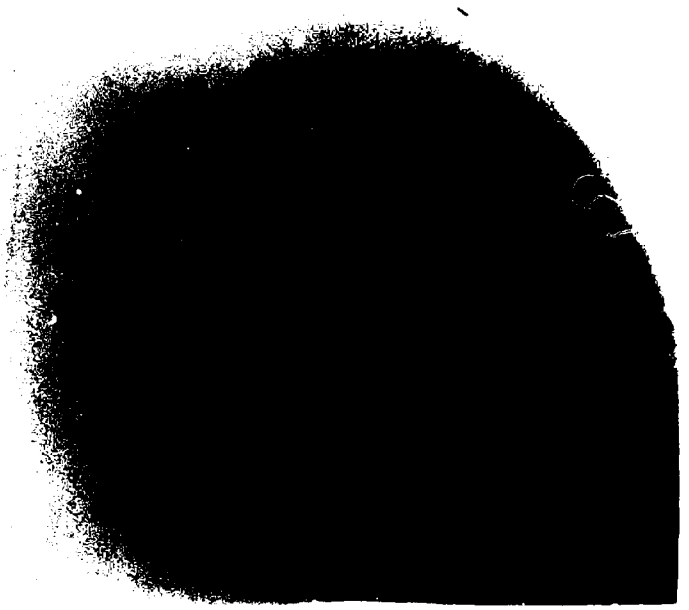
Fig. 4. Formaldehyde-agarose gel electrophoresis of DEN-2 RNA labeled at its 3' end by ^{32}pCp.

The labeling reaction was carried out as described in the text, the RNA was purified as described in 4a, and the electrophoresis was carried out on a 1.5% agarose gel containing formaldehyde as described by Lehrach et al.

Fig. 5. Size determination of complementary DNA of DEN-2 RNA synthesized in vitro by reverse transcriptase.

The labeled cDNA (Lane 2) from G-75 column (Fig. 3b) was electrophoresed in a 1.2% alkaline agarose gel as described by McDonnel et al. (1977). Lanes 1 and 2 contained 5' labeled λ DNA digested by a mixture of EcoRI and HindIII as molecular weight standards.

