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De novo synthesis of minus strand RNA by the rotavirus RNA polymerase in a cell-free system involves a novel mechanism of initiation

DAYUE CHEN and JOHN T. PATTON

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

ABSTRACT

The replicase activity of rotavirus open cores has been used to study the synthesis of (–) strand RNA from viral (+) strand RNA in a cell-free replication system. The last 7 nt of the (+) strand RNA, 5′-UGUGACC-3′, are highly conserved and are necessary for efficient (–) strand synthesis in vitro. Characterization of the cell-free replication system revealed that the addition of NaCl inhibited (–) strand synthesis. By preincubating open cores with (+) strand RNA and ATP, CTP, and GTP prior to the addition of NaCl and UTP, the salt-sensitive step was overcome. Thus, (–) strand initiation, but not elongation, was a salt-sensitive process in the cell-free system. Further analysis of the requirements for initiation showed that preincubating open cores and the (+) strand RNA with GTP or UTP, but not with ATP or CTP, allowed (–) strand synthesis to occur in the presence of NaCl. Mutagenesis suggested that in the presence of GTP, (–) strand synthesis initiated at the 3′-terminal C residue of the (+) strand template, whereas in the absence of GTP, an aberrant initiation event occurred at the third residue upstream from the 3′ end of the (+) strand RNA. During preincubation with GTP, formation of the dinucleotides pGpG and ppGpG was detected; however, no such products were made during preincubation with ATP, CTP, or UTP. Replication assays showed that pGpG, but not GpG, pApG, or ApG, served as a specific primer for (–) strand synthesis and that the synthesis of pGpG may occur by a template-independent process. From these data, we conclude that initiation of rotavirus (–) strand synthesis involves the formation of a ternary complex consisting of the viral RNA-dependent RNA polymerase, viral (+) strand RNA, and possibly a 5′-phosphorylated dinucleotide, that is, pGpG or ppGpG.

Keywords: RNA elongation; RNA initiation; RNA polymerase; RNA synthesis; rotavirus

INTRODUCTION

With the exception of RNA viruses using a reverse transcriptase in their replication cycle, all RNA viruses encode an RNA-dependent RNA polymerase (RdRP), and this enzyme is responsible for both genome replication and transcription. Specific recognition of *cis*-acting elements by the RdRP is crucial for initiation of (+) and (−) strand RNA synthesis at appropriate sites on RNA templates (Buck, 1996). Not only does successful initiation involve interactions between the RdRP and the RNA template, but is also likely to involve interaction of the polymerase with the initiating nucleotide, and a protein-mediated stabilization between the initiating nucleotide and the 3′ end of the RNA template. Besides the viral RdRP, other proteins of either viral or host origin may also be involved in the initiation of viral RNA synthesis. Despite the availability of several cell-free systems that support the *de novo* synthesis of viral RNAs from exogenous RNA templates, our understanding of the events that are involved in the initiation of RNA synthesis remains limited.

Rotaviruses, members of the *Reoviridae*, possess a genome of 11 segments of double-stranded (ds)RNA surrounded by three concentric layers of capsid proteins (Estes, 1996). The 11 segments share no sequence homology except for short highly conserved regions at their 5′ and 3′ termini. The 5′ and 3′ terminal conserved sequences are 5′-GGC(A/U)$_{6–8}$ and 5′-UGUGACC-3′, respectively. Rotavirus mRNAs possess 5′ caps but lack 3′ poly (A) tails and function as templates for two events in infected cells: the synthesis of viral protein and the synthesis of (−) strand RNA to produce dsRNA (Imai et al., 1983; McCrae & McCor-

Reprint requests to: John T. Patton, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 7 Center Drive, MSC 0720, Room 117, Bethesda, Maryland 20892, USA; e-mail: jpatton@niaid.nih.gov.

quodale, 1983). Treatment with EDTA and $CaCl_2$ removes the outer two layers of capsid proteins from virions, generating cores consisting of three proteins: VP1 (125 kDa), VP2 (98 kDa), and VP3 (88 kDa). Several lines of evidence indicate that VP1 is the viral RdRP: (1) VP1 contains four consensus amino acid motifs shared by RdRPs of other RNA viruses, including the GDD hallmark motif (Mitchell & Both, 1990). (2) VP1 possesses NTP-binding activity, and crosslinking of the nucleotide analog, 8-azido-ATP, to VP1 inhibits viral RNA transcription (Valenzuela et al., 1991). (3) The RNA-binding activity of VP1 specifically recognizes the 3′ end of viral mRNAs (Patton, 1996). (4) Recombinant VP1 can direct template-dependent (−) strand synthesis in vitro, but only in the presence of VP2 (Zeng et al., 1996; Patton et al., 1997). VP2 makes up the icosahedral shell of rotavirus cores and possesses nonspecific RNA-binding activity (Boyle & Holmes, 1986). VP3 contains both guanylyltransferase and methyltransferase activities (Pizarro et al., 1991; Liu et al., 1992; Chen et al., 1999) and is responsible for capping of viral mRNAs. In the core, one copy each of VP1 and VP3 are located in channels present at the 5′-fold axes of the VP2 shell.

