*Proc. Natl. Acad. Sci. USA*
Vol. 80, pp. 373–377, January 1983
Biochemistry

# Molecular cloning of double-stranded RNA virus genomes

(rotavirus/reovirus/RNA ligase/3′ tailing)

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*Contributed by Aaron J. Shatkin, October 7, 1982*

**ABSTRACT** **Genome double-stranded RNAs isolated from purified human reovirus (serotype 3) and rotavirus (Wa strain) were modified at the 3′ termini by addition of oligo(C)$_{\sim 15}$ with T4 RNA ligase. These RNAs were transcribed into cDNA by oligo(dG)-primed reverse transcriptase and cloned after insertion into pBR322 at the *Pst* I site. Hybridization of plasmid-transformed *Escherichia coli* RR1 colonies with $^{32}$P-labeled viral genome RNAs demonstrated the presence of DNA clones representative of each of the 10 reovirus RNAs and 10 of the 11 constituent segments of the rotavirus genome. Analyses of the size and terminal nucleotide sequences of insert DNAs indicated that some clones contained a full-length copy of the virus genome segment. The complete nucleotide sequence of rotavirus genome segment 11 double-stranded RNA was obtained by using this procedure. It provides a general method for cloning double-stranded RNAs and also nonpolyadenylylated single-stranded RNAs.**

Double-stranded (ds) RNAs that function as genetic elements are widely distributed in nature (1). They include the genomes of eukaryotic members of the order Reoviridae (2, 3), prokaryotic $\phi$6 phage (4), and the recently reported trout infectious pancreatic necrosis, chicken infectious bursal disease, and *Drosophila* X viruses (5). In addition, yeast killer factors (6) and virus-like particles in *Penicillium* species (7) apparently occur as ds RNA plasmids in these and other fungi.

Genome ds RNA segments of the Reoviridae consist of a monocistronic mRNA (+ strand) and its complement (− strand) in an end-to-end base-paired duplex structure, except for the overlapping 5′ cap in the + strand. The segments are transcribed by virus-associated RNA polymerase to form capped mRNAs which in turn also function as templates for a putative replicase in virus-infected cells (2, 3). Thus, each genome subunit probably contains recognition sites for genome transcription, viral mRNA translation, and duplex segment replication and correct assembly into virions.

We have been attempting to identify some of these important structure/function relationships by elucidating more completely the nucleotide sequences of Reoviridae genomes. As part of this effort we have developed a general method for preparing cDNAs from a mixture of ds RNAs (Fig. 1). Duplexes rather than mRNAs were used as templates for cDNA synthesis by reverse transcriptase because ample amounts of ds RNAs can be isolated from purified viruses, and the individual genome segments are easily separated. By taking advantage of the efficient 3′-terminal tailing of duplex RNAs by RNA ligase (8), we prepared oligo(C)$_{15}$-tailed genome segments of human reovirus and rotavirus, and the corresponding oligo(dG)-primed cDNAs were cloned in pBR322. A full-length copy of the virus dsRNA genome segment was obtained in some clones including rotavirus genome segment 11 whose complete sequence has been determined.

## MATERIALS AND METHODS

**Preparation of 5′-$^{32}$P-Phosphorylated Oligo(C)$_{15}$.** Poly(C) (P-L Biochemicals, 100 mg) was dissolved in 10 ml of 0.1 M $NaHCO_3$ (adjusted to pH 10 with 14 M $NH_4OH$) and partially hydrolyzed by boiling for 30 min. Oligo(C) chains of various lengths were separated by DEAE-cellulose chromatography, and $A_{260\ nm}$ peaks corresponding to oligo(C)$_{12-17}$ [referred to as oligo(C)$_{15}$] were pooled and desalted (9).

Oligo(C)$_{15}$ (25 nmol) was 5′-$^{32}$P phosphorylated by incubation for 45 min at 37°C in a reaction mixture (50 $\mu$l) which consisted of 50 mM Tris·HCl buffer (pH 8.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 nmol of [$\gamma$-$^{32}$P]ATP (Amersham; 40 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ Bq) and 10 units of polynucleotide kinase (P-L Biochemicals). The mixture was chilled and phenol extracted, and the $^{32}$P-labeled oligo(C) was purified by Sephadex G-50 gel filtration and ethanol precipitation.

