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# *cis*-Acting Signals That Promote Genome Replication in Rotavirus mRNA

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**A previous study has shown that rotavirus cores have an associated replicase activity which can direct the synthesis of double-stranded RNA from viral mRNA in a cell-free system (D. Y. Chen, C. Q.-Y. Zeng, M. J. Wentz, M. Gorziglia, M. K. Estes, and R. F. Ramig, J. Virol. 68:7030–7039, 1994). To define the *cis*-acting signals in rotavirus mRNA that are important for RNA replication, gene 8 transcripts which contained internal and terminal deletions and chimeric transcripts which linked gene 8-specific 3′-terminal sequences to the ends of nonviral sequences were generated. Analysis of these RNAs in the cell-free system led to the identification of a *cis*-acting signal in the gene 8 mRNA which is essential for RNA replication and two *cis*-acting signals which, while not essential for replication, serve to enhance the process. The sequence of the essential replication signal is located at the extreme 3′ end of the gene 8 mRNA and, because of its highly conserved nature, is probably a common feature of all 11 viral mRNAs. By site-specific mutagenesis of the gene 8 mRNA, residues at positions −1, −2, −5, −6, and −7 of the 3′ essential signal were found to be particularly important for promoting RNA replication. One of the *cis*-acting signals shown to enhance the replication in the cell-free system was located near the 5′ end of the 3′ untranslated region (UTR) of the gene 8 mRNA, while remarkably the other was located in the 5′ UTR of the message. The existence of an enhancement signal in the 5′ UTR raises the possibility that the 5′ and 3′ ends of the rotavirus mRNA may interact with each other and/or with the viral replicase during genome replication.**

Rotaviruses, members of the family *Reoviridae*, are the most important cause of acute infantile diarrhea causing an estimated one million deaths annually (2). The rotavirion consists of three concentric layers (shells) of protein and 11 segments of double-stranded (ds) RNA. The outermost shell is made up of the glycoprotein, VP7, and the trypsin-activated spike protein, VP4, while the intermediate shell is formed from trimers of VP6 (39, 48). The core of the virion includes the major inner shell protein, VP2 (20); the putative RNA-dependent RNA polymerase, VP1 (6, 28, 45); the putative guanylytransferase, VP3 (12, 22, 36); and the 11 genome segments. Cores surrounded by VP6 (double-shelled particles) have an associated transcriptase activity able to synthesize viral mRNA in vitro (1, 7). The ability of double-shelled particles to carry out transcription is dependent on VP6, as removal of this protein produces cores that are transcriptionally inactive (42).

During rotavirus replication, viral mRNA serves two functions: to direct the synthesis of viral protein and as templates for the asymmetrical synthesis of dsRNA (29, 31). In contrast to most eukaryotic mRNAs, rotavirus mRNAs are unique in that they possess 5′-cap structures but lack 3′-poly(A) tails (18, 26). Except for short sequences at their extreme 5′ and 3′ termini, the viral mRNAs have no obvious nucleotide sequence homology (9). The consensus sequence at the 5′ end is 5′-GGuuUuaaa-3′ and at the 3′ end is 5′-AUgUGaCC-3′, with those residues that are fully conserved shown in uppercase and those found in at least 8 of the 11 viral mRNAs shown in lowercase. Among the different rotavirus isolates, the entire 5′ and 3′ untranslated regions (UTRs) of homologous genome segments are highly conserved, sometimes more so than the open reading frames (ORFs) of the genes (10, 11, 17, 40). In addition to forming stem-loop secondary structures (17), RNA folding programs predict that the 5′ and 3′ UTRs may stably interact, giving an overall panhandle shape to the viral mRNAs (34). A direct demonstration of the importance of the conserved termini and the predicted secondary structures in RNA replication has not been provided.

The mechanism of rotavirus genome replication and the role that the viral proteins play in the process remain unclear. Analysis of the replication intermediates recovered from cells infected with rotavirus temperature-sensitive mutants has indicated that VP2, but not VP6, is essential for RNA replication and that core-like particles are the simplest replication intermediates with replicase activity (mRNA→dsRNA) (24). More recently, in vitro studies of the polymerase activity associated with baculovirus-expressed core-like particles showed that, while VP1 is a necessary component of complexes with replicase activity, VP3 and VP6 are not (46). Additional studies of the properties of replication intermediates with replicase activity have suggested that the viral mRNA template moves into cores during dsRNA synthesis and, hence, the packaging and replication of viral mRNA are concurrent events (33). While it is not known when assortment takes place in the replication process, the absence of free pools of nonpackaged dsRNA and the equimolar replication of the 11 genome segments in the infected cell imply that it is viral mRNA and not dsRNA that undergoes assortment (30, 31).

To perform their multiple roles in the infected cell, the 11 viral mRNAs must contain *cis*-acting signals that not only control their translational efficiency but also direct their assortment, packaging, and replication. While assortment perhaps requires that each species of mRNA possess a unique signal that allows it to be distinguished from the other 10 species of

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TABLE 1. Oligonucleotide primers used in the preparation of reporter RNAs[a]

| Primer | Sequence | Reporter RNA |
|---|---|---|
| T7g8R | taatacgactcactataGGCTTTTAAAGCGTC | g8R |
| g83′Sac | ccgcGGTCACATAAGCGCT | g8R |
| T7g8RM$^+$ | taatacgactcactataGGTCACATAAGCGCTTTC | g8RM |
| g8RM$^-$ | ccgcGGCTTTTAAAGCGTCTC | g8RM |
| PCRg8R3GG | GGTCACATAAGCGCTTTCTAT | T7g8d1-502/3′CC |
| PCRg8R3GGG | gGGTCACATAAGCGCTTTCTAT | T7g8d1-502/3′CC+C |
| PCRg8R3G | GTCACATAAGCGCTTTCTAT | T7g8d1-502/3′CdC |
| PCRg8R3AA | AATCACATAAGCGCTTTCTAT | T7g8d1-502/3′UU |
| PCRg8R3GA | AGTCACATAAGCGCTTTCTAT | T7g8d1-502/3′CU |
| PCRg8C1→A | TGTCACATAAGCGCTTTCTAT | T7g8d1-502/3′C1→A |
| PCRg8R3AG | GATCACATAAGCGCTTTCTAT | T7g8d1-502/3′UC |
| PCRg8C2→A | GTTCACATAAGCGCTTTCTAT | T7g8d1-502/3′C2→A |
| PCRg8R3CCC | GGGCACATAAGCGCTTTCTAT | T7g8d1-502/3′A3→C |
| PCRg8R3GVC | GGCCACATAAGCGCTTTCTAT | T7g8d1-502/3′A3→G |
| PCRg8A3→U | GGACACATAAGCGCTTTCTAT | T7g8d1-502/3′A3→U |
| PCRg8R3CCG | GGTGACATAAGCGCTTTCTAT | T7g8d1-502/3′G4→C |
| PCRg8AG4→U | GGTAACATAAGCGCTTTCTAT | T7g8d1-502/3′G4→U |
| PCRg8R3GCC | GGTCGGATAAGCGCTTTCTAT | T7g8d1-502/3′U5G6→CC |
| PCRg8G6→C | GGTCAGATAAGCGCTTTCTAT | T7g8d1-502/3′U5→C |
| PCRg8U5→C | GGTCGCATAAGCGCTTTCTAT | T7g8d1-502/3′G6→C |
| PCRg8U7→C | GGTCACGTAAGCGCTTTCTAT | T7g8d1-502/3′U7→C |
| PCR3g8d1 | GGTCACATCCAACTTGAGAAACTTCGT | g8R3′d(−9)-(−66) |
| PCR3g8d2 | GGTCACATCCTCAAATTGATAGCGAAT | g8R3′d(−9)-(−41) |
| T7g8-5′d1-10 | taatacgactcactataGCGTCTCAGTCGCCGTTTGAGC | g8R5′d1-10 |
| T7g8-5′d1-24 | taatacgactcactataGTTTGAGCCTTGCGTGTAGCC | g8R5′d1-24 |
| T7g8-5′d1-50 | taatacgactcactataGAGCTAGCTTGCTTTTGCTATCC | g8R5′d1-50 |
| T7g8-5′d4-50 | taatacgactcactataGGGAGCTAGCTTGCTTTTGCTATCC | g8R5′d4-50 |
| T7g8-5′d11-51 | taatacgactcactataGGCTTTTAAAGAGCTAGCTTGCTTTTGCTA | g8R5′d11-51 |
| T7g8-5′d26-51 | taatacgactcactataGGCTTTTAAAGCGTCTCAGTCGCCGAGCTAGCTTGCTTTTGCT | g8R5′d26-51 |

[a] Gene 8-specific sequences are given in uppercase letters. Primers designated T7 begin with a T7 promoter sequence. Altered residues present in the gene 8-specific sequences of the PCRg8 primers are underlined.

