J. gen. Virol. (1983), **64**, 2585–2595. *Printed in Great Britain*

Key words: *human rotavirus/rotavirus cloning/pBR322*

Molecular Cloning of a Human Rotavirus Genome

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(*Accepted 5 August 1983*)

SUMMARY

The genes of a field isolate of human rotavirus were cloned into pBR322. The strategy used involved polyadenylation of denatured double-stranded (ds) genomic RNA, followed by cDNA synthesis using reverse transcriptase. Oligo(dC) tails were added to the 3′ end of the single-stranded cDNA and then separated by alkaline agarose electrophoresis. Sized cDNA of both polarities were allowed to hybridize and inserted into the *Pst*I site of pBR322. Transformations done with sized cDNA always resulted in the selection of hybrid plasmids carrying inserts with a size representative of the original cDNA. Four individual clones were selected for preliminary characterization. One clone has an insert of 1360 base pairs (bp) and corresponds to gene 6. The second clone has an insert of 1140 bp and corresponds to one of the genes in the triplet 7-8-9. The other two genes, with inserts of 780 and 660 bp, were identified as corresponding to dsRNA segments 10 and 11.

INTRODUCTION

Rotaviruses, a major cause of neonatal diarrhoea in humans and many other animal species (Flewett & Woode, 1978; Viera de Torres *et al.*, 1978; Holmes, 1979), are typical members of the *Reoviridae*. They possess a genome composed of eleven segments of double-stranded (ds)RNA (Kalica *et al.*, 1978; Rodger & Holmes, 1979) which can be separated by polyacrylamide gel electrophoresis. Analysis of different human and animal rotavirus isolates have revealed considerable intra- and inter-species variability in the migration of many of the dsRNA segments, which remains to be explained in the terms of molecular epidemiology (Espejo *et al.*, 1980; Spencer *et al.*, 1983).

The genomic RNA is surrounded by two concentric protein shells (Esparza & Gil, 1978; Roseto *et al.*, 1979) formed by at least five structural polypeptides (Dyall-Smith & Holmes, 1981*b*; Espejo *et al.*, 1981; Novo & Esparza, 1981; Erickson *et al.*, 1982). An outer capsid glycoprotein, with an apparent mol. wt. of 37K (VP7), appears to be the major neutralization antigen (Killen & Dimmock, 1982; Matsuno & Inouye, 1983). The polypeptides encoded by each of the dsRNA segments of the simian rotavirus SA-11 (Smith *et al.*, 1980; Dyall-Smith & Holmes, 1981*a*; Arias *et al.*, 1982) and of the U.K. strain of bovine rotavirus (McCrae & McCorquodale, 1982*b*) have been determined using the technique of *in vitro* translation of denatured dsRNA (McCrae & Joklik, 1978).

Methods for the molecular cloning of dsRNA virus genomes have been described previously (Cashdollar *et al.*, 1982; Imai *et al.*, 1983), including the production of DNA clones from the Wa strain of human rotavirus (Imai *et al.*, 1983), from the U.K. strain of bovine rotavirus (McCrae & McCorquodale, 1982*a*) and from the simian rotavirus SA-11 (Both *et al.*, 1982).

In this paper we describe the molecular cloning and preliminary characterization of the genes of a human rotavirus isolate.