**Preparation of Oligo(C)-Tailed Viral ds RNAs.** Purified virus ds RNAs (100–276 $\mu$g; 140–386 pmol) were incubated for 18 hr at 4–6°C in reaction mixtures (75 $\mu$l) containing 50 mM Hepes buffer (pH 7.5), 20 mM $MgCl_2$, 3 mM dithiothreitol, 0.8 $\mu$g of bovine serum albumin, 10% (vol/vol) dimethyl sulfoxide, 60 $\mu$M ATP, 5′-$^{32}$P-labeled oligo(C)$_{15}$ (4.2 nmol, $1.8 \times 10^7$ cpm), and 30 units of T4 RNA ligase. To prevent self-joining of oligomers, nonphosphorylated molecules potentially present in the oligo(C) preparation before ligation were converted to the 3′-phosphorylated form by periodate oxidation and aniline-catalyzed $\beta$-elimination of 3′-terminal cytosine (10). Oligo(C)-tailed ds RNAs were isolated after phenol extraction by exclusion from Sephadex G-120-200 (0.6 × 40 cm column) in 20 mM Tris·HCl, pH 8.0/7 M urea and ethanol precipitation.

**Synthesis of cDNAs from Oligo(C)-Tailed, Denatured Viral RNAs.** Oligo(C)-tailed ds RNAs (30 $\mu$g, 42 pmol) dissolved in 0.45 ml of $H_2O$ containing oligo(dG)$_{10}$ (3 $\mu$g, 820 pmol) were denatured (5 min, 100°C) and added to mixtures (total, 0.5 ml) containing 50 mM Tris·HCl buffer (pH 8.0), 7.5 mM $MgCl_2$, 10 mM dithiothreitol, 20 $\mu$g of actinomycin D, dATP, dCTP, and dGTP at 2 mM each, 0.1 mM dTTP including 100 $\mu$Ci of [$\alpha$-$^{32}$P]dTTP (Amersham; 3,000 Ci/mmol), and 800 units of reverse transcriptase (kindly provided by Joseph Beard). After incubation at 40°C for 120 min, the mixtures were extracted with phenol and then twice with chloroform and treated with 0.3 M NaOH (37°C, 18 hr) to digest RNA before precipitation of DNA by addition of 3 vol of cold ethanol. Precipitates were rinsed twice with 80% ethanol and dissolved in 0.5 ml of 20 mM Hepes, pH 7.0/0.6 M NaCl/5 mM EDTA/0.1% $NaDodSO_4$.

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Abbreviations: ds, double-stranded; CPV, cytoplasmic polyhedrosis virus.

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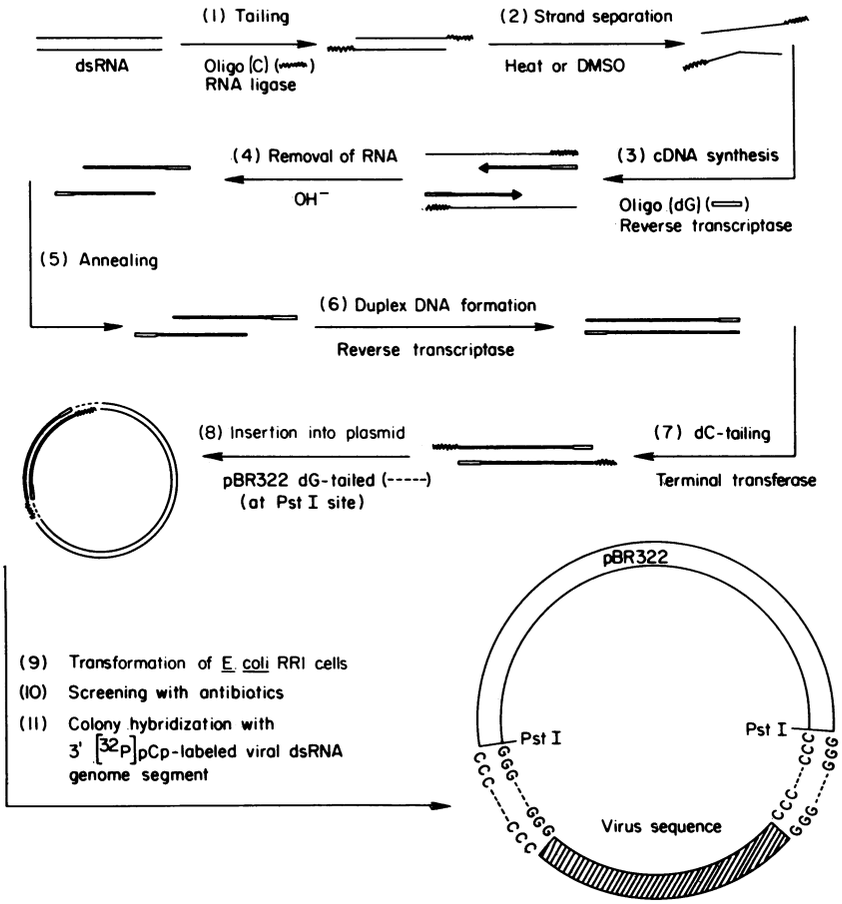