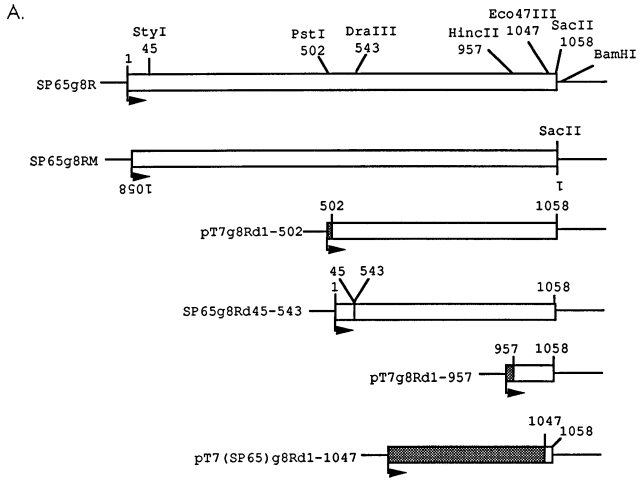
mRNA, the 11 mRNAs may be expected to share signals that serve to promote recognition and replication of the RNA by the viral replicase. Our ability to identify and characterize *cis*-acting replication signals in viral mRNAs has been aided by the recent development of a rotavirus template-dependent in vitro replication system (4). This system utilizes open cores as a source of replicase activity and is able to synthesize dsRNA from input rotavirus mRNAs. By testing the replication efficiency of reporter RNAs in the system, Chen et al. (4) mapped a *cis*-acting signal required for replication to the 3′-terminal 26 nucleotides of the gene 9 RNA of porcine rotavirus OSU. In this study, we have used the cell-free system to further define the *cis*-acting signals in the viral mRNAs that promote minus-strand synthesis. We have localized an essential replication signal to the 3′-terminal consensus sequence of the gene 8 mRNA and have identified two other signals which, although not required for replication, act to promote maximal levels of replication. Interestingly, one of the enhancement signals maps to the 5′ end of the gene 8 mRNA.

## MATERIALS AND METHODS

**Preparation of open cores.** MA104 cells were grown in Eagle's minimal essential medium containing 10% fetal bovine calf serum. Approximately 50 175-cm$^2$ flasks containing confluent monolayers of MA104 cells were infected with 0.1 PFU of trypsin-activated rotavirus strain SA11-4F per cell, and the infection was allowed to proceed until all the cells exhibited cytopathic effects. Following repeated freeze-thawing, the infected cell lysates were extracted with trichlorotrifluoroethane and the virus was recovered by pelleting through a cushion of 40% sucrose in Tris-buffered saline (TBS) (3). The virus was centrifuged to equilibrium at 4°C in a 33.3% (wt/wt) solution of CsCl in TBS, and the triple-shelled particles were recovered and dialyzed overnight against TBS at 4°C. After treatment with 50 mM $Na_2EDTA$, pH 7.0, for 1 h at 37°C to remove the outer shell of the virus, the sample was again centrifuged to equilibrium in CsCl and the double-shelled virus was recovered and dialyzed against TBS. To disrupt the VP6 shell, the sample was adjusted to 1 M $CaCl_2$ and incubated for 45 min at 37°C with constant gentle rocking. The cores were recovered from the sample by pelleting through 40% sucrose in TBS, resuspended in TBS, and then centrifuged to equilibrium at 4°C in a solution of 42.8% (wt/wt) CsCl in TBS. The cores (density of 1.44 to 1.49 g/cm$^3$) were recovered and dialyzed initially against TBS for 6 h and then against LSB (2 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 0.5 mM dithiothreitol) for 18 h to open the cores (4). The open cores were stored on ice and retained high levels of replicase activity for at least 2 months.

**Construction of transcription vectors.** The sequences of oligonucleotide primers used in the construction of transcription vectors are presented in Table 1. The primers were purchased from Keystone Laboratories (Menlo Park, Calif.). *Taq* DNA polymerase (Boehringer-Mannheim) was used to amplify DNA by PCR under the following conditions: 1 min at 94°C, 2 min at 42 to 48°C, and 2 min at 72°C (30 cycles) (5). Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and alkaline phosphatase were obtained from New England BioLabs and were used according to the supplier's protocols. Ligation products were introduced into competent *Escherichia coli* DH5α (Bethesda Research Laboratories) by the $CaCl_2$ method (41). Transformants containing the desired plasmid were selected on the basis of antibiotic resistance and digestion with restriction enzymes. Plasmid DNA was recovered by the alkaline lysis procedure and purified by centrifugation on CsCl-ethidium bromide density gradients (41). The expected nucleotide sequences in the transcription vectors were confirmed by dideoxynucleotide sequencing, using a Sequenase version 2.0 kit (United States Biochemical Corp.) and appropriate oligonucleotides as primers (43).

**SP65g8R.** The vector pSP72g8R, which contains a full-length cDNA clone of gene 8 of simian rotavirus SA11, was prepared as described previously (19). To construct a T7 transcription vector that could be used to produce gene 8 plus-sense RNA containing authentic 5′ and 3′ termini, the gene 8 cDNA of pSP72g8R was amplified by PCR with primers T7g8R and g8-3′Sac (5). The PCR product, which contains an upstream T7 promoter and downstream *Sac*II site, was gel purified and ligated into the PCR cloning vector, pT7Blue (Novagen), generating pT7B-g8R. This intermediate plasmid contains two T7 promoters, one which derives from the vector pT7Blue and the other which was linked by




B.

| | 1040 | 1058 |
|---|---|---|
| g8R/SacII (wt)... | GGC UUA GCA AGA AUA GAA AGC GCU UAU GUG ACC | |
| g8R/Eco47III ... | GGC UUA GCA AGA AUA GAA AGC | |
| q8R/BamH1 ... | GGC UUA GCA AGA AUA GAA AGC GCU UAU GUG ACC AAU CGG AUC | |

FIG. 1. Transcription vectors for the synthesis of gene 8 reporter RNAs. (A) The locations of selected restriction sites in the vectors are given. Sequences located between the T7 promoter and the *Sac*II site of the vectors that are not rotavirus specific have been shaded. (B) The 3′-terminal sequences of the T7 transcripts made from SP65g8R cleaved with *Sac*II, *Eco*47III, and *Bam*HI and treated with T4 DNA polymerase are shown. Runoff transcripts made from *Sac*II-cleaved SPg65g8R possess authentic 3′ termini. wt, wild type.

PCR to the gene 8 cDNA and ligated into pT7Blue. In the vector pT7B-g8R, the gene 8 cDNA is positioned in the positive orientation with respect to both T7 promoters.

The gene 8 insert of pT7B-g8R was released by digestion with *Bam*HI and *Hin*dIII, gel purified, and then ligated into the *Bam*HI and *Hin*dIII sites of pSP65. The resulting construct pSP65g8R (Fig. 1), when cleaved with *Sac*II and blunt ended with T4 DNA polymerase, was used in T7 transcription reactions to produce wild-type gene 8 mRNA.

**SP65g8RM.** To generate a T7 transcription vector for the synthesis of gene 8 minus-sense RNA containing authentic 5′ and 3′ termini, the gene 8 cDNA insert of pSP72g8R was amplified by PCR with the primers T7g8RM$^+$ and g8RM$^-$ (Table 1). The PCR product contains the gene 8 cDNA in the reverse orientation relative to an upstream T7 promoter and downstream *Sac*II site. The amplified DNA was gel purified and treated with T4 polynucleotide kinase. The construct SP65g8RM (Fig. 1) was then generated by ligating the amplified DNA into pSP65 which had been cleaved with *Sma*I and treated with alkaline phosphatase.

**pT7g8Rd1-502.** By cleavage with *Pst*I, a fragment of pT7B-g8R that extended from the *Pst*I site of the pT7Blue polylinker to position 502 of the gene 8 insert was released. To produce pT7g8Rd1-502 (Fig. 1), the residual vector fragment was gel purified and self-ligated. Transcripts made from the pT7g8Rd1-502 contain a nonviral 5′ leader sequence of 16 nucleotides derived from the polylinker of pT7Blue.

**SP65g8Rd45-543.** The vector SP65g8R was digested with *Sty*I and *Dra*III, which cut the gene 8 cDNA insert at nucleotides 45 and 543, respectively (Fig. 1). The residual vector fragment was gel purified and treated with T4 DNA polymerase to blunt end the DNA. Afterwards, the fragment was self-ligated, producing the vector SP65g8Rd45-543.