0022-1317/83/0000-5784 $02.00

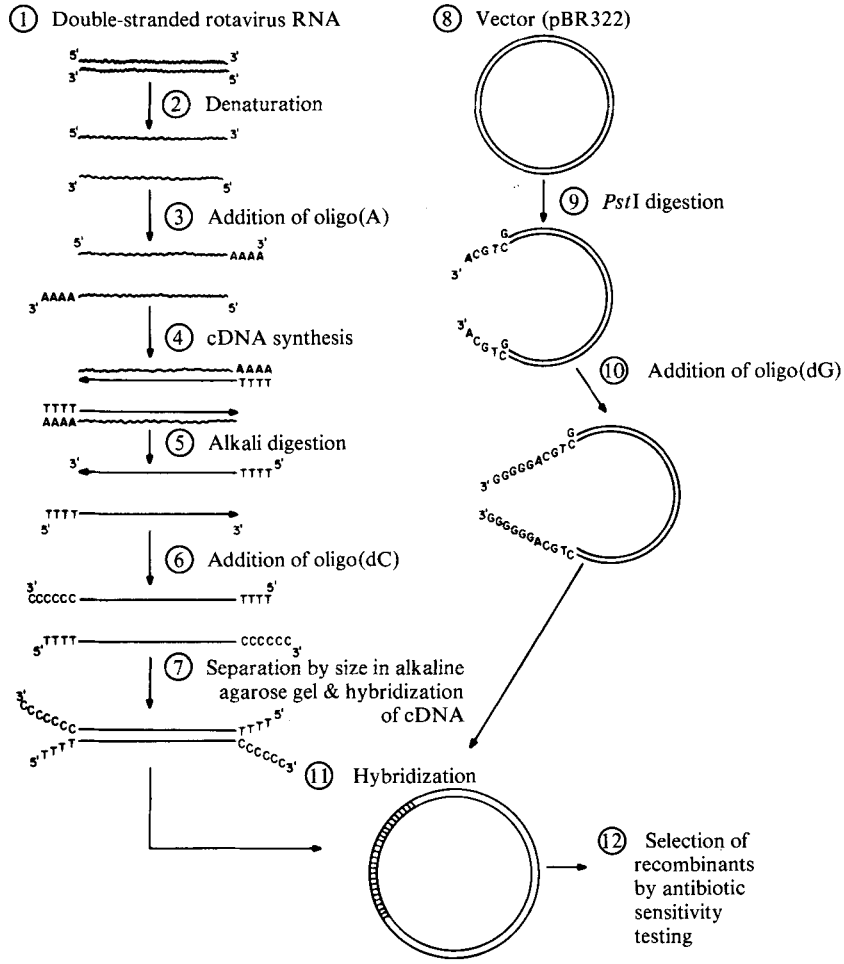

Fig. 1. Strategy for cloning human rotavirus genes.

METHODS

Virus purification and isolation of RNA. Human rotavirus was purified from 25 g of stools collected from a single patient (K.F.) in Durham, N.C., U.S.A., in March 1981. A 10% suspension of faecal material was homogenized in a buffer containing 10 mM-Tris–HCl pH 8 and 0·25 M-NaCl, followed by Freon 113 extraction. The aqueous phase was layered over a 10 ml cushion of 45% (w/v) sucrose in 20 mM-Tris–HCl pH 7·4, and ultracentrifuged in an SW27 Spinco rotor at 20000 rev/min for 3 h at 4 °C. The virus pellet was resuspended in a buffer containing 20 mM-Tris–HCl pH 7·4 and 15 mM-EDTA, and kept at 37 °C for 2 h to remove the outer capsid (Cohen *et al.*, 1979). Single capsid viruses were further purified by isopycnic centrifugation in a CsCl gradient. The band corresponding to single-capsid particles was recovered and the amount of virus was estimated to be in the order of 600 μg, assuming that $1A_{260}$ is equal to 185 μg of virus per ml (Smith *et al.*, 1969). For RNA extraction, purified particles were resuspended in 1% SDS and incubated at 55 °C for 1 h, followed by phenol–chloroform extraction and ethanol precipitation in the presence of 0·2 M-sodium acetate. Approx. 90 μg of RNA were obtained, corresponding to 15% of the total particle weight (Novo & Esparza, 1981).

Synthesis and cloning of human rotavirus cDNA. The strategy used to clone the human rotavirus genes was essentially similar to that previously used to clone reovirus genes (Cashdollar *et al.*, 1982), with some modifications that will be explained in more detail (see Fig. 1). A 60 μg amount of dsRNA was melted by incubation at 50 °C for 45 min in 90% dimethyl sulphoxide (DMSO) and the 3′ ends of both strands were polyadenylated using the *Escherichia coli* poly(A) polymerase (Bethesda Research Laboratories) (Sippel, 1973) as described (Cashdollar *et al.*, 1982). A 25 μg amount of polyadenylated RNA was obtained after phenol extraction and chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972). cDNA copies of rotavirus genes were synthesized using avian

myeloblastosis virus reverse transcriptase and oligo(dT) as primer. The RNA template was removed by alkali treatment (2 h at room temperature in 0·5 M-KOH) and the cDNA was separated by filtration through a Sephadex G-75 column, eluting with 1 mM-Tris–HCl pH 7·5. Fractions containing the cDNA (approx. 6·25 μg) were pooled and lyophilized. At this stage we introduced some modifications to the protocol used for the reovirus cloning (Cashdollar *et al.*, 1982). The first modification was to add oligo(dC) tails to the 3′ ends of the single-stranded cDNA, using terminal transferase (Roychoudhury & Wu, 1980), followed by separation of individual species of oligo(dC)-tailed cDNAs in preparative alkaline agarose gel electrophoresis (McDonnell *et al.*, 1977). Individual ^{32}P-labelled cDNAs were localized by wet-gel autoradiography, excised with a scalpel and the cDNA eluted with the powdered glass technique (Vogelstein & Gillespie, 1979). Sized DNA of both polarities were then allowed to hybridize in 20 mM-Tris–HCl pH 8, 100 mM-NaCl, 1 mM-EDTA and 50% formamide, by incubating for 3 min at 65 °C and then for 24 h at 20 °C. Formamide was removed by dialysis against 1 mM-Tris–HCl pH 8, and the double-stranded cDNA was lyophilized and used for cloning into the *Pst*I site of pBR322, previously tailed with oligo(dG). The hybrid plasmids were used to transform *E. coli* HB101 and transformants containing recombinant plasmids were identified by antibiotic sensitivity testing.