FIGURE 1



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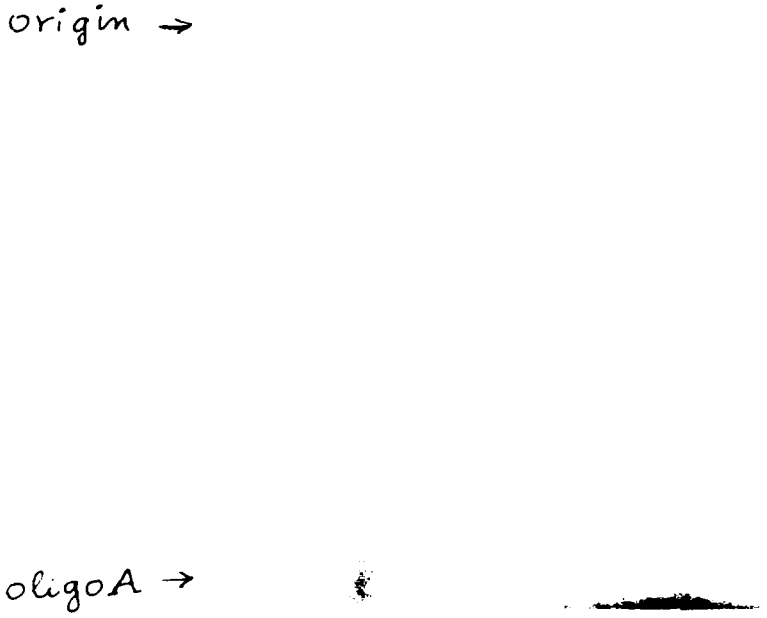
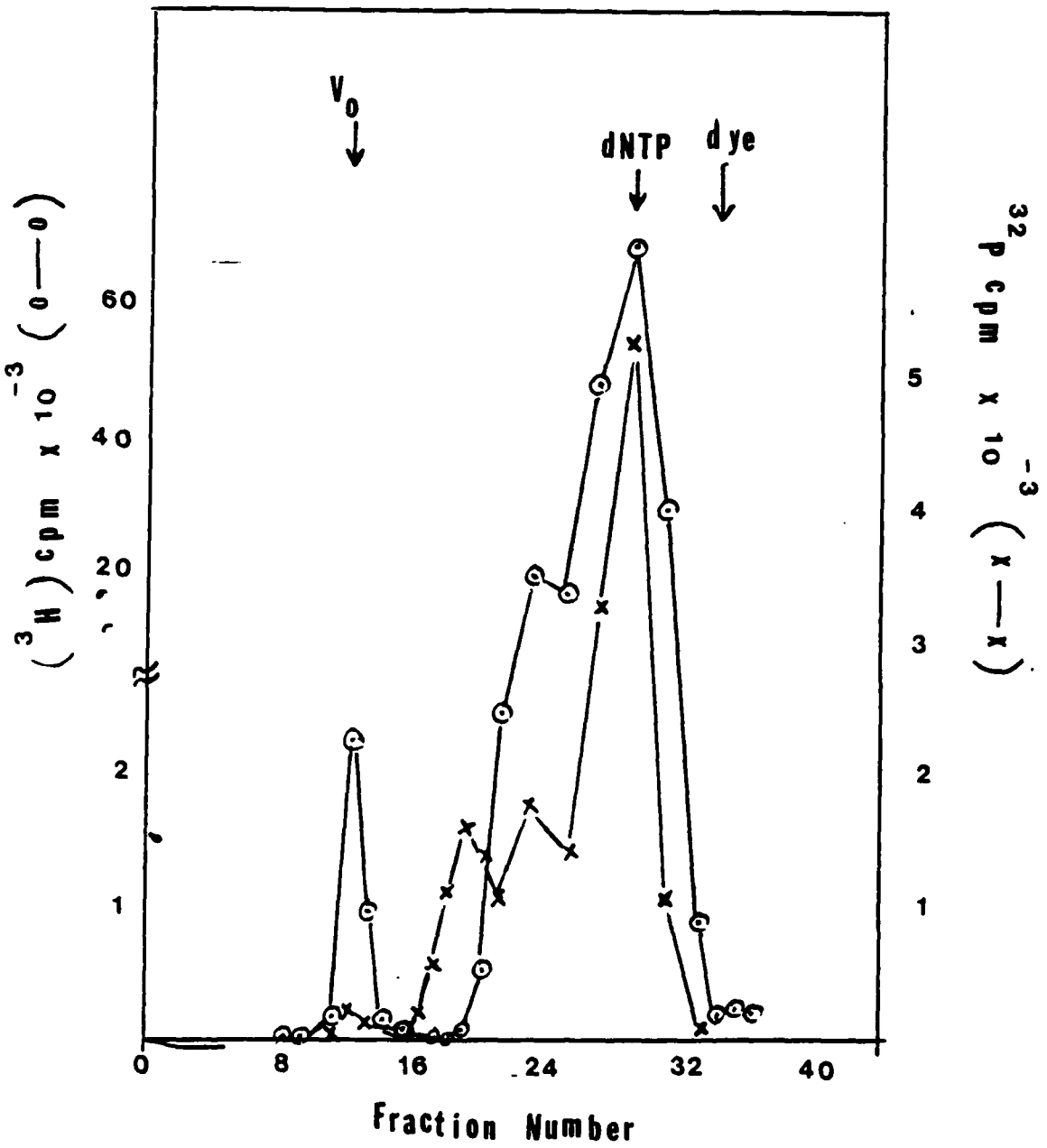