Extensive dialysis of cores in hypotonic buffer results in the disruption of the VP2 shell and the release of the dsRNA genome. When supplied with exogenous viral (+) strand RNA and the four NTPs, such "open" cores catalyze (−) strand RNA synthesis in vitro, producing full-length genomic dsRNA (Chen et al., 1994). Several features make the open core replication system an excellent tool to understand the mechanism of RNA synthesis: (1) The system contains only three viral proteins and, thus, is well defined in composition. (2) The RNA polymerase activity exhibits stringent template specificity and catalyzes *bona fide de novo* (−) strand synthesis. (3) The RNA polymerase is efficiently recycled following (−) strand synthesis and catalyzes RNA synthesis in a near linear manner for several hours. (4) The protein and RNA components of the system can be derived from cloned cDNA, which makes the system particularly useful for identifying and characterizing the domains and signals involved in the RNA replication.

Previous studies using the open core replication system have indicated that the 3′ consensus sequence, 5′-UGUGACC-3′, of viral (+) strand RNA is the minimal *cis*-acting signal essential for specific (−) strand synthesis (Patton et al., 1996; Wentz et al., 1996) and that base pairing of complementary sequences near the 5′ and 3′ termini of the viral (+) strand RNAs in cis leads to formation of panhandles that promote the synthesis of (−) strand RNA (Chen & Patton, 1998; Patton et al., 1999). The replicase activity of the open core replication system is inhibited even when low concentrations of salt are included in the reaction mixtures (Chen et al., 1994). We report here that the salt inhibition of (−) strand synthesis can be overcome by preincubating open cores, (+) strand RNA, and selected NTPs prior to the addition of NaCl. These results indicate that (−) strand RNA synthesis can be separated into a salt-sensitive initiation phase and a salt-resistant elongation phase. Taking advantage of this finding, initiation of (−) strand synthesis was investigated using the open core replication system. The data indicate that initiation of (−) strand RNA synthesis involves the formation of a ternary complex consisting of RdRP and possibly other core proteins, (+) strand RNA, and (p)pGpG, a dinucleotide that is synthesized by the core proteins in the absence of exogenous (+) strand RNA template.

RESULTS

Salt inhibits (–) strand synthesis by open cores

Reaction mixtures of the open core replication system typically contained 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 20 U of RNasin, 200 μM each of the four NTPs, 0.2 μg of gene 8 (+) strand RNA (1.1 kB), 0.4 μg of open cores, and 10 μCi [α-^{32}P]-UTP, and were incubated at 32 °C. Under these conditions, where (+) strand RNA template is in excess, the synthesis of full-length gene 8 dsRNA by the RdRP activity of the open cores in the system occurred in a near liner manner for 3 h and was initially detected by 10 min of incubation (Fig. 1). Initiation of (−) strand synthesis to form dsRNA takes place throughout the incubation period, as indicated by experiments showing that when a second viral (+) strand template RNA is added to the reaction mixture, but after the onset of incubation with the first template RNA, the second template also undergoes replication to produce dsRNA (data not shown). These findings indicated that when the (+) strand RNA template is in excess in the open core replication system, the RdRP complex is recycled upon reaching the 5′ end of (+) strand RNA and is able to re-initiate (−) strand synthesis on a new molecule of (+) strand RNA.

The effect of salt on (−) strand synthesis was examined by including 200 mM NaCl in the standard reaction mixtures after 1 or 2 h of incubation (Fig. 1). The synthesis of (−) strand RNA was monitored by measuring the level of dsRNA products in aliquots taken at different times of the incubation. As shown in Figure 1, the level of dsRNA products increased continuously in the absence of NaCl (reaction mixture A) or prior to the addition of NaCl (reaction mixtures B and C). However, the level of dsRNA products no longer increased 10–20 min after NaCl was added to the reaction mixtures. These results indicated that either initiation or elongation of (−) strand synthesis, or both, by the viral RdRP was a salt-sensitive event in the open core replication system.