Preliminary characterization of human rotavirus clones. The size of the inserts was determined by rapid lysis of transformants (Birnboim & Doly, 1979) followed by agarose gel electrophoresis and by electrophoresis of *Pst*I excised inserts. A partial restriction map of some clones was constructed using restriction enzymes (New England Biolabs). Cloned rotavirus genes were identified by hybridization of ^{32}P-labelled nick-translated plasmids to genomic RNA immobilized to diazotized aminophenylthioether (APT) paper (Schleicher & Schüll) as described by Cashdollar *et al.* (1982).

RESULTS

Synthesis of rotavirus cDNA

The product of the reverse transcription of total polyadenylated rotavirus dsRNA was analysed in a 1·5% alkaline agarose gel, and the result is shown in Fig. 2 side by side with an electropherogram of the original viral dsRNA. Several discrete bands of cDNA were identified, with sizes ranging from 3500 to 690 bases. The size distribution of cDNA suggests that they represent full-length copies of the individual species of the viral dsRNA. The band of cDNA corresponding to 1100 bases may contain copies of genes 7, 8 and 9. It is also possible that the band of 1450 bases includes both genes 5 and 6, and that the band of 1700 bases represents incomplete synthesis of larger genes, which are reverse transcribed very inefficiently. It is important to mention that a smear of low mol. wt. cDNA was frequently observed at the bottom of the gel and may represent incomplete cDNA copies of all genes.

Cloning of double-stranded cDNA

Since one of the steps of cloning dsRNA genomes would be the hybridization of cDNA corresponding to both RNA strands, and knowing that many of the cDNA species present in the reaction mixture represent incomplete copies of the genes, it was desirable to size the cDNAs before hybridization, to avoid duplex formation between complete and incomplete cDNA copies, which would result in the frequent cloning of incomplete genes. The sizing of cDNA in preparative alkaline agarose gel was carried out after the addition of oligo(dC) to the 3′ ends of the single-stranded cDNA because we found that impurities in the gel prevented the proper functioning of the terminal transferase after the cDNA was separated and eluted.

Oligo(dC)-tailed cDNA migrates in alkaline gel as the original cDNA. Five regions of the gel, as indicated with letters A to E in Fig. 2(*b*), were excised, the cDNA eluted and allowed to hybridize as described in Methods. In this way we attempted to obtain full-length cDNA duplex molecules.

Double-stranded cDNA corresponding to each of the five regions of the gel were annealed with oligo(dG)-tailed pBR322 and each mixture of hybrid plasmids was used to transform four different preparations of competent *E. coli* HB101 cells. Transformed cells were selected overnight in LB broth containing 15 μg of tetracycline per ml. The total DNA of tetracycline-resistant plasmids was isolated by rapid lysis and analysed in agarose gels. Fig. 3 shows the results obtained with four different transformations done with cDNA obtained from the five regions of the sizing alkaline gel. It can be seen that no recombinant plasmids were identified in cells transformed with cDNA of preparations A and B, corresponding to an insert size of more