FIG. 1. General scheme for ds RNA cloning. DMSO, dimethyl sulfoxide.

Base pairing of complementary DNA strands was effected by annealing the samples at 68°C for 6 hr followed by gradual cooling to 20°C. Partially ds cDNA molecules were completed by incubation for 60 min at 42°C in (0.25 ml) 50 mM Tris·HCl, pH 8.0/7.5 mM $MgCl_2$/2 mM dATP/2 mM dGTP/2 mM dTTP/0.8 mM dCTP including 200 $\mu$Ci of [$^3$H]dCTP (Amersham; 19 Ci/mmol)/10 mM dithiothreitol/40 mM NaCl/160 units of reverse transcriptase. cDNA products were isolated by exclusion from Sephadex G-100 or repeated precipitation with ethanol after extraction with phenol. The sizes of the cDNAs were determined by electrophoresis in 2% agarose gels; for single-stranded cDNAs, gels contained 4 mM methylmercury hydroxide (11).

**Colony Hybridization to Detect Genome Segment-Specific Sequences.** Transformation of *Escherichia coli* RRI cells with pBR322 containing virus cDNA inserts was carried out according to Norgard *et al.* (12). Plasmid DNAs fixed on Millipore filters (13) were hybridized with individual segments of viral genome RNA 3'-end-labeled with [$^{32}$P]pCp (8, 14).

## RESULTS

**Tailing of ds RNA 3' Termini with Oligo(C)$_{15}$.** Genome RNAs extracted from purified human reovirus, rotavirus, and insect cytoplasmic polyhedrosis virus (CPV) were "tailed" at 3' termini with [5'-$^{32}$P]$\overset{*}{p}$(Cp)$_{15}$ and separated from unreacted oligo(C) by polyacrylamide gel electrophoresis (Fig. 2 *Left*). From measurements of the radioactivity in the individual bands excised from the gel it was calculated that 51%, 48%, and 40% of the total 3' termini of the ds RNAs of reovirus, CPV, and rotavirus were oligo(C)-tailed, assuming that each ds RNA segment contained two 3' termini and an average of 1,500 base pairs. The addition of oligo(C)$_{15}$ was confirmed by polyacrylamide gel analysis of the $^{32}$P-labeled oligonucleotides released from tailed reovirus RNAs by RNase T1 digestion (data not shown). Ligand chain length apparently had relatively little effect on ligase-catalyzed tailing because oligo(C)s consisting of 8, 12, 14, or 16 nucleotides were ligated to the 3' termini of reovirus ds RNA with similar efficiencies (data not shown). Tailing was enhanced by increasing the concentrations of ds RNA and oligo(C) and by using optimal enzyme concentration (133 units/ml), temperature (6°C), and time (18 hr).