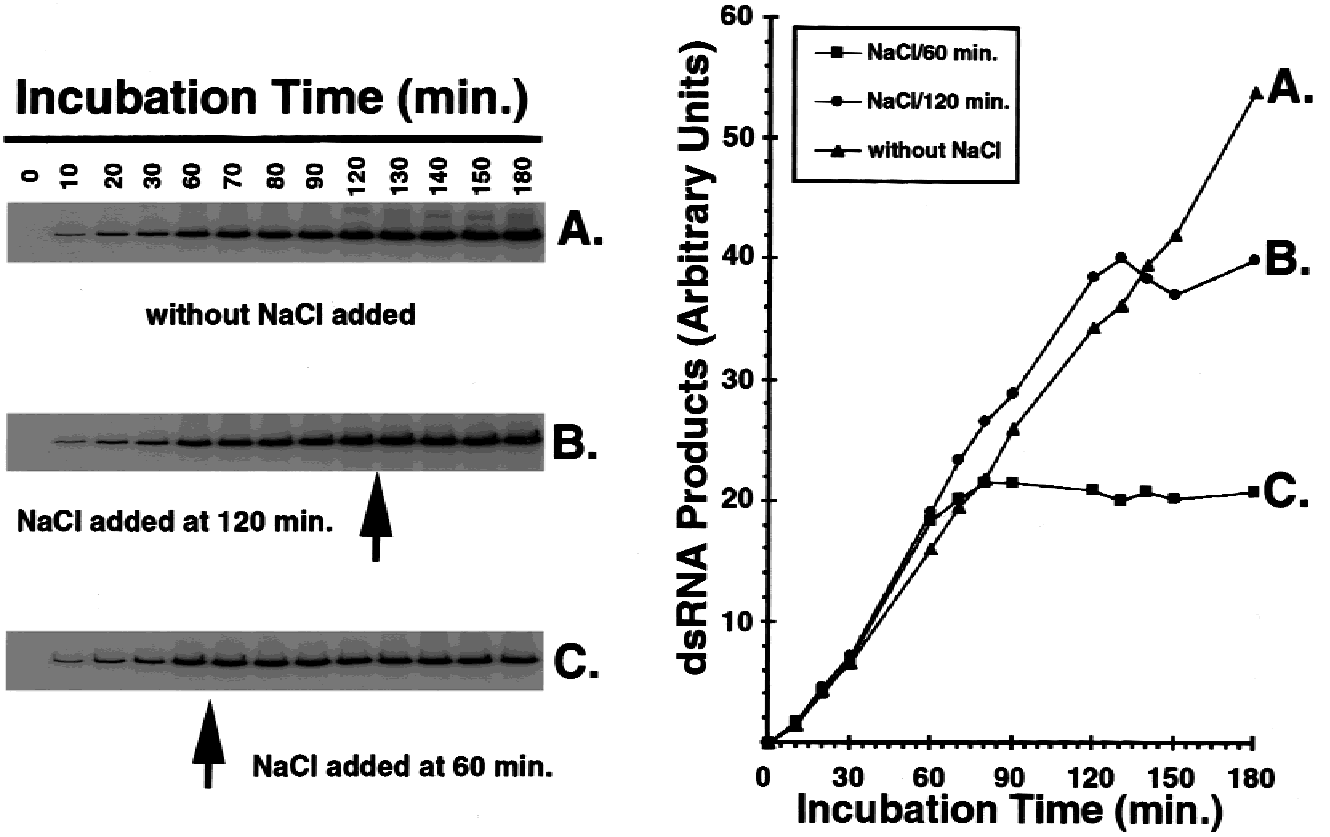

FIGURE 1. Kinetics of (−) strand RNA synthesis in the absence and presence of salt. NaCl was added to the reaction mixtures to a final concentration of 200 mM at 1 h (C) or 2 h (B) after beginning of the incubation at 32 °C. The synthesis of (−) strand RNA was monitored by taking aliquots from the reaction mixtures at the indicated times. ^{32}P-labeled dsRNA products in the aliquots were resolved by electrophoresis on an SDS-polyacrylamide gel, visualized by autoradiography, and quantified by phosphorimaging.

Initiation of (–) strand synthesis is salt sensitive

The impact of salt on (−) strand synthesis was further evaluated by including 0–500 mM NaCl in reaction mixtures. The results showed that (−) strand synthesis was completely inhibited at ≥150 mM NaCl and, even at a low concentration of NaCl, that is, 25 mM, its synthesis was inhibited by one-half (Fig. 2A).