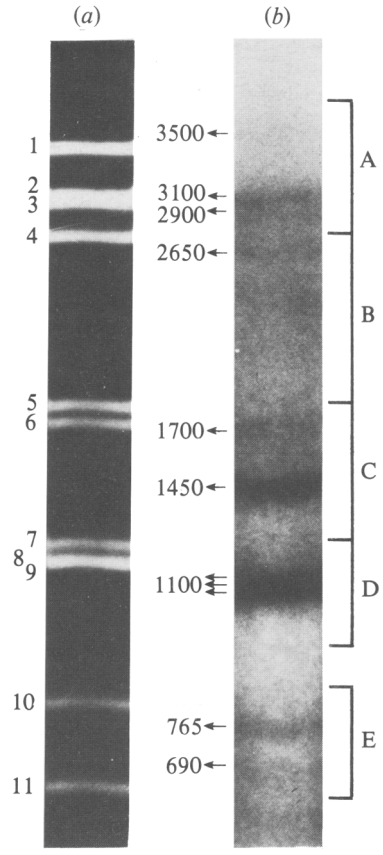

Fig. 2. Electrophoretic analysis of the viral genome and its cDNA. (*a*) Polyacrylamide gel electrophoresis of the dsRNA used for molecular cloning. Total viral dsRNA was electrophoresed in a 10% polyacrylamide gel using the buffer system of Maizel (1971) as described by Gaillard & Joklik (1982). (*b*) Electropherogram of cDNA transcribed from the rotavirus dsRNA, analysed in 1·5% alkaline agarose (McDonnell *et al.*, 1977). The number of bases on the cDNA was calculated using ^{32}P-3′-end-labelled *Hae*III fragments of ϕX174 DNA and *Hpa*I fragments of lambda DNA as size markers (not shown). Regions A to E are discussed in the legend to Fig. 3.

than 1700 base pairs (bp). Even when the only visible band corresponds to empty pBR322 it is possible that recombinant plasmids are present in a very low proportion that precludes detection by ethidium bromide staining.

One of the four transformations done with preparation C (cDNA of 1450 bp) resulted in a homogeneous band of a recombinant plasmid migrating slightly above the S_2 cloned reovirus gene, which has an insert of 1329 bp (Cashdollar *et al.*, 1982). Cells transformed with preparation D (cDNA of 1100 bp) resulted in one or two discrete bands of plasmids carrying inserts of approx. 1000 bp, and cells transformed with preparation E (cDNA of approx. 700 bp) showed bands of plasmids carrying slightly shorter inserts.

In summary, transformation with sized cDNA always resulted in the selection of hybrid plasmids carrying inserts with a size representative of the original cDNA.

Isolation and preliminary characterization of individual clones

Four individual clones (one from preparation C, one from preparation D and two from preparation E) were selected for preliminary characterization. Fig. 4(*a*) shows an agarose gel of the complete plasmids and Fig. 4(*b*) shows the results obtained after excision of the insert by *Pst*I