**pT7g8Rd1-957.** The intermediate vector pT7-g8R was digested with *Hin*cII, releasing a fragment extending from the *Hin*cII site in the pT7Blue polylinker to position 957 of the gene 8 insert. To produce pT7g8Rd1-957, the residual vector was gel purified and self-ligated. Transcripts made from pT7g8Rd1-957 contain a nonviral 5′ leader sequence of 22 nucleotides originating from the polylinker of pT7Blue (Fig. 1).

**pT7(SP65)g8Rd1-1047.** The vector pT7g8Rd1-502 was digested with *Pst*I and *Eco*47III, releasing a DNA fragment extending from the *Pst*I site of the pT7Blue polylinker to position 1047 of the gene 8 insert. The residual vector fragment was then gel purified and ligated to the 0.3-kb DNA fragment produced upon digestion of SP65 with *Nac*I and *Pst*I. When transcribed with T7 RNA polymerase, the resulting vector pT7(SP65)g8Rd1-1047 produces RNA that contains a nonviral 5′ leader sequence of 352 nucleotides linked to the 3′-terminal 12 nucleotides of the gene 8 mRNA (Fig. 1).

**pCITEg8R.** The construction of the pCITEg8R vectors is shown in Fig. 2.

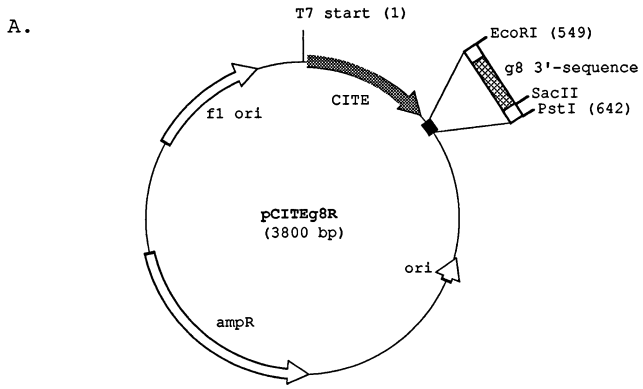
**Site-specific mutagenesis of the 3′ end of gene 8 RNA.** To introduce site-specific changes in the 3′ end of gene 8 transcripts, gene 8 cDNA was amplified by PCR with the oligonucleotides indicated in Table 1 as the reverse primers and a T7 promoter oligonucleotide as the forward primer. The template DNA included in the reactions was T7g8d1-502.

**PCR-directed deletion mutagenesis of the 5′ and 3′ UTRs of gene 8 RNA.** To prepare gene 8 RNA containing deletions at the 5′ terminus, gene 8 cDNA was amplified by PCR with the T7g8-5′d oligonucleotides described in Table 1 as the forward primers and PCRg8R3GG as the reverse primer. DNA used for the synthesis of the RNAs g8R5′d1-10, g8R5′d1-24, g8R5′d1-50, and g8R5′d4-50 was amplified from the gene 8 *Eco*RI-*Hin*dIII cDNA fragment (1.1 kb) of the vector PCRg8R (34). The gene 8 *Sty*I-*Sac*II fragment (1.0 kb) of SP65g8R (Fig. 1) was used as template to amplify DNA for the synthesis of the RNAs g8R5′d11-51 and g8R5′d26-51. DNA used for the synthesis of g8R RNA was amplified in reactions containing supercoiled SP65g8R, the T7 promoter primer, and the reverse primer PCRg8R3GG.

Gene 8 RNA containing 3′-terminal deletions was prepared by transcription of template DNAs synthesized by PCR. The amplification reaction mixtures contained a T7 promoter oligonucleotide as the forward primer; PCR3g8d1, PCR3g8d2, or PCRg8R3GG as the reverse primers (Table 1); and SP65g8R or pT7g8Rd1-502 as the template DNA (Fig. 1).

**Synthesis of reporter RNAs.** Prior to runoff transcription with T7 RNA polymerase (27), DNA templates produced by PCR were treated with T4 DNA polymerase to remove overhang A residues and then were gel purified. DNA templates prepared by linearizing vectors with restriction enzymes were also treated with T4 DNA polymerase before transcription.

RNAs were synthesized by runoff transcription with Ambion MEGAscript T7




B.

| | |
|---|---|
| g8R40 | aatTGATGATGGCTTAGCAAGAATAGAAAGCGCTTATGTGACCgcggtgca |
| | CTACTACCGAATCGTTCTTATCTTTCGCGAATACACTGGcgcc |
| g8R20 | aatTAGAAAGCGCTTATGTGACCgcggtgca |
| | TCTTTCGCGAATACACTGGcgcc |
| g8R10 | aaTTATGTGACCgcggtgca |
| | TACACTGGcgcc |
| g8R7 | aatTGTGACCgcggtgca |
| | CACTGGcgcc |
| g8R6 | aattccccGTGACCgcggtgca |
| | ggggCACTGGcgcc |
| g8R4 | aattccccccGACCgcggtgca |
| | gggggCTGGcgcc |

FIG. 2. Construction of the pCITEg8R vectors. (A) The pCITE-2a(+) vector (Novagen) contains a cDNA clone of a region of the 5′ UTR of the encephalomyocarditis virus, which functions as a cap-independent translation enhancer (CITE). The 0.5-kb cap-independent translation enhancer is situated in the vector between the T7 promoter and the cloning site. To produce the pCITEg8R vectors, complementary oligonucleotides were annealed, forming short DNA hybrids that contained *Eco*RI and *Pst*I cohesive ends and an internal sequence representing the 3′-terminal 4 to 40 nucleotides of the gene 8 mRNA. A *Sac*II site was positioned immediately downstream of the rotavirus-specific sequence. The annealed oligonucleotides were ligated into the *Eco*RI and *Pst*I sites of pCITE-2a(+). The pCITEg8R vectors were cleaved with *Sac*II and blunt ended with T4 DNA polymerase prior to runoff transcription with T7 RNA polymerase, producing RNAs of 550 to 600 nucleotides. (B) Sequences of oligonucleotide hybrids inserted into the pCITE vector. Virus-specific sequences are shown in uppercase, and the *Sac*II sites are underlined.

transcription kits. Each 20-μl reaction mixture contained 1 μg or less of linearized plasmid DNA or gel-purified PCR-amplified DNA and typically yielded 50 to 100 μg of RNA product. After transcription, 2 U of RNase-free DNase was added to reaction mixtures to remove the template DNA. The RNA was purified by repeated phenol-chloroform extraction and precipitated with isopropanol to remove unincorporated nucleotides. The quality of the RNA products was assessed by electrophoresis on 5% polyacrylamide gels containing 7 M urea (4), and the concentrations of the RNA preparations were calculated from their optical densities at 260 nm.

**Cell-free replication assay and analysis of replication products.** The ability of reporter RNAs to serve as templates for dsRNA synthesis was evaluated with the cell-free system developed by Chen et al. (4). Unless otherwise noted, reaction mixtures contained 50 mM Tris-HCl (pH 7.1); 10 mM magnesium acetate; 1.5% polyethylene glycol; 1.5 mM dithiothreitol; 1.5 U of RNasin; 1.25 mM (each) ATP, CTP, GTP, and UTP; 15 μCi of [$^{32}$P]UTP; 4 to 8 μg of reporter RNA; and 1.6 μg of open cores, and the final volume was 20 μl. Reaction mixtures were incubated for 3 to 4 h at 37°C.

Prior to electrophoretic analysis of $^{32}$P-labeled dsRNA products on nondenaturing polyacrylamide gels, 1 μl of 20 mM $CaCl_2$ and 2.5 μl of 10-mg/ml micrococcal nuclease were added to reaction mixtures. After incubation at 37°C for 15 min, 2.5 μl of 10-mg/ml proteinase K and 100 μl of sample buffer were added and the reaction mixtures were incubated for an additional 30 min at 37°C. The samples were then electrophoresed on 12% polyacrylamide gels containing 0.5% sodium dodecyl sulfate (21).

To analyze the polarity of the RNA products of the cell-free system, the reaction mixtures were deproteinized by phenol-chloroform extraction and the RNAs were recovered by ethanol precipitation. The purified RNAs were then electrophoresed on 1.75% agarose gels containing 6 M urea and 25 mM sodium citrate buffer (pH 3.0) at 175 V for 24 h (35).

After electrophoresis, gels were dried and the $^{32}$P-labeled products were identified by autoradiography with Kodak MR film. The intensity of bands detected on film was quantitated with a Scanmaster 3+ densitometer and Bio Image Whole Band Analyzer package, version 3.2.