**cDNAs Synthesized from Oligo(C)-Tailed RNAs by Reverse Transcriptase.** Oligo(C)-tailed RNAs of human reovirus and rotavirus were heat-denatured (Fig. 1, step 2) and used for cDNA synthesis by oligo(dG)$_{10}$-primed reverse transcriptase (step 3). The reaction mixtures included actinomycin D to prevent formation of a terminal hairpin in the product cDNAs (15). Equivalent yields of cDNA (28 $\mu$g) were obtained from 100 $\mu$g of reovirus and rotavirus RNAs that contained oligo(C) on 51% and 48% of their 3' termini. After incubation with reverse transcriptase, the template RNAs were hydrolyzed by treatment with alkali (step 4), and the resulting mononucleotides were removed by repeated ethanol precipitation of the product cDNAs. The cDNAs were self-hybridized to effect base pairing of overlapping sequences in molecules that had been copied from the two complementary strands of individual genome segments (step 5). Single-stranded ends of the hybridized DNAs were completed by reincubation with reverse transcriptase (step 6). [$^3$H]dCTP was included in the filling reaction; from the [$^3$H]dCMP incorporated, it was calculated that about 6 $\mu$g and 4 $\mu$g of reovirus and rotavirus cDNAs, respectively was syn-

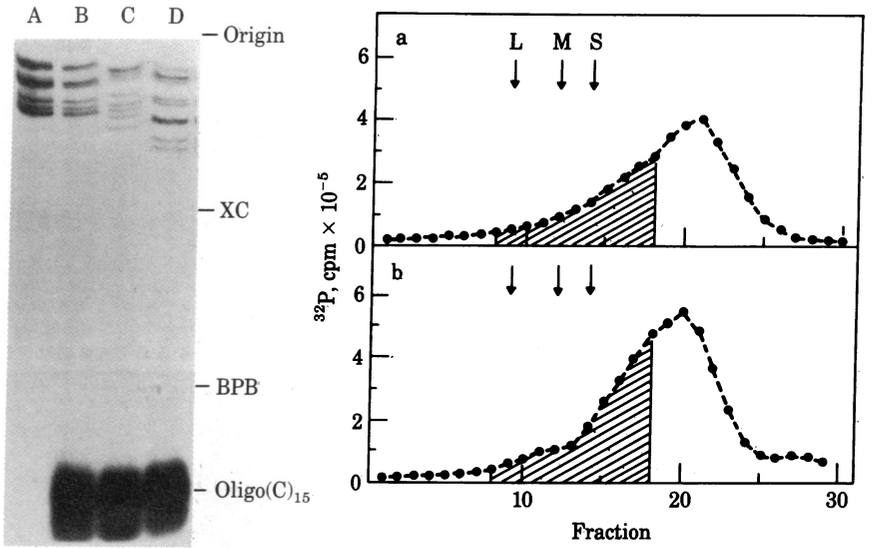


FIG. 2. Analysis of viral genome ds RNAs and cDNAs. (*Left*) Viral ds RNAs were oligo(C)-tailed by T4 RNA ligase. Aliquots (1 μl) were analyzed by electrophoresis (30 mA, 7 hr, 4°C) in a 5% polyacrylamide gel in 40 mM Tris acetate, pH 8.0/20 mM sodium acetate/2 mM EDTA/18 mM NaCl. Lanes: A, marker reovirus ds RNA 3′-labeled with [$^{32}$P]pCp; B,C,D, Oligo(C)$_{15}$-tailed ds RNAs of reovirus, CPV, and rotavirus, respectively. XC and BPB, positions of xylene cyanol and bromophenol blue. (*Right*) Duplex cDNAs transcribed from reovirus RNA (*a*) and rotavirus RNA (*b*) were centrifuged in 5–30% glycerol gradients (SW 40 rotor, 17 hr, 39,000 rpm). Fractions were collected, assayed by Cerenkov radiation, and pooled as indicated by the shaded regions. [$^{32}$P]pCp 3′-labeled reovirus ds RNA was analyzed in a parallel gradient, and the positions of the L, M, and S genome segment size classes are indicated.

thesized on 28 μg of the corresponding hybridized cDNA primer template. The molecular sizes of the completed reovirus and rotavirus dsDNAs were estimated by sedimentation in 5–30% glycerol gradients (Fig. 2 *Right*). cDNA molecules that sedimented in the region of the L, M, and S classes of reovirus dsRNA (≈8–18 S, shaded region) were combined, precipitated with ethanol, and used for insertion into pBR322 and molecular cloning.