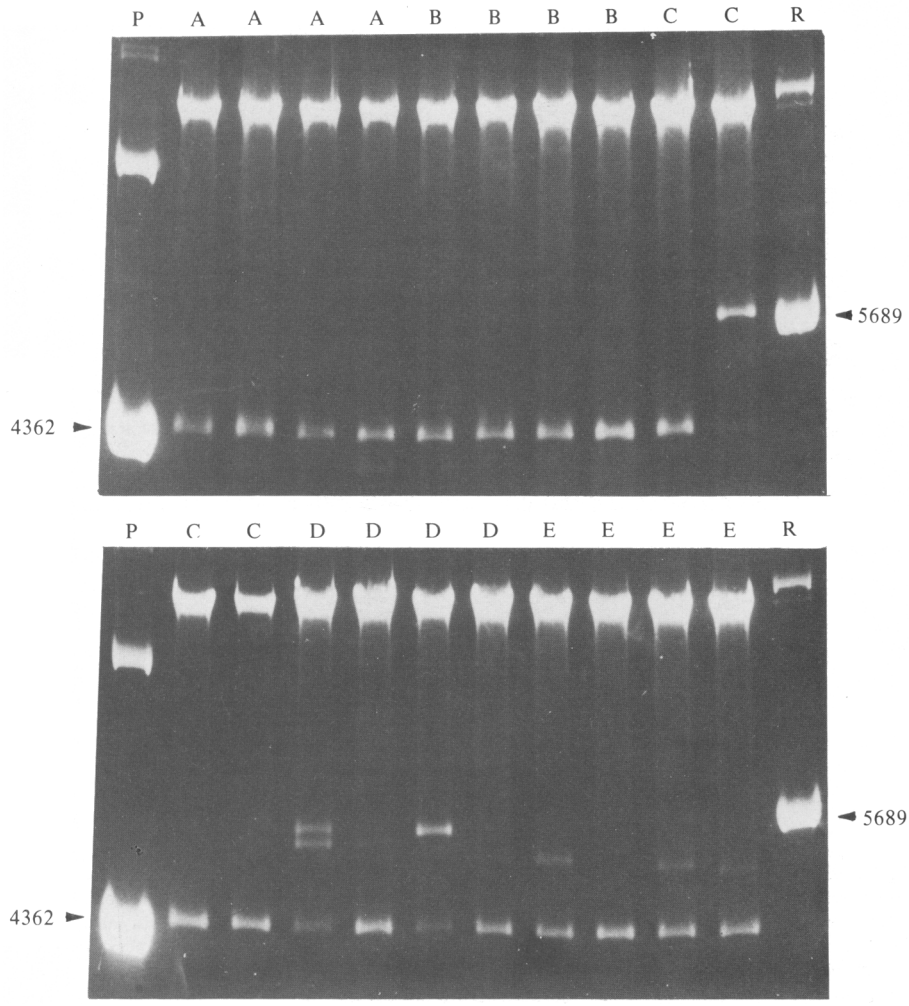

Fig. 3. Screening of recombinant plasmids obtained by transformation with sized cDNA. Total DNA was isolated (Birnboim & Doly, 1979) and analysed by neutral 1% agarose gel electrophoresis. Letters A to E correspond to cells transformed with cDNA obtained from regions A to E in Fig. 2(*b*); P, pBR322 marker (4362 bp); R, S132 plasmid, carrying a cloned S2 reovirus gene (5689 bp) (Cashdollar *et al.*, 1982). In both P and R the top band corresponds to open circles and the bottom band corresponds to covalently closed circular molecules. The uppermost band in lanes A to E corresponds to cell DNA, and the lowest band corresponds to supercoiled pBR322 carrying no insert. The intermediate bands correspond to pBR322 carrying inserts of different sizes.

digestion. The plasmid in lane 2 has an insert of 1360 bp, with an internal *Pst*I site that divides it into two fragments of 1000 and 360 bp; the size of this insert is compatible with a complete copy of gene 5 or 6. Plasmids in lanes 3, 4 and 5 have inserts of 1140, 780 and 660 bp. The size of the insert in the plasmid shown in lane 3 is compatible with any of the genes 7, 8 or 9. The other two plasmids may carry complete or near-complete copies of genes 10 and 11.

A more positive identification of the clones was obtained by hybridizing ^{32}P-labelled nick-translated plasmids to genomic segments immobilized in APT paper. Most of the experiments were done using heterologous dsRNA obtained from tissue culture-grown bovine rotavirus, separated in polyacrylamide gels and blotted on to the APT paper. One such experiment is shown in Fig. 5, in which the plasmid carrying the 780 bp insert hybridized to genome segment

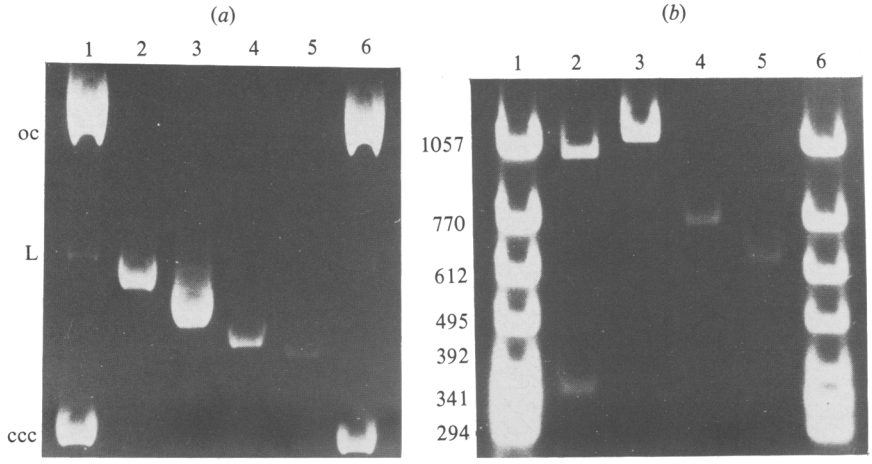

Fig. 4. Neutral agarose gels of individual clones selected for preliminary characterization. (*a*) Electropherogram of complete plasmids. Lanes 1 and 6, pBR322 marker (ccc, covalently closed circular molecules; L, linear molecules; oc, open circular molecules). Lane 2, clone obtained from cDNA preparation C in Fig. 2 and 3. Lane 3, clone obtained from preparation D in Fig. 2 and 3. Lanes 4 and 5, clones obtained from preparation E in Fig. 2 and 3. (*b*) Electropherogram of *Pst*I digest plasmids. Lanes 1 and 6, *Hin*cII digest of ϕX174 replicative form DNA. Lanes 2 to 5, as in (*a*). Inserts have fragments with the following sizes: lane 2, 1000 + 360 bp; lane 3, 1140 bp; lane 4, 780 bp; lane 5, 660 bp.