To investigate whether the initiation and elongation phases of (−) strand synthesis differed in their salt sensitivity, reaction mixtures that lacked UTP were preincubated at 32 °C for 1 h. Afterwards, 100 to 1,000 mM NaCl, 10 μCi [α-^{32}P]-UTP, and 200 μM UTP were added sequentially to the reaction mixtures, which were then incubated at 32 °C for 1 h. If initiation was salt sensitive, but elongation was not, the preincubation step would allow the RdRP to bind to the (+) strand RNA and to initiate (−) strand synthesis. However, the RdRP would have to pause after synthesis of a guanosine dinucleoside on the (+) strand RNA template, 5′-.....UGU GACC-3′, because of the lack of UTP in the preincubation mixture. Furthermore, if only initiation was salt sensitive, the paused RdRP complex should be able to resume (−) strand synthesis once UTP and NaCl were added to the preincubation mixture. On the other hand, if NaCl inhibited the elongation step, or if NaCl inhibited both the initiation and elongation steps, (−) strand synthesis would not occur after the addition of NaCl and UTP, even if the other components of the system were preincubated. As shown in Figure 2B, the analysis showed that dsRNA products were made in the presence of 100–1,000 mM NaCl, but only if the preincubation step was carried out. Thus, NaCl inhibited the production of dsRNA in the open core replication system by interfering with the initiation of (−) strand synthesis, most likely by impeding the formation of initiation complexes. From studies evaluating the relationship between time of preincubation and level of dsRNA synthesis after salt was added to the reaction mixture, the putative initiation complexes were able to form within 10 min of preincubation. However, the number of functional initiation complexes formed by 1 h of preincubation was much higher than that formed by 10 min (data not shown).

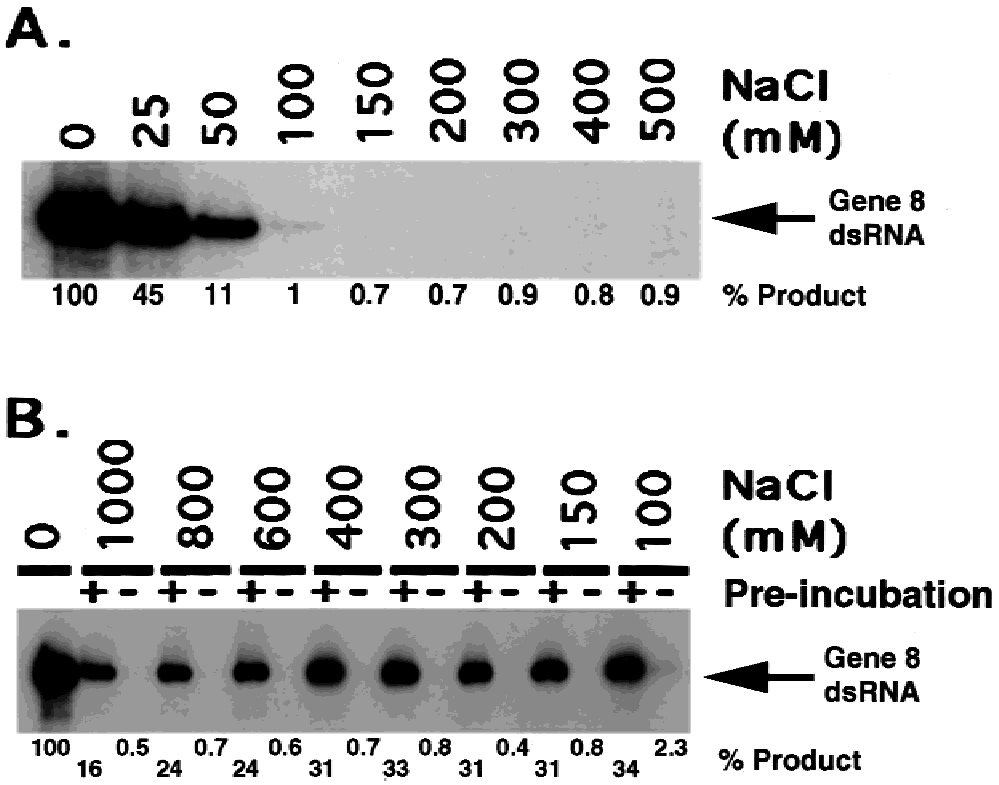

FIGURE 2. Effect of salt on (−) strand initiation. **A:** The standard in vitro replication assays were carried out in the presence of the indicated concentration of NaCl. The ^{32}P-labeled dsRNA products were resolved by electrophoresis on an SDS-polyacrylamide gel, visualized by autoradiography, and quantified with a phosphorimager. The amount of dsRNA synthesized in the absence of NaCl was normalized to 100%. **B:** NaCl was added to standard reaction mixtures at a final concentration of 100–1,000 mM (−: preincubation). Alternatively, gene 8 (+) strand RNA (0.2 μg) was incubated with 0.4 μg of open cores in the presence of 200 μM each of ATP, GTP, and CTP at 32 °C for 1 h prior to the addition of NaCl (+: preincubation). The amount of dsRNA synthesized in the absence of NaCl was considered to be 100%.