**Transformation of *E. coli* RR1 Cells by pBR322 DNA Containing Viral cDNA Inserts.** Reovirus cDNAs (200 ng) and rotavirus cDNAs (50 ng) were prepared for plasmid insertion by 3′-tailing with dC (Fig. 1, step 7), according to Norgard *et al.* (12). dC-tailed DNAs mixed with 50 ng of pBR322 DNA that had been cut with *Pst* I and dG-tailed at the cleavage site were used to transfect *E. coli* RR1 cells. Reovirus cDNA-treated RR1 cells (200 ng of DNA per 6 × 10$^9$ cells) yielded 250 tetracycline-resistant colonies of which 167 were also ampicillin-sensitive. For rotavirus cDNAs (50 ng), 47 ampicillin-sensitive colonies were isolated from a total of 113 tetracycline-resistant transformants. As described (12), the yield of transformants was de-

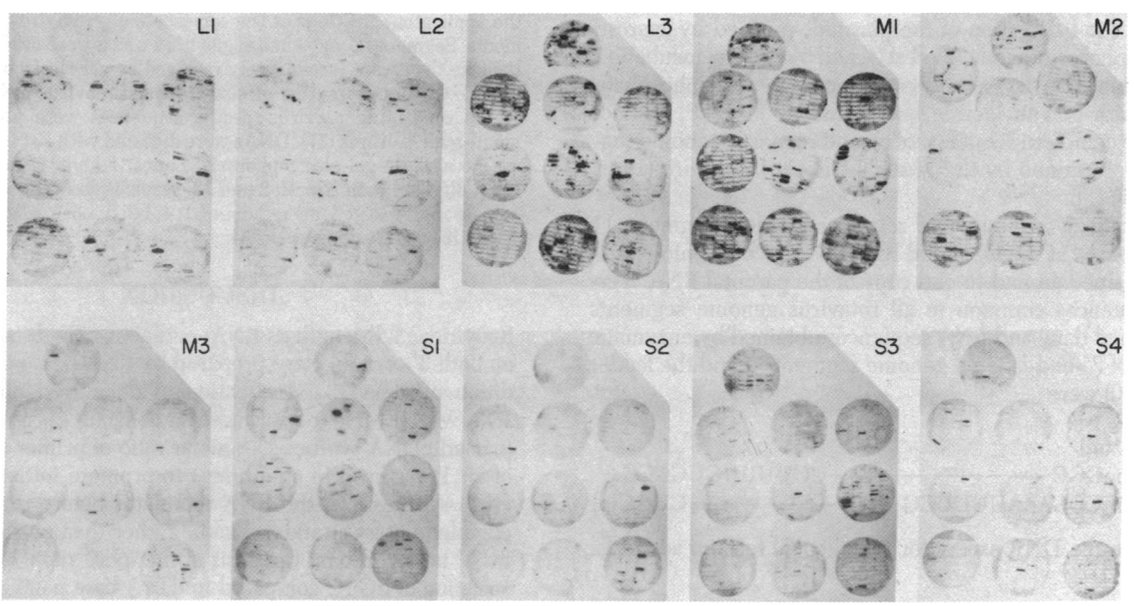


FIG. 3. Colony hybridization with [$^{32}$P]pCp 3′-labeled ds RNA genome segments as probes. Each panel shows a replicate set of 10 filters (≈50 colonies each), and each set was probed with the indicated L, M, or S species of $^{32}$P-labeled reovirus RNA. Individual ds RNA segments (2 × 10$^5$ cpm) mixed with 2.5 mg of yeast tRNA in 1.8 ml of $H_2O$ were denatured (100°C, 5 min) before hybridization. Plasmid DNAs fixed on filters were incubated overnight at 37°C in 0.30 M NaCl/0.03 M Na citrate/50% (vol/vol) formamide/20 mM Tris·HCl, pH 7.4/0.5% $NaDodSO_4$ containing 5 mg of yeast tRNA (five discs per 10 ml), drained, and annealed in 10 ml of this solution containing one of the denatured, radiolabeled RNA segments. After 48 hr at 37°C in the dark on a rocking platform, filters were washed four times for 30 min each by gentle shaking at 37°C in 0.30 M NaCl/0.03 M Na citrate (five discs per 50 ml) and dried before autoradiography.

pendent on the ratio of dG-tailed pBR322 DNA to dC-tailed viral cDNA. Although the yield varied among different preparations, on the average one tetracycline-resistant ampicillin-sensitive colony was obtained per ng of input cDNA.