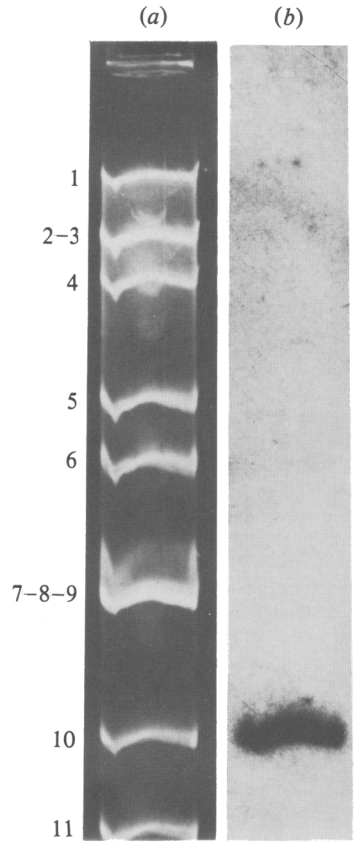

Fig. 5. Identification of the 780 bp cloned rotavirus gene. (*a*) Total bovine rotavirus dsRNA was electrophoresed in a 10% polyacrylamide gel using the buffer system described by Laemmli (1970); bands were stained with ethidium bromide and photographed under u.v. light. The RNA was transferred to diazotized APT paper and used for hybridization to the ^{32}P-labelled nick-translated plasmid carrying the 780 bp insert. (*b*) Autoradiogram showing that the labelled probe hybridizes only to dsRNA segment 10.

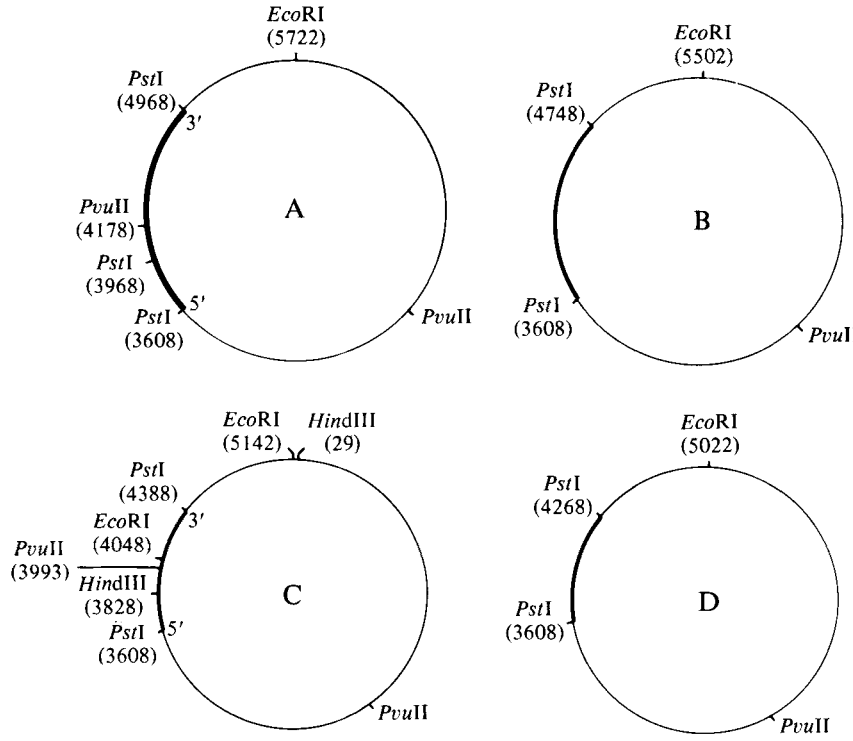

Fig. 6. Preliminary restriction maps of cloned human rotavirus genes. The figure shows plasmids carrying inserts with 1360 bp (A), 1140 bp (B), 780 bp (C) and 660 bp (D). All plasmids were digested with *Eco*RI, *Hin*dIII, *Bam*HI, *Pvu*II and *Pst*I, and only the relevant sites are indicated. The order numbers assigned to the restriction enzyme sites are estimates taken from the approximate size of fragments on gels when compared to size markers.

10. Likewise, the plasmids carrying the 660 and 1360 bp inserts were identified as corresponding to segment 11 and 6 respectively. The plasmid with the 1140 bp insert hybridized to the triple band of segments 7, 8 and 9.

Restriction maps and orientation of the insert in relation to the corresponding mRNA

Preliminary restriction maps of the four recombinant plasmids are shown in Fig. 6. The relative position of the cDNA in relation to the corresponding viral mRNA was determined using a specific probe for the 3′ end of rotavirus mRNA (Fig. 7). For this, bovine rotavirus (Novo & Esparza, 1981) mRNA was synthesized *in vitro* (Cohen, 1977) and polyadenylated as described above. Short reverse transcripts, complementary to the 3′ end of the mRNAs , were synthesized in a 3 min reverse transcription reaction, using [^{32}P]dATP as radioactive label.