## RESULTS

**Template activity of viral plus- and minus-strand RNA.** To characterize the *cis*-acting signals that promote rotavirus RNA replication, a transcription vector that contained an SA11 gene 8 cDNA positioned immediately downstream from a T7 promoter (SP65g8R) was constructed (Fig. 1). Following cleavage with *Sac*II, the linearized vector was used in runoff transcription reactions to produce full-length gene 8 mRNA containing authentic 5′ and 3′ termini. The ability of the wild-type gene 8 mRNA to serve as a template for replication was assayed in the template-dependent cell-free replication system developed for the rotaviruses by Chen et al. (4). In addition to saturating levels of the input gene 8 mRNA, the system contained open core particles as a source of replicase activity and [$^{32}$P]UTP to label new synthesized RNA products. Analysis of the $^{32}$P-labeled products by electrophoresis on a nondenaturing polyacrylamide gel showed that the gene 8 mRNA served as an efficient template for the synthesis of dsRNA (Fig. 3A, lane 1). To verify that the gene 8 dsRNA product represented a noncovalently linked duplex of unlabeled plus-strand RNA and radiolabeled minus-strand RNA, the product was also subjected to electrophoresis on a low-pH agarose-urea gel, a system which electrophoretically resolves rotavirus plus- and minus-strand RNAs (35). The analysis demonstrated that the gene 8 mRNA was used as a template by the replicase activity of the open cores for the de novo synthesis of minus-strand RNA (Fig. 3B, lane 1).

Because rotavirus mRNAs have multiple functions in the infected cell, the level of mRNA synthesis in vivo exceeds that of minus-strand RNA synthesis (44), and hence, the promoter for transcription, i.e., plus-strand synthesis, must be more active than the promoter for RNA replication, i.e., minus-strand synthesis. However, this is not reflected in the cell-free system. Instead, while mRNA is efficiently used as a template for RNA replication in vitro, the endogenous dsRNA of the open cores is a poor template for transcription. To examine this phenomenon further, a transcription vector containing a gene 8 cDNA positioned in the reverse orientation (SP65g8RM) was used to produce gene 8 minus-sense RNA (Fig. 1). The minus-sense RNA was then assayed for the ability to serve as the template for RNA synthesis in the cell-free system. Despite being single-stranded and containing authentic 5′ and 3′ termini, electrophoretic analysis showed that the minus-strand RNA, like dsRNA, was a poor template for RNA synthesis (Fig. 3A, lane 2, and 3B, lane 2). Specifically, the amount of dsRNA product made from the minus-strand template was at least 10-fold lower than the amount of product made from the plus-strand template. The poor template activity of the minus-strand RNA rules out the possibility that the failure of dsRNA to act as a template for RNA synthesis stemmed from the lack of an unwindase or helicase activity to melt the duplex RNA and thereby allow the polymerase to gain access to the minus-strand template of the duplex. Rather, the inability of the minus-strand RNA to function as a template for RNA synthesis suggests that there are fundamental differences between the promoter recognized by the replicase and the promoter recognized by the transcriptase.

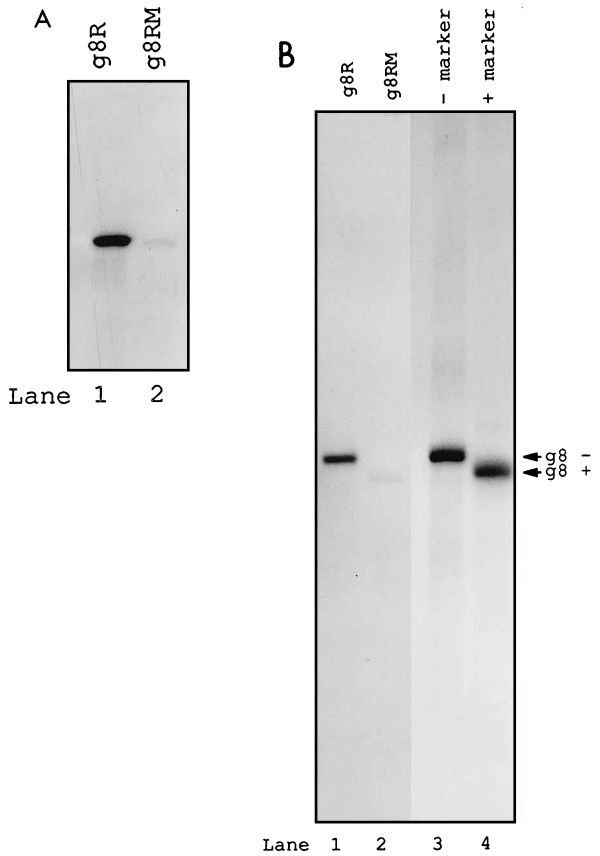



FIG. 3. Template activity of plus- and minus-strand gene 8 RNAs. The plus-sense g8R (lanes 1) and minus-sense g8RM (lanes 2) RNAs were made by T7 transcription and assayed for the ability to serve as template for the synthesis for dsRNA in vitro. The dsRNA products were either treated with micrococcal nuclease and proteinase K and then analyzed by electrophoresis on a nondenaturing polyacrylamide gel (A) or left untreated, deproteinized by chloroform extraction, and then analyzed by electrophoresis on a strand-separation agarose-urea gel (B) and by autoradiography. As markers for the strand separation gel (B), $^{32}$P-labeled minus-strand (lane 3) and plus-strand (lane 4) RNAs were prepared by runoff transcription of *Sac*II-cleaved SP65g8RM and SP65g8R, respectively, and electrophoresed in parallel lanes.

**Detection of an essential 3′ replication signal.** To analyze the importance of the 3′ terminus of viral mRNA in replica-

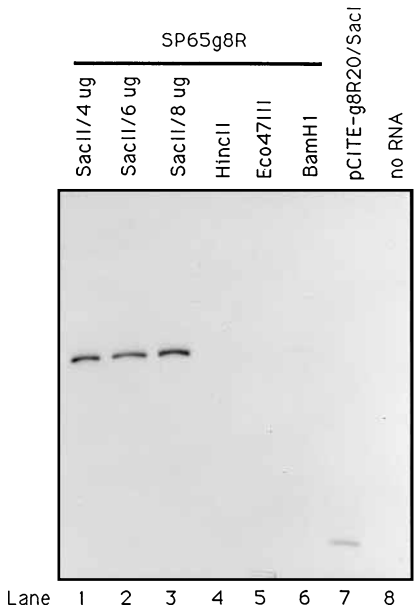



FIG. 4. Importance of 3′-terminal sequences in the replication of gene 8 mRNA. T7 transcripts were made by runoff transcription of SP65g8R cleaved with *Sac*II, *Hin*cII, *Eco*47III, or *Bam*HI and treated with T4 DNA polymerase. The RNAs and pCITE-g8R20 (Fig. 2) were then assayed for template activity in the cell-free system. Unless indicated otherwise, the reaction mixtures contained 6 μg of RNA and the $^{32}$P-labeled dsRNA products were detected by polyacrylamide gel electrophoresis and autoradiography.

tion, SP65g8R was cleaved with *Hin*cII or *Eco*47III (Fig. 1) and then used in runoff transcription reactions to produce gene 8 mRNAs that lacked the last 100 or 12 nucleotides, respectively, of the wild-type message. The 3′-truncated gene 8 mRNAs and the wild-type gene 8 mRNA were then assayed in the cell-free replication system, and the products were electrophoretically resolved (Fig. 4). While the wild-type gene 8 mRNA (lanes 1 to 3) and a reporter RNA containing only the 3′-terminal 20 nucleotides of the gene 8 RNA (pCITE-g8R20) (lane 7) underwent efficient replication in the system, the analysis showed that the 3′-truncated mRNAs were replicated to undetectable or barely detectable levels (lanes 4 and 5). Given that the truncated mRNA derived from *Eco*47III-cleaved SP65g8R lacked only the last 12 nucleotides of the wild-type mRNA (Fig. 1), these data indicate that the 3′ terminus of the gene 8 mRNA contains a *cis*-acting signal that is essential for the synthesis of minus-strand RNA, i.e., a 3′ essential replication signal.

The effect on replication of adding a nonviral sequence to the 3′ end of viral mRNA was tested by cleaving SP65g8R with *Bam*HI and then transcribing the linearized vector with T7 RNA polymerase (Fig. 1). The gene 8 transcripts produced by the runoff transcription were identical in sequence to wild-type gene 8 mRNA except that they contained the 3′-terminal extension, 5′-AAUCGGAUC-3′. The ability of the 3′-extended mRNA to serve as a template for replication was evaluated by in vitro assay and gel electrophoresis (Fig. 4, lane 6). As was observed for the 3′-truncated gene 8 mRNAs (lanes 4 and 5), the 3′-extended gene 8 mRNA was not an effective template for replication in the cell-free system. Hence, the addition of nonviral sequences to the end of the gene 8 mRNA interferes with the activity of the 3′ essential replication signal.