**Colony Hybridization with Individual [$^{32}$P]pCp 3′-Labeled Virus Genome RNA Segments.** To detect virus-specific sequences in transformed bacteria, colony hybridizations were carried out with each of the separated genome RNA segments (Fig. 3). Of 500 colonies tested, about 20% hybridized unequivocally with $^{32}$P-labeled reovirus RNAs, and about 10 positive colonies specific for each genome segment were obtained.

Human rotavirus sequences similarly detected by colony hybridization were confirmed by preparing small-scale cultures of plasmid DNAs containing virus sequences, $^{32}$P-labeling the isolated recombinant DNAs by nick-translation, and hybridizing the $^{32}$P-labeled DNA probes to separated genome RNA segments by a dot-blot procedure (16). Ten plasmid insert DNAs which were characterized as corresponding to 10 different genome segments by colony hybridization also hybridized specifically with the individual denatured dsRNAs (Fig. 4*A*). Cloned DNAs containing sequences of all 10 reovirus segments and 10 of 11 rotavirus segments were identified by these procedures.

**Size Determination of Cloned DNAs.** Plasmid DNAs containing reovirus DNA inserts were prepared from RR1 cells (17), digested with *Pst* I, and analyzed by agarose gel electrophoresis together with reovirus ds RNA and *Hin*cII-digested $\phi$X174 DNA as size markers (Fig. 4*B*). DNA inserts of apparent sizes 1,100–1,300 base pairs were obtained for DNA copies of viral genome segments in the small size class (lanes 9–12); S3 and S4 DNAs migrated in positions close to parental ds RNA segments (lanes 11 and 12). The excised DNA insert copies from M1, M2, M3, and the three L species of genome segments all were much shorter than the parental ds RNAs (lanes 6–8 and 3–5). Incomplete copies of the rotavirus genome also were obtained for segments 1–9, but the DNA inserts corresponding to the shortest genome segments—namely, numbers 10 and 11—were apparently full-length (data not shown).

**Sequence Analysis of Cloned Viral DNA.** Insert DNA was excised by *Pst* I digestion of the plasmid, purified by agarose gel electrophoresis, and labeled at 3′ and 5′ ends by incubation with terminal transferase and cordycepin [$\alpha$-$^{32}$P]triphosphate (18) and with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP, respectively. The complete sequence of cloned rotavirus genome segment 11 determined by the Maxam–Gilbert procedure (19) is shown in Fig. 5.

The terminal sequences of rotavirus genome segment 11 were compared at the DNA and RNA levels to determine if the clone contained an end-to-end copy of the parental RNA. Terminal sequences common to all rotavirus genome segments (unpublished data) and RNA sequences obtained by enzymatic digestion of 3′-end-labeled genome segment 11 and the ladder method (20) were

$$\begin{array}{l} \overset{(m)}{} \\ (+)\ m^7GpppGGC\ \text{———————}\ CGUUUUGUGACC\ 3', \\ (-)\qquad 3'\ CCGAAAUUUUCG\ \text{———————}\ CUGG(p) \end{array}$$

identical to the DNA except for the oligo(C) termini added for cloning:

$$\begin{array}{l} \qquad\qquad\qquad\qquad \text{——} CGTTTTGTGACC\text{-}(C)_{16} \\ (C)_{18}\text{-}CCAGTGTTTTGC\ \text{——} \end{array}$$

These sequence results together with the insert size measurements demonstrate that full-length DNA copies of a specific genome segment can be cloned from a mixture of ds RNAs by the method outlined in Fig. 1.