FIGURE 3A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dNTP
dye
(^3H) cpm x 10^{-3} (o—o)
^{32}P cpm x 10^{-3} (x—x)
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5
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1
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8
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24
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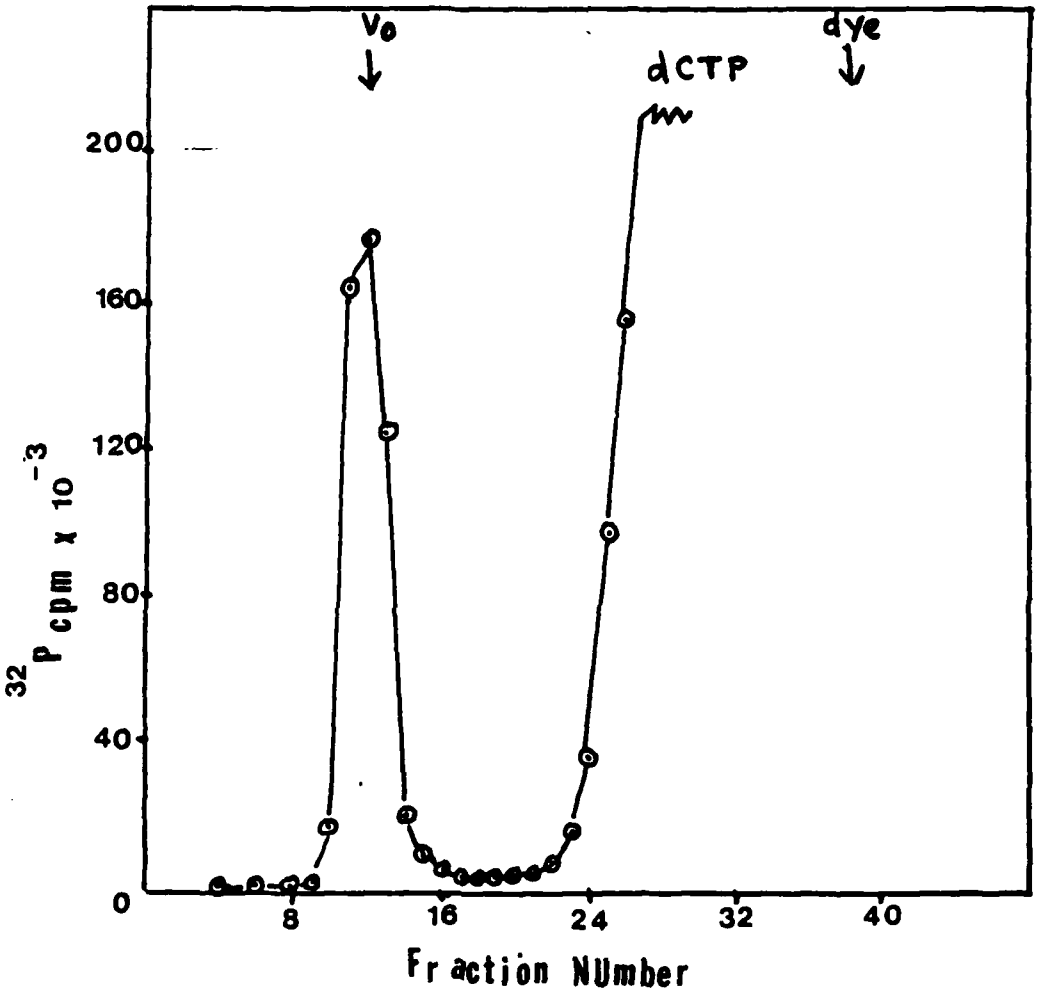
40
Fraction Number

FIGURE 3B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dye
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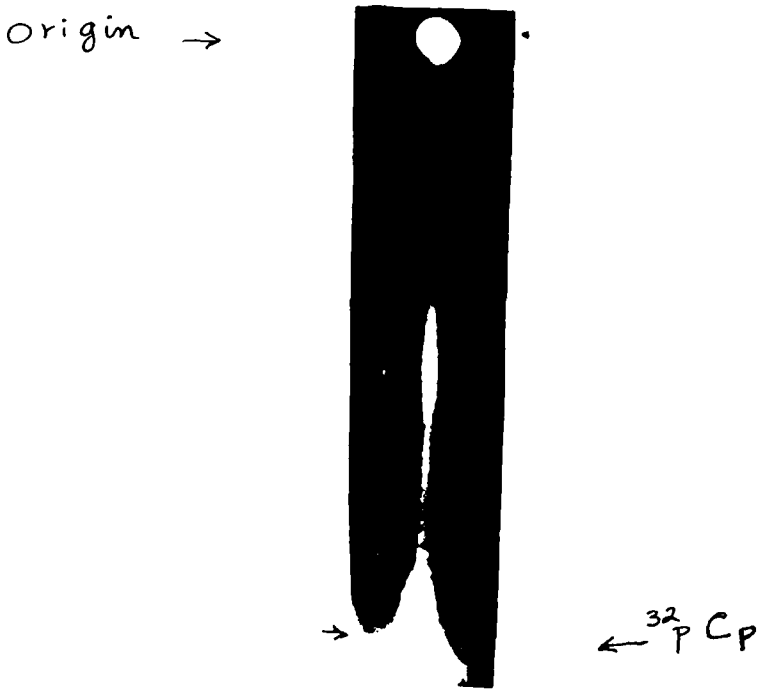
FIGURE 4