**Defining the minimum essential signal for replication.** To obtain additional information on the location of signals essential for the replication of rotavirus mRNA, gene 8 cDNAs containing 5′-terminal or internal deletions were introduced into T7 transcription vectors. After linearization with *Sac*II, the vectors were then used to synthesize gene 8 RNAs that lacked the first 502 (g8Rd1-502), 957 (g8Rd1-957), or 1,046 (g8Rd1-1047) nucleotides or the internal sequence from nucleotides 45 to 543 (g8Rd45-543) of the wild-type message (Fig. 1). As shown in Fig. 5, all the 5′-truncated and internally deleted RNAs were replicated successfully in the cell-free system, even g8Rd1-957, an RNA of only 120 nucleotides in length. On a molar basis, the g8Rd1-502, g8Rd1-957, and g8Rd45-543 RNAs were replicated at least as efficiently as the wild-type gene 8R message. Therefore in this initial analysis, signals which promote mRNA replication did not appear to be present at the 5′ end of gene 8 mRNA. In contrast to the other modified RNAs, the g8Rd1-1047 RNA, which contains only the last 12 nucleotides of the gene 8 RNA, replicated less well than the full-length message (Fig. 5). Albeit it is a somewhat less active template for minus-strand synthesis, the ability of the g8Rd1-1047 mRNA to replicate demonstrated that the last 12 nucleotides of the gene 8 mRNA contain a signal which is sufficient to promote mRNA replication. However, since the g8Rd1-1047 RNA replicates less efficiently than wild-type gene 8R mRNA, sequences in the message upstream of the 3′-terminal 12 nucleotides are also likely to contribute to the replication efficiency of the RNA.

In order to define the minimum size of the 3′ essential replication signal in the gene 8 mRNA, appropriate oligonucleotides were used to insert gene 8 3′-terminal sequences of 4 to 40 nucleotides into the polylinker of the transcription vector pCITE (Fig. 2). Following linearization with *Sac*II, the vectors were then employed as templates for the synthesis of a set of

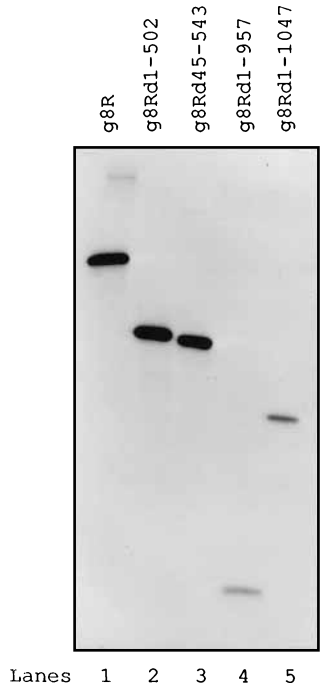



FIG. 5. Deletion mapping of the gene 8 RNA promoter sequence. The transcription vectors SP65g8R (lane 1), pT7g8Rd1-502 (lane 2), SP65g8Rd45-543 (lane 3), pT7g8Rd1-957 (lane 4), and pT7(SP65)g8Rd1-1047 (lane 5) (Fig. 1) were treated with *Sac*II and T4 DNA polymerase and used in T7 transcription reactions to produce reporter RNAs. The RNAs were then assayed for the ability to serve as templates for dsRNA synthesis in vitro, and the products were analyzed by polyacrylamide gel electrophoresis.

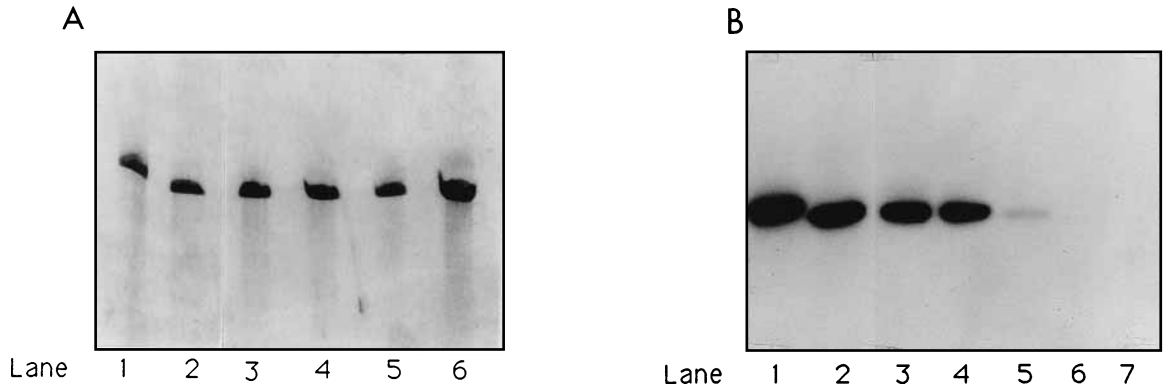



FIG. 6. Replication of chimeric reporter RNAs ending with gene 8 3′-terminal sequences. Following cleavage of the appropriate transcription vectors with *Sac*II and treatment with T4 DNA polymerase, the pCITEg8R-40 (lanes 1), −20 (lanes 2), −10 (lanes 3), −7 (lanes 4), −6 (lanes 5), and −4 (lanes 6) RNAs were synthesized by T7 transcription. (A) Prior to performance of the replicase assay, the quality of the T7 transcripts was assessed by electrophoresis on a denaturing polyacrylamide-urea gel. The gel was then silver stained. (B) The ability of the transcripts to serve as templates for the synthesis of dsRNA was determined with the cell-free replication system, and the $^{32}$P-labeled dsRNA products were detected by polyacrylamide gel electrophoresis and autoradiography. Lane 7, no RNA.

chimeric pCITEg8 RNAs (550 to 600 nucleotides) of which only the last 4 to 40 nucleotides were rotavirus specific (Fig. 6A). As shown in Fig. 6B, pCITEg8 RNAs terminating with the last 7, 10, 20, or 40 nucleotides of the wild-type message (pCITEg8R-7, -10, -20, and -40 RNAs, respectively) all were replicated efficiently in the cell-free system, although there was a corresponding increase in the level of replication of approximately twofold as the length of the 3′-terminal rotavirus-specific sequence increased from 7 to 40 nucleotides (Table 2). In contrast, the level of replication associated with pCITEg8 RNAs terminating with only the last four or six nucleotides of the wild-type gene 8 mRNA (pCITEg8-4 and -6 RNAs, respectively) was at least 25 times lower than that obtained with pCITEg8R7 (Table 2). Together, these data indicate that the last seven nucleotides of the gene 8 mRNA, i.e., 5′-UGUG ACC-3′, constitute the minimum *cis*-acting signal required to promote efficient replication of the message. Given that this signal falls within the 3′ consensus sequence of rotavirus mRNAs, the 3′ essential replication signal detected in the gene 8 message is likely a common feature of all rotavirus mRNAs. The location of the 3′ essential replication signal in the gene 8 mRNA is consistent with the results of previous experiments which indicated that an essential *cis*-acting signal for replication was located in the last 26 nucleotides of the OSU gene 9 mRNA (4).

**Mutational analysis of the essential 3′ replication signal.** The importance of each of the last seven nucleotides of the gene 8 mRNA, i.e., 5′-UGUGACC-3′, in promoter function was evaluated by using PCR-based site-specific mutagenesis to generate a battery of viral RNAs that differed only in their 3′-terminal sequences (Fig. 7A). The ability of the RNAs to serve as templates for dsRNA synthesis was then assayed with the cell-free system, and the dsRNA products were analyzed by polyacrylamide gel electrophoresis (Fig. 7B). As shown in Table 3, mutagenesis of residues located at positions −1, −2, −5, −6, and −7 of the template RNA reduced the levels of dsRNA synthesis by 50% or more. Indeed, the mutations C(−1)→A or U, C(−2)→U, and U(−5)→C reduced dsRNA synthesis to levels that were 25% or less of control RNA (Table 3). Notably, the residues C(−1), C(−2), and U(−5) are fully conserved among the rotavirus mRNAs, indicating their importance in the optimal performance of the 3′ essential replication signal. Unlike mutation of residues at positions −1, −2, −5, −6, and −7, mutations of residues at −3 and −4 were remarkable in that they had little or no inhibitory affect on dsRNA synthesis. Instead, two mutations at these positions, A(−3)→G and G(−4)→U, significantly increased the levels of dsRNA synthesis in the cell-free system (Table 3). Given that in some cases the naturally occurring residue at the −3 position in rotavirus mRNA is a G, e.g., SA11 genes 3 and 7, it is not particularly surprising to find that the replication of the gene 8 RNA remained efficient despite the introduction of the A(−3)→G mutation.