This labelled probe was then hybridized to restriction fragments of selected clones which had been previously transferred to nitrocellulose (Southern, 1975). The restriction fragment hybridizing with the 3′ end-specific probe was identified after autoradiography. This procedure is illustrated in Fig. 7 and Fig. 8 with the plasmid carrying the 1360 bp insert. It is clear that the larger *Pst*I fragment hybridized with the probe, identifying the 3′ end of the corresponding mRNA. Likewise, the 3′ end of the plasmid carrying the 780 bp insert was identified by hybridization of the labelled probe with plasmid double-digested with *Pst*I and *Hin*dIII (data not shown).

DISCUSSION

In this paper we describe the molecular cloning of human rotavirus genes using a strategy that resulted in production of apparently full-length clones. The use of dsRNA as template for cDNA synthesis has been described recently in the molecular cloning of *Reoviridae* genes (Cashdollar *et*

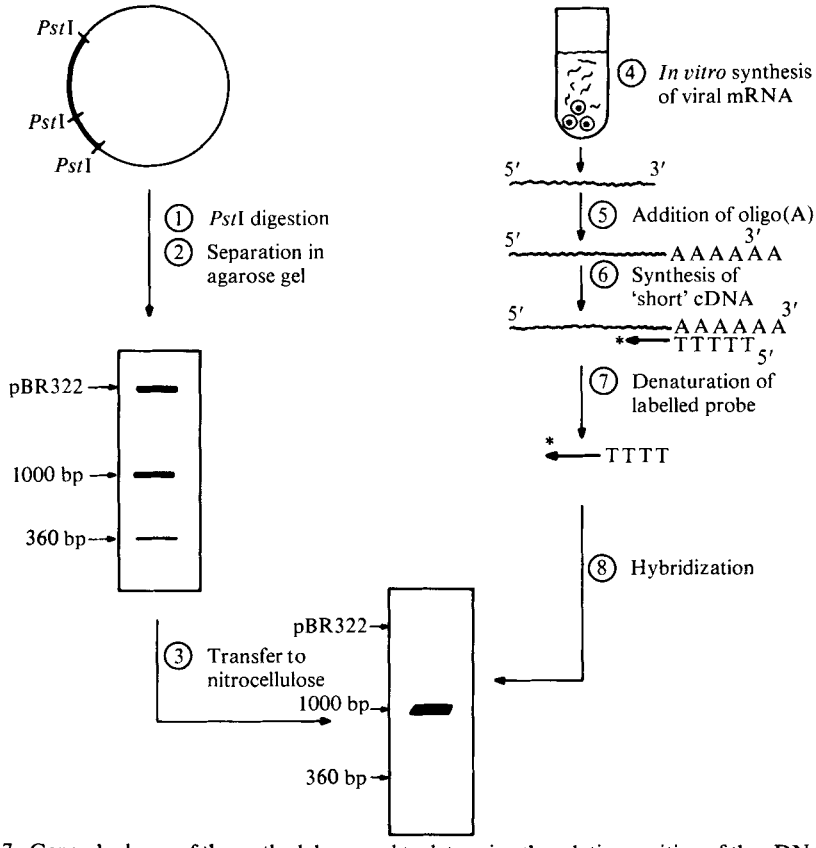

Fig. 7. General scheme of the methodology used to determine the relative position of the cDNA in the recombinant plasmids in relation to the corresponding viral mRNA.

al., 1982; McCrae & McCorquodale, 1982*b*; Both *et al.*, 1982; Imai *et al.*, 1983). Reverse transcription of dsRNA is relatively inefficient when compared with single-stranded RNA and results in the synthesis of incomplete transcripts. However, the obvious advantage of using dsRNA as template is that incomplete plus and minus cDNA strands can be annealed, and complementary strands repaired using the Klenow fragment of DNA polymerase I (Cashdollar *et al.*, 1982) or reverse transcriptase (Both *et al.*, 1982; Imai *et al.*, 1983).

In the strategy described in this paper, instead of trying to repair incomplete complementary cDNA transcripts, we selected for cloning full-length cDNA separated in alkaline agarose gels (Fig. 1). This procedure resulted in the production of hybrid plasmids carrying inserts with a size representative of the original cDNA. Hybridization of ^{32}P-labelled nick-translated plasmids to genomic segments immobilized in APT paper confirmed the identity of the two smaller inserts as corresponding to genes 10 and 11, and provided evidence that one of the larger inserts corresponds to gene 6 and the other to one of the genes in the triplet 7–8–9. At this point it is important to mention that hybridization to genomic segments does not provide an absolute identification of the cloned genes since altered migration of dsRNA segments has been described among different human and animal rotavirus isolates. A striking example occurs with the migration pattern of segments 10 and 11 of human rotavirus. Isolates belonging to antigenic subgroup 1 have slower-moving segments 10 and 11 ('short' electropherotype) when compared with viruses belonging to subgroup 2 ('long' electropherotype) (Kalica *et al.*, 1981; Rodger *et al.*, 1981). Moreover, it has been shown by *in vitro* translation that segment 10 of viruses with 'short' electropherotype corresponds to segment 11 of viruses with 'long' electropherotype (Dyall-Smith & Holmes, 1981*a*).