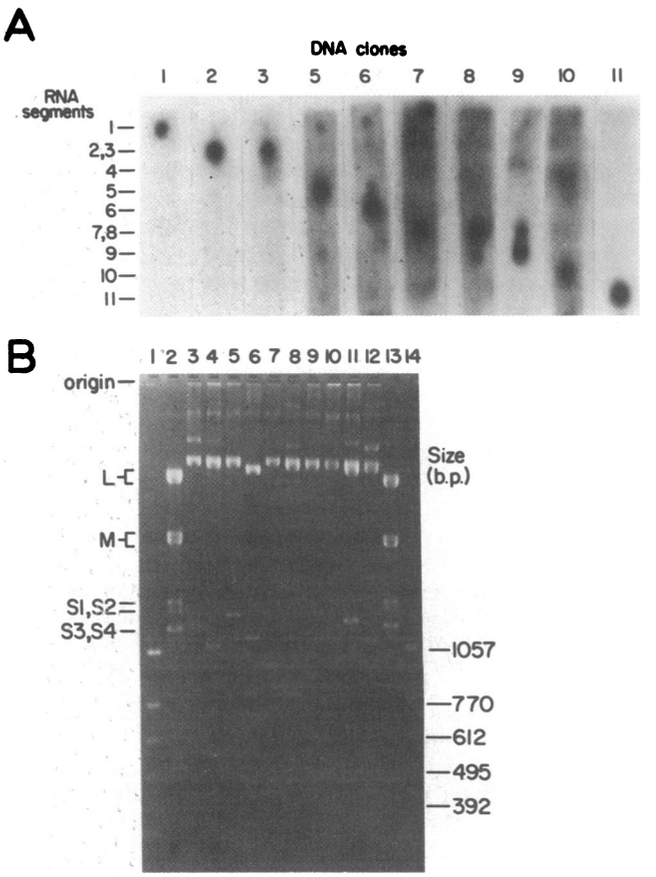


FIG. 4. (*A*) Dot-blot hybridization of separated rotavirus genome ds RNAs with $^{32}$P-labeled nick-translated pBR322 DNAs containing rotavirus-specific sequences. Rotavirus genome segments were separated by electrophoresis in a 5% polyacrylamide gel, and individual segments 1–11 were recovered by electroelution. All of the purified RNAs (0.1 $\mu$g each of segments 1–11) were spotted individually and fixed on a nitrocellulose strip which was hybridized with nick-translated, cloned DNAs as described by Thomas (16). Numerals indicate the application positions of the corresponding rotavirus genome segments. Segments 2 and 3 and segments 7 and 8 were used as mixtures because they were incompletely resolved by gel electrophoresis (Fig. 2 *Left*). (*B*) Determination of size of cloned reovirus DNAs. Plasmid DNAs containing reovirus-specific sequences were prepared from small-scale cultures (17). DNAs were digested with *Pst* I and analyzed by 2% agarose gel electrophoresis. Lanes: 1 and 14, marker $\phi$X174 DNA digested with *Hin*cII; 2 and 13, reovirus ds RNA; 3, cloned DNA corresponding to genome segment L1; 4, L2; 5, L3; 6, M1; 7, M2; 8, M3; 9, S1; 10, S2; 11, S3; and 12, S4.

## DISCUSSION

Reoviridae full-length ds RNAs containing single-stranded tails on both 3′ termini were prepared by RNA ligase-catalyzed addition of oligo(C)$_{15}$. Incubation of denatured oligo(C)-tailed RNA with reverse transcriptase and oligo(dG)$_{10}$ resulted in dG-primed cDNA synthesis. A molar ratio of primer to 3′ termini of 20:1 was used in an attempt to promote initiation of DNA chain synthesis on the RNA templates before renaturation of complementary strands; oligo(C) rather than oligo(A) was used for 3′ tailing also on the basis of the more rapid and stable association of dG·rC compared to dT·rA base pairs. Oligo(dG)$_{10}$-primed cDNA synthesis was initiated from some of the oligo(C)-tailed 3′ termini as demonstrated by sequence analysis of the cloned full-size DNA from rotavirus genome segment 11 (Fig. 5).

Although incomplete transcripts were obtained with reverse transcriptase, presumably due to endonuclease contamination, we used this polymerase for both DNA synthetic reactions (Fig.