While deletion of the 3′-terminal C(−1) residue had no significant effect on replication of the gene 8 RNA, the addition of an extra C residue to the end of the RNA decreased replication by approximately one-half (Table 3). This latter finding is consistent with the results described above, indicating that the presence of nonviral sequences downstream of the 3′ end of gene 8 mRNA interferes with the function of the 3′ essential replication signal. The importance of the RNA template terminating with a C residue for efficient replication is

TABLE 2. Minimum essential sequence for RNA replication

| RNA species | 3′ sequence | % dsRNA product[a] |
|---|---|---|
| | −30 −20 −10 −1 | |
| pCITEg8R40 | UGAUGAUGGCUUAGCAAGAAUAGAAAGCGCUUAUGUGACC | 100 |
| pCITEg8R20 | UAGAAAGCGUUAUGUGACC | 88 |
| pCITEg8R10 | UUAUGUGACC | 69 |
| pCITEg8R7 | UGUGACC | 51 |
| pCITEg8R6 | GUGACC | <2 |
| pCITEg8R4 | GACC | 0 |

[a] % dsRNA product = (integrated intensity of the band of dsRNA product/integrated intensity of the band of dsRNA product for pCITEg8R40) × 100.

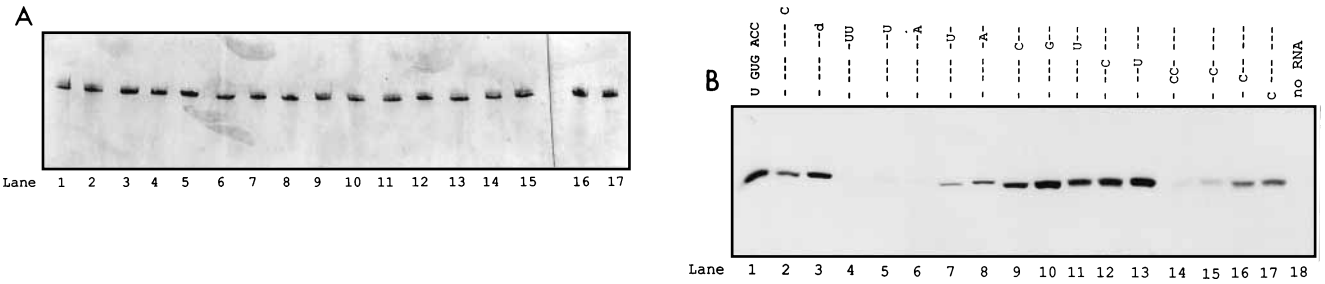



FIG. 7. Mutagenesis of the 3′ essential replication signal. PCR was used to introduce changes in the 3′ consensus sequence of a gene 8 cDNA. The amplification reaction mixtures included pT7g8d1-502 as template, a T7 promoter oligonucleotide as the forward primer, and the appropriate reverse primer (Table 1). (A) The quality of the T7 transcripts prepared from the amplified DNAs was examined by electrophoresis on a polyacrylamide-urea gel and by silver staining. (B) The ability of the T7 transcripts to function as templates for replication was assayed in vitro, and the $^{32}$P-labeled dsRNA products of the reactions were detected by polyacrylamide gel electrophoresis and autoradiography. The lanes of panel A correspond to those of panel B, and the 3′-terminal sequences of the mutant gene 8 transcripts are presented in panel B. Lanes 1 represent the authentic 3′ end of gene 8 mRNA, 5′-UGUGACC-3′. Sequence identity is indicated with dashes. d, deletion.

reflected in the observation that the mutations C(−1)→U, C(−1)→A, and C(−1)C(−2)→UU decreased the level of dsRNA synthesis by greater than 10-fold (Table 3).

**Detection of a 5′ signal that enhances replication.** The results of initial experiments designed to evaluate the role of the 5′ end of gene 8 mRNA in replication indicated that the deletion of 5′ sequences did not alter the ability of the RNA to function as a template for minus-strand synthesis. In particular, the 5′-modified RNAs g8Rd1-502 (Fig. 5) and g8R5′d4-50 (Fig. 8B, lanes 1 and 2) were replicated as efficiently as the wild-type message when assayed in the cell-free system in separate but parallel reactions. However, in subsequent competition experiments, it was found that when equal amounts of g8R5′d4-50 RNA and wild-type gene 8 RNA were combined and then assayed in the cell-free system, the 5′-modified RNA replicated significantly less efficiently than the wild-type RNA (Fig. 8B, lane 3). To further examine the possible role of the 5′ end of the gene 8 mRNA in dsRNA synthesis, PCR-based deletion mutagenesis was used to prepare a set of gene 8 RNAs that contained various deletions within their first 51 nucleotides (Fig. 8A). With the exception of the g8R5d1-10 RNA, these RNAs were then combined with equal amounts of the wild-type gene 8 mRNA and assayed for the ability to support dsRNA synthesis with the cell-free system. Gel electrophoresis was used to distinguish between the dsRNA products derived from the wild-type RNA and the 5′-modified RNA (Fig. 8B). The g8R5d1-10 RNA was not analyzed by competitive assay with the wild-type RNA, since it was not possible to electrophoretically resolve the replication products of these two templates.

TABLE 3. Effect of the 3′-terminal sequence on replication

| RNA species | 3′ sequence | % dsRNA product[a] | |
|---|---|---|---|
| | | Expt 1 | Expt 2 |
| | −9 −1 | | |
| T7g8d1-502/3′CC (wt[b]) | UAU GUG ACC | 100 | 100 |
| T7g8d1-502/3′CC+C | --- --- --- C | 41 | 51 |
| T7g8d1-502/3′CdC | --- --- --d[c] | 88 | 127 |
| T7g8d1-502/3′UU | --- --- -UU | <1 | 3 |
| T7g8d1-502/3′CU | --- --- --U | 5 | 9 |
| T7g8d1-502/3′C1→A | --- --- --A | 2 | 3 |
| T7g8d1-502/3′UC | --- --- -U- | 13 | 25 |
| T7g8d1-502/3′C2→A | --- --- -A- | 29 | 36 |
| T7g8d1-502/3′A3→C | --- --- C-- | 78 | 63 |
| T7g8d1-502/3′A3→G | --- --- G-- | 149 | 222 |
| T7g8d1-502/3′A3→U | --- --- U-- | 86 | 83 |
| T7g8d1-502/3′G4→C | --- --C --- | 107 | 135 |
| T7g8d1-502/3′G4→U | --- --U --- | 141 | 171 |
| T7g8d1-502/3′U5G6→CC | --- CC- --- | 7 | 9 |
| T7g8d1-502/3′U5→C | --- -C- --- | 17 | 14 |
| T7g8d1-502/3′G6→C | --- C-- --- | 40 | 26 |
| T7g8d1-502/3′U7→C | --C --- --- | 50 | 45 |

[a] % dsRNA product = (integrated intensity of the band of dsRNA product/integrated intensity of the band of dsRNA product for T7g8d1-502/3′CC) × 100. The results are given for two independent experiments.
[b] wt, wild type.
[c] d, deletion.

The analysis showed that deletions made anywhere within the first 51 nucleotides of the gene 8 mRNA reduced the replication efficiency of the RNA to 22 to 50% of wild type (Fig. 8A). Hence, while the 5′ end of the viral mRNA does not contain a *cis*-acting signal that is essential for minus-strand synthesis, these data indicate that the 5′ end may contain a signal that enhances the ability of the RNA to function as a template for the synthesis of minus-strand RNA. The fact that the deletion of nucleotides 1 to 10 and 25 to 51 both decreased the replication efficiency of the RNA suggests that various sequences within the first 51 nucleotides contribute to the enhancement activity of the 5′ end. The precise location of the 3′ end of 5′-enhancement signal has yet to be defined but may include sequences extending downstream of nucleotide 51. Except for the 5′-terminal trinucleotide, GGC, the 11 rotavirus mRNAs do not exhibit nucleotide identity at their 5′ end. As a result, the primary sequence of the 5′-enhancement signal can be presumed to be unique to the gene 8 mRNA. However, we cannot rule out the possibility that the 5′-enhancement signal is associated with a 5′-terminal secondary structure that is common to all the viral mRNAs.