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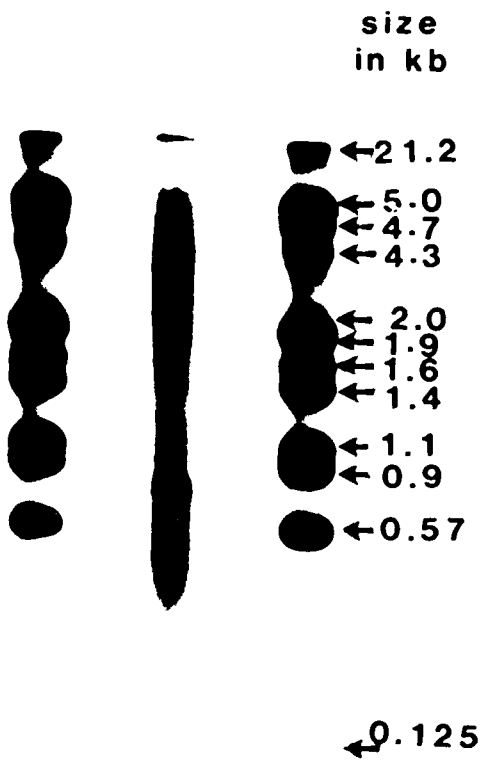

LITERATURE CITED

England, T. E., and Uhlenbeck, O. C. (1978) 3'-terminal labeling of RNA with T_4 RNA ligase. Nature 275, 560-561.

Lehrach, H., Diamond, D., Wozney, J. M., Boedtker, H. (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16, 4743-4750.

McDonnell, M. W., Simon, M. N., Studier, F. W. (1977) Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. 110, 119-146.

Russell, P. K., Brandt, W. E., and Dalrymple, J. M. (1980) Chemical and antigenic structure of Flaviviruses. In The Togaviruses: Biology, Structure and Replication, chapter 18 (W. Schlesinger, ed.), Academic Press, New York, pp. 503-529.

Stollar, V., Schlesinger, R. W., and Stevens, T. M. (1967) Studies on the nature of the Dengue viruses. III. RNA synthesis in cells infected with type 2 Dengue virus. Virology 33, 650-658.

Svitkin, Y. V., Ugarova, T. Y., Chernovskaya, T. V., Lyapustin, V. N., Lashkevich, V. A., and Agol, V. I. (1981) Translation of TBE virus (Flavivirus) genome in vitro: Synthesis of two structural polypeptides. Virology 110, 26-34.

Wengler, G., Beato, M., and Wengler, G. (1979) In vitro translation of 42S virus-specific RNA from cells infected with the flavivirus West Nile Virus. Virology, 96, 516-529.

Westaway, E. G., and Reedman, B. M. (1969) Proteins of the group B arbovirus Kunjin. J. Virol. 4, 688-693.

Westaway, E. G. (1977) Strategy of the flavivirus genome: Evidence for multiple internal initiation of translation of proteins specified by Kunjin virus in mammalian cells. Virology 80, 320-335.

Personnel Supported from March 1, 1982 to September 1, 1982

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Dr. Masatoshi Nohara	Research Associate	May, 1982-present
Maj. John R. Lowe*	Graduate Student	March, 1982-present
Mr. Stuart Litwer	Summer Undergraduate Student	June, 1982-August, 1982
Mr. David Coussens	Summer Undergraduate Student	June, 1982-July, 1982
Mr. C. Subramaniyam	Summer Graduate Student	June, 1982-August, 1982
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