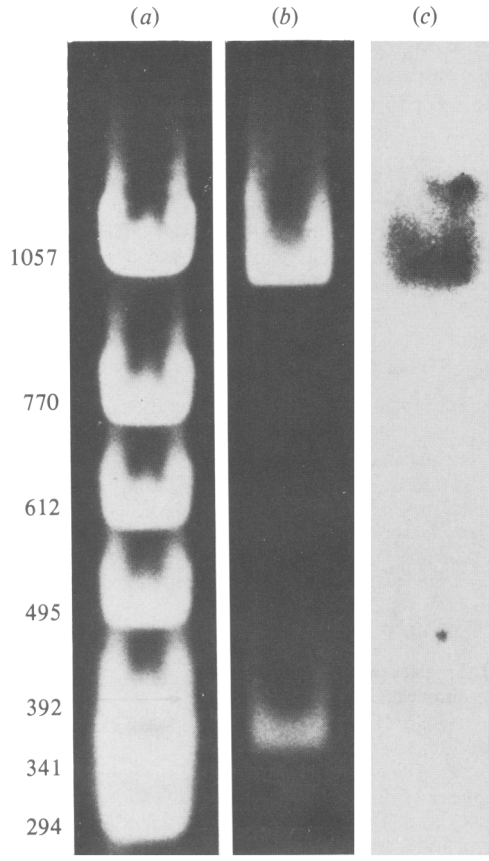

Fig. 8. Identification of the orientation of the cDNA in relation to the mRNA in the recombinant plasmid carrying the 780 bp insert (gene 10). (*a*) *Hinc*II digest of ϕX174 replicative form DNA. (*b*) *Pst*I-digested plasmid showing the 1000 bp and 360 bp virus-specific fragments. (*c*) Autoradiogram showing that only the 1000 bp fragment hybridizes with the 3′ end-specific probe.

The human rotavirus used in the present study has a 'short' electrophoretic pattern, whereas the Wa strain, cloned by Imai *et al.* (1983) is the prototype of viruses with the 'long' pattern. Since Imai *et al.* (1983) published the complete nucleotide sequence of segment 11, we attempted to compare it with our preliminary restriction map of cloned gene 10, which has restriction sites for *Hin*dIII (220 bp from the 5′ end), *Pvu*II (at 385 bp) and *Eco*RI (at 440 bp). Even when those sites were not located in the published sequence it was possible to identify five of the six nucleotides of each one of the restriction enzyme recognition sites very close to the position observed in our cloned gene 10 (*Hin*dIII at approx. 178, *Pvu*II at approx. 358 and *Eco*RI at approx. 419). A comparison of the complete nucleotide sequence of gene 11 of the Wa strain of human rotavirus with our cloned gene 10 may provide information on the molecular basis of the antigenic differences observed among human rotavirus isolates belonging to different antigenic types (Dyall-Smith & Holmes, 1981*a*).

In any case, unequivocal assignment of cloned genes requires the identification of the encoded protein, using methods of hybrid-arrested translation (Paterson *et al.*, 1977) or hybridization selection (Goldberg *et al.*, 1979), which are under way.

A collection of cloned human and animal rotavirus genes may prove useful to understand better their structure, function and epidemiological significance, such as has been done with the influenza viruses (Gething *et al.*, 1980). On the other hand, the expression of selected cloned genes in *E. coli* may provide an avenue to the synthesis of viral immunogens, particularly

important in view of the fact that rotaviruses are difficult to grow to high titres in cell cultures (Esparza *et al.*, 1980; Wyatt *et al.*, 1980; Sato *et al.*, 1981). The knowledge of the orientation of the insert, in relation to the corresponding viral mRNA, would facilitate the construction of expression vectors with the appropriate promoters.

We are grateful to Dr W. K. Joklik for providing in his laboratory the favourable environment in which three of the authors (L.W.C., G.R.H. and J.E.) discussed many of the experiments presented in this paper. We wish to thank Mrs Teresa Avendaño for her help in preparing the manuscript and Mr M. Díaz for his cooperation with the photographic work. This work was supported in part by Grant S1-1311 from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT), Caracas, Venezuela.

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(Received 2 June 1983)