**Location of an upstream 3′-enhancement signal.** Assay of the pCITE g8 RNAs in the cell-free system not only mapped the 3′ essential replication signal to the last seven nucleotides of the gene 8 mRNA but also indicated that sequences from positions −8 to −40 of the 3′ UTR could be important for maximal replication (Table 2). To further explore the importance of the upstream sequences of the 3′ UTR in replication, PCR-based deletion mutagenesis was used to produce gene 8 mRNAs that lacked residues −9 to −66 and −9 to −41 (Fig. 9A). The ability of the 3′-modified RNAs to support minus-strand synthesis was then compared with that of wild-type mRNA by in vitro assay (Fig. 9B). The results showed that deletion of residues −9 to −66 reduced the level of dsRNA synthesis by at least fivefold whereas deletion of residues −9 to −41 reduced the efficiency by less than twofold. Hence, the

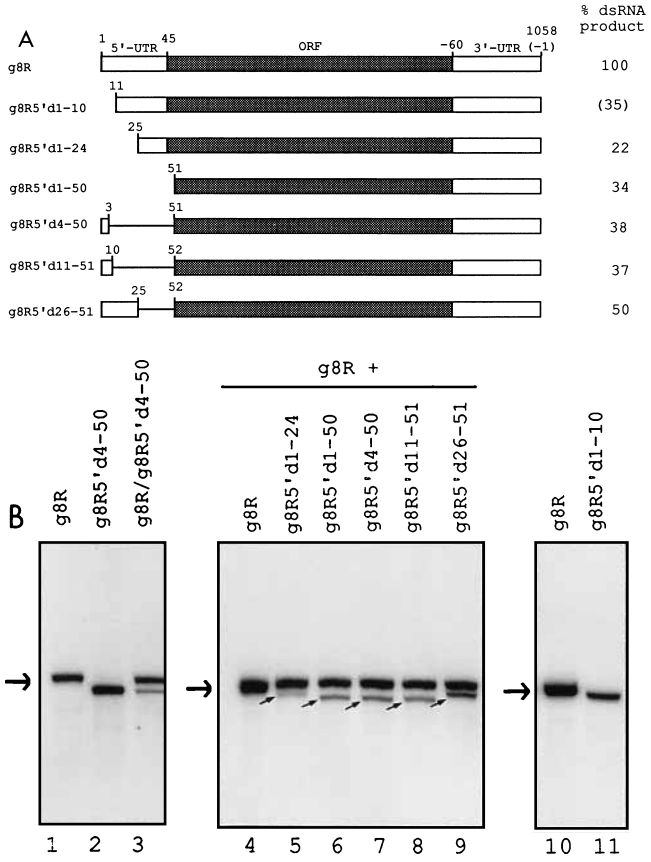



FIG. 8. Comparison of the replication efficiency of wild-type gene 8 mRNA and gene 8 mRNA containing 5′ deletions. (A) RNAs containing the deletions indicated were synthesized by runoff transcription of PCR-generated DNA. (B) To test their ability to function as templates for RNA replication, RNAs were added alone (lanes 1, 2, 4, 10, and 11) or together with wild-type mRNA (lanes 5 to 9) to the cell-free system. Reaction mixtures contained a total of 10 μg of RNA, and when two RNAs were included in a reaction mixture, 5 μg of each was added. The $^{32}$P-labeled dsRNA products were resolved by polyacrylamide gel electrophoresis, and the intensity of bands was determined by densitometry. The position of wild-type gene 8 dsRNA is indicated by the large arrows while the dsRNA products of gene 8 RNA containing 5′ deletions are indicated with the small arrows (lanes 5 to 9). Except for the reaction mixture containing g8R5′d1-10, the percent dsRNA product = (amount of dsRNA product made from the 5′-deleted gene 8 RNA/amount of dsRNA product made in the same reaction from the wild-type gene 8 mRNA) × 100. The calculated values are given in panel A.

data indicate that a *cis*-acting signal exists near the junction of the COOH end of the ORF for NSP2 and the 5′ end of the 3′ UTR (residues −42 and −67), a signal which strongly enhances the replication efficiency of the gene 8 mRNA, i.e., a 3′-enhancement signal. The minimal effects (less than twofold) of deleting residues −9 to −41 on replication are consistent with the results obtained from assay of the pCITE g8RNAs, in which it was found that residues −8 to −40 are responsible for an approximately twofold enhancement in the replication efficiency of an RNA (Table 2). The extent of RNA replication achieved with the 3′-modified RNAs g8R3d(−9)-(−66) and g8R3d(−9)-(−41) was not significantly altered by deleting the 5′-terminal halves of the RNAs [g8Rd1-502/3d(−9)-(−66) and g8Rd1-502/3d(−9)-(−41)]. This result indicates that the 3′-enhancement signal operates independently of the 5′-enhancement signal and does not require the formation of a 5′-3′ panhandle structure for activity.

## DISCUSSION

In this study, we have taken advantage of the replicase activity associated with rotavirus open cores to identify *cis*-acting signals that promote the replication of viral gene 8 mRNA. By analyzing the replication efficiency of modified gene 8 RNAs and chimeric RNAs ending with short gene 8 3′-terminal sequences, three *cis*-acting signals were detected, only one of which was found to be essential for efficient replication of the mRNA. The essential replication signal, 5′-UGUGACC-3′, is formed by the last seven nucleotides of the gene 8 mRNA and, despite its small size, contains all the information necessary for specific recognition and replication of an RNA by the viral replicase (Fig. 10). Given that the essential replication signal is located within the 3′ consensus sequence of the gene 8 mRNA (8), all 11 rotavirus mRNAs are likely to contain a similar 3′-terminal *cis*-acting signal that functions to promote minus-strand synthesis. As expected, site-specific mutagenesis confirmed the importance of the last seven nucleotides of the gene 8 mRNA in dsRNA synthesis. The introduction of mutations in the essential region generally decreased replication efficiency,

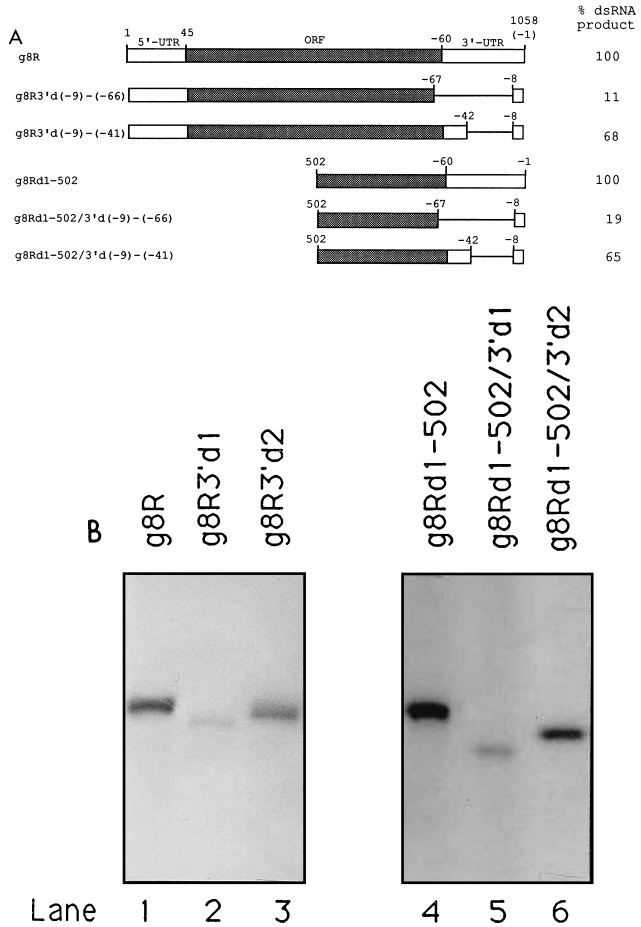



FIG. 9. Detection of an enhancement signal in the 3′ UTR by deletion mutagenesis. (A) Gene 8-specific RNAs containing deletions within the 3′ UTR were synthesized by runoff transcription. (B) The RNAs were assayed for the ability to serve as templates for dsRNA synthesis in the cell-free system, and the products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The integrated intensities of the bands of dsRNA product were used to calculate the percent dsRNA product. The percent dsRNA product = (amount of dsRNA made from the RNA containing a deletion within the 3′ UTR/amount of dsRNA made from the RNA containing no deletion in the 3′ UTR) × 100. The calculated values are given in panel A.

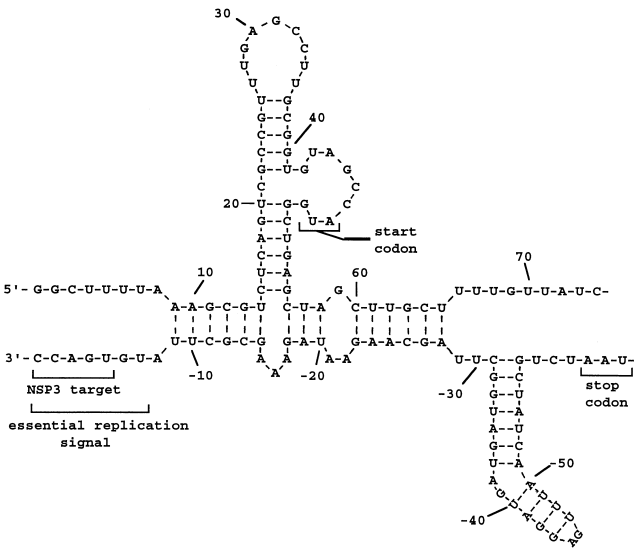



FIG. 10. Possible panhandle structure formed by the 5′ and 3′ termini of the gene 8 mRNA. The recognition signal for NSP3 and the 3′ essential replication signals are indicated. Sequences in the 3′-terminal region −42 to −67 and 5′-terminal region 1 to 52 enhance the replication of gene 8 mRNA in vitro.

sometimes by as much 20-fold [C(−1)→A]; however, in at least one instance, mutation of the region significantly increased replication efficiency [A(−3)→G].