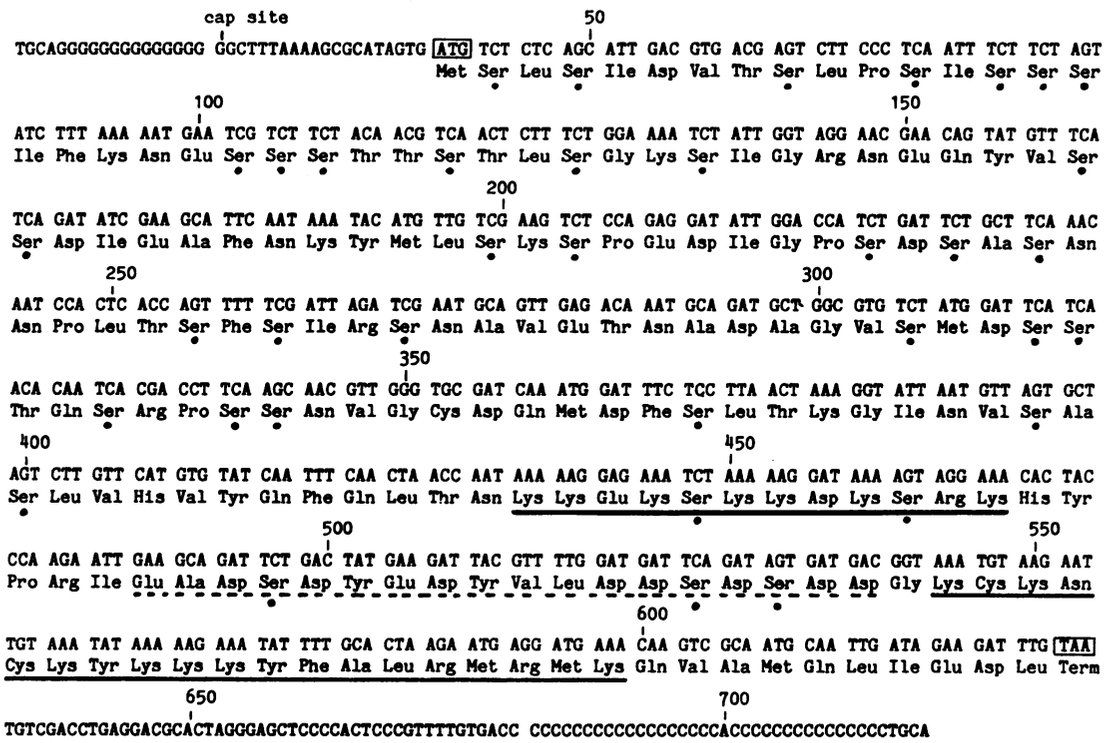


FIG. 5. Sequence of cloned DNA of rotavirus genome segment 11. The + sense strand (corresponding to mRNA) is shown. Also indicated are transcription cap (19) and termination (631) sites, the predicted amino acid sequence, serine residues (Ser), and basic (——) and acidic (– – –) polypeptide domains.

1, steps 3 and 6) because the two-step procedure should yield full-size ds DNAs by transcription of overlapping, complementary strands even if the primary products are incomplete or interrupted (see Fig. 1). By including the Klenow fragment of DNA polymerase I in the second phase of DNA synthesis (21) and dimethyl sulfoxide for ds RNA denaturation it may be possible to obtain complete DNA copies of high molecular weight ds RNAs including reovirus L and M genome segments. Alternatively, cloned incomplete DNAs can be used as primers for chain elongation (and completion) because they all contain one of the genome RNA terminal sequences.

Genome segment 11 of human rotavirus consists of 663 base pairs and the 5′ end of the duplex RNA + strand is capped (unpublished data). The $m^7G$ cap is followed by 20 residues that are probably noncoding because the first AUG precedes an uninterrupted potential coding region of 591 bases; the other two possible reading frames contain multiple termination codons. No A-A-U-A-A-A processing signal (22) is present in the 3′-terminal 52-base putative noncoding sequence, consistent with the rotavirus RNA 3′ sequence and the absence of polyadenylylation in Reoviridae mRNA (2, 3). A striking potential for formation of a highly stable [$\Delta G = -21$ kcal/mol (1 Cal = 4.184 J)] base-paired structure between the 5′-terminal residues 29–82 and the 3′-terminal residues 609–661 of the + sense strand presumably influences rotavirus expression at the level of RNA replication or mRNA translation.

The inferred amino acid sequence also implies some interesting features for the rotavirus polypeptide coded for by segment 11. It has an unusually high serine content—37 of 197 total residues. In addition, three sequence domains can be distinguished near the carboxyl terminus of the polypeptide. Each consists of clusters of basic or acidic amino acids (Fig. 5). A viral polypeptide of molecular weight 21,560 with the above properties is predicted for the rotavirus gene 11 product. Expression studies of this rotavirus gene and of other cloned dsRNAs should provide new insights into the structure and function of a large and diverse group of RNA genomes.

We thank Dr. J. Monahan, Ms. A. J. LaFiandra, and Mr. K. Akatani for advice and assistance.

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