Two *cis*-acting signals which markedly enhanced the replication of the gene 8 mRNA were also identified, one located at the 5′ terminus of the mRNA and the other near the junction of the gene 8 ORF and 5′ end of the 3′ UTR. The nucleotide sequences within the regions of the gene 8 mRNA that contain the enhancement signals are not conserved among the 11 viral mRNAs, and hence, it is not known whether the other mRNAs also contain such signals and whether they are positioned at similar sites on the mRNA molecule. While the nucleotide sequences of the enhancement signals are not conserved, it may be possible that these sequences form secondary structures which are conserved among all the mRNAs and which function as the enhancement signals. In fact, the regions of the gene 8 RNA that contain the 5′- and 3′-enhancement signals have been predicted to form secondary structures (34) (Fig. 10). In our analysis, we have yet to systematically explore the possibility that the ORF of the gene 8 mRNA contains additional enhancement signals. Clearly, since the 3′ UTRs of rotavirus mRNAs vary in length from 17 to 182 nucleotides, if all the mRNAs are like the gene 8 mRNA and contain a 3′-enhancement signal approximately 50 nucleotides upstream from the 3′ terminus, then for those RNAs with short 3′ UTRs (<50 nucleotides), the signal would fall within the ORF.

While this report is the first to provide evidence that rotavirus mRNAs contain *cis*-acting signals affecting RNA synthesis at both their 5′ and 3′ termini, similar observations have been made for the RNAs of influenza virus (9, 23). In particular, recent studies have indicated that panhandle structures formed from the 3′- and 5′-terminal nucleotides of influenza virion RNA are an integral part of the RNA promoter for transcription (11, 15). Because RNA folding programs have also predicted that sequences within the 5′ and 3′ UTR of the gene 8 mRNA are able to stably basepair (34), it follows that such an interaction may in a similar manner lead to the generation of a panhandle structure that promotes the replication of the gene 8 mRNA (Fig. 10). However, since we have shown that the 5′-terminal nucleotides of the gene 8 RNA are not essential for replication, it follows that the formation of a 5′-3′ panhandle structure is not required for minus-strand synthesis, at least in vitro. Interestingly, the 5′- and 3′-terminal sequences that are proposed to anneal to form the gene 8 panhandle structure do not include the 3′-terminal seven nucleotides that constitute the 3′ essential replication signal (Fig. 10).

A number of RNA-binding proteins, including NSP3, are produced in rotavirus-infected cells (32). This abundant nonstructural protein accumulates on the cytoskeleton and is a component of rotavirus early replication intermediates (13, 16, 25). Poncet et al. (37, 38) have shown by gel retardation assay and UV cross-linking that the last five bases of rotavirus mRNAs form a specific recognition signal for NSP3 (Fig. 10). Hence, the recognition signal for NSP3 resides within the 3′ essential replication signal identified in this study, and thus, NSP3 and the viral replicase apparently recognize overlapping signals within the mRNA. A common feature of the NSP3 recognition signal and 3′ essential replication signal is that the addition of extra nucleotides to the 3′ ends of either markedly inhibits their function (38). It seems doubtful that NSP3 and the replicase can both stably interact with their 3′-terminal recognition signals simultaneously, given that site-specific mutagenesis indicates that both recognition signals share residues that are essential for their function. For example, mutagenesis of residues −1 and −2 indicates that the 3′-terminal CC residues are critical for recognition of viral mRNA by NSP3 and for replication of the mRNA by the replicase. However, the presence of NSP3 on its 3′ signal does not preclude the replicase from interacting with other recognition signals on the same mRNA. In fact, on the basis of models generated for replication of the dsRNA genome of yeast L-A virus (47), it may be that the replicase interacts with rotavirus mRNAs via enhancement signals, and then when NSP4 is displaced from the 3′ terminus of the mRNAs, the replicase may reach back to the 3′ essential replication signal and there initiate minus-strand synthesis. What would displace NSP3 from its 3′ signal? Certainly, previous studies indicating that the RNA-binding protein VP2 is required for RNA replication raise the possibility that VP2 might disrupt the NSP3-RNA interaction, allowing RNA replication to commence (20, 24).

While the RNA polymerase activity associated with open cores is able to use mRNA as a template for minus-strand synthesis, the polymerase activity is unable to effectively use either dsRNA or minus-strand RNA as a template for mRNA synthesis. This phenomenon indicates that the open cores contain replicase activity but not transcriptase activity and therefore that the viral replicase and transcriptase are functionally separable enzymatic activities. The idea that the replication of the rotavirus genome involves two unique polymerase activities is not novel, given earlier experiments which revealed that the minimum protein components of particles with transcriptase and replicase activity differ from one another (1, 24, 46). More precisely, although cores surrounded by a layer of VP6 have transcriptase activity, cores alone do not, thus indicating that VP6 is an essential component of functional transcriptase particles (42). In addition to VP6, the polymerase core VP1, the guanylyltransferase VP3, and the major core protein VP2 are probably also essential components of particles with transcriptase activity (32). In comparison, studies of baculovirus-expressed rotavirus-like particles have indicated that, while VP1 and VP2 are essential components of replicase particles, VP3 and VP6 are not and, therefore, replicase particles seem to be structurally less complex than transcriptase particles (46). In part, the complexity of the transcriptase particles may stem from the additional activities involved in synthesis of mature

mRNAs that are not involved in the synthesis of dsRNA. In particular, capping of the progeny mRNA, melting of the dsRNA template, and displacement of the daughter plus-strand RNA from the dsRNA template are all activities that would be unique to the transcriptase particle.

Not only do the viral replicase and transcriptase differ in their minimum protein composition, but the replicase and transcriptase also appear to differ in the *cis*-acting signals that they recognize in viral RNA. For instance, while the replicase in the open core preparations can efficiently utilize viral plus-strand RNA as a template for RNA synthesis, it cannot effectively utilize viral minus-strand RNA as a template for RNA synthesis, despite the fact that the transcriptase efficiently uses the minus-strand RNA as the template for plus-strand synthesis during transcription. On the basis of inspection of the last seven nucleotides of the minus-strand RNA of gene 8, it is not surprising that the RNA is a poor template for the replicase, as its 3′-terminal sequence 5′-AAAAGCC-3′ differs from the 3′ essential replication signal of the plus-strand RNA 5′-UGUG ACC-3′ at residues that site-specific mutagenesis indicates are key for the signal to fully promote replication. Overall, the data seem to suggest that the proteins associated with the transcriptase and replicase alter the specificity of these polymerase activities such that they each recognize unique and noninterchangeable *cis*-acting signals. Because rotavirus mRNAs are capped and capping and elongation are often linked processes during viral transcription, it may be that *cis*-acting signals on the minus strand function not only to catalyze the initiation of plus-strand synthesis but also to stall elongation until the nascent transcript has been capped.

In previous efforts to identify replication signals in rotavirus mRNAs, Gorziglia and Collins (14) constructed synthetic analogs of gene 9 in which the viral ORF was replaced with the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. These investigators observed that when plus-sense gene 9-CAT RNA was introduced into rotavirus-infected cells, the CAT signal was amplified, suggesting that the viral replicase successfully replicated the reporter RNA. By deletional analysis, Gorziglia and Collins (14) also found that the last 12 to 17 nucleotides of the gene 9 RNA are necessary and sufficient for amplification, which is consistent with our results indicating that the last seven bases of the gene 8 RNA are necessary and sufficient to promote replication of the gene 8 RNA in the cell-free system. Analogous to what we observed for gene 8 minus-sense RNA, minus-sense gene 9-CAT RNA was not amplified when transfected into cells, suggesting that *cis*-acting signals for the replicase lie exclusively on viral plus-sense RNA. During the rotavirus infection process, the synthesis of minus-strand RNA on the mRNA template and the packaging of the dsRNA product appear to be concurrent events. In our study and in the study by Gorziglia and Collins (14), it is not yet known whether the reporter RNA was packaged into cores as it was replicated. Thus, we do not know whether the *cis*-acting signals identified in these studies strictly represent those involved with minus-strand synthesis or whether they also include signals that participate in RNA packaging.

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