Essential components of the initiation complex

To determine which components were involved in the formation of the putative initiation complexes, we investigated whether omitting any of the three major components from the preincubation mixtures, namely, open cores, template RNA, and NTPs (ATP, GTP, and CTP), would affect the level of dsRNA products. As shown in Figure 3A, lanes 1, 3, and 4, dsRNA products were not detected in any of the reaction mixtures in which any of the three components was omitted. These results suggested that the interaction between (+) strand RNA and one or more of the proteins of open cores were probably essential, yet not sufficient for the formation of a functional initiation complex. The requirement of NTPs in the preincubation mixture implied either that hydrolysis of NTPs was required for the formation of the initiation complex or that NTPs were an essential component of the initiation complex. However, the fact that open cores lack any detectable NTPase activity argues against the first possibility (Chen et al., 1999). Given that the 3′ terminus of all rotavirus (+) strand RNAs ends with two C residues, the second possibility predicts that GTP alone would be sufficient for the formation of a functional initiation complex. To test this, individual NTPs were preincubated with open cores and gene 8 (+) strand RNA prior to the addition of NaCl, [α-^{32}P]-UTP, and the other three NTPs. As predicted, dsRNA products were detected in the preincubation mixture which contained GTP, demonstrating that the presence of GTP alone was sufficient to support the formation of a functional initiation complex (Fig. 3B, lane 1). Few or no dsRNA products were synthesized in the preincubation mixtures which contained ATP or CTP (Fig. 3B, lanes 2 and 4). Unexpectedly, relatively high levels of dsRNA products were detected when the preincubation reaction mixture contained UTP alone (Fig. 3B, lane 3). Because A (−3) is the third residue upstream from the 3′ end of the (+) strand RNA template, 5′-……UGUGACC-3′, it was possible that the UTP may have promoted initiation of (−) strand synthesis at an internal site of the RNA template. To address this possibility, site-specific mutagenesis was used to change the A residue at the third position of the wild-type gene 8 (+) strand RNA, generating three mutant RNAs, G8–3C (A → C), G8–3U (A → U), and G8–3G (A → G). The 3′ terminal sequences of these mutant gene 8 (+) strand RNAs are shown in Figure 4.

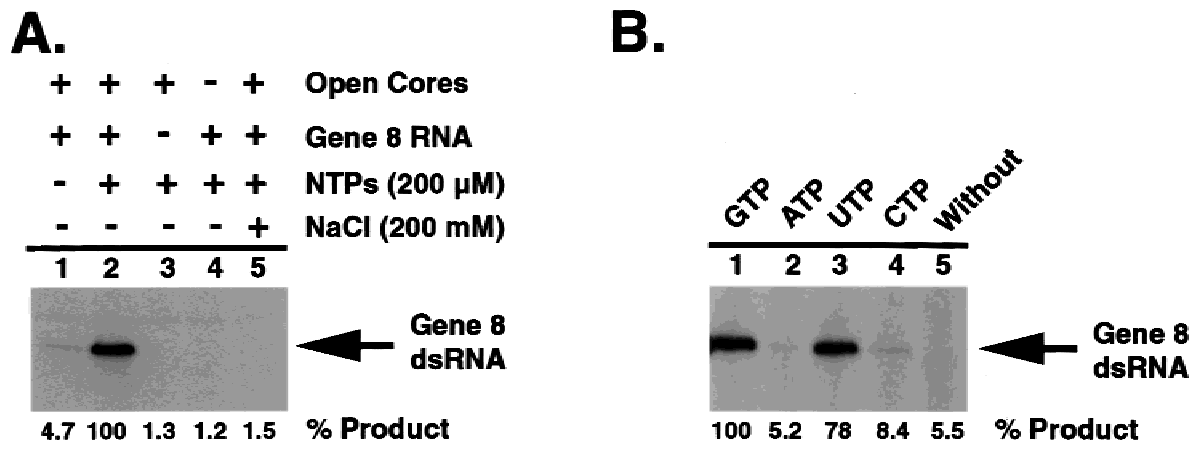

FIGURE 3. Components in the preincubation mixture necessary for supporting (−) strand synthesis in salt. **A:** Preincubation mixtures were assembled with various combinations of open cores, gene 8 (+) strand RNA, and NTPs (mixture of ATP, CTP, and GTP) and were incubated at 32 °C for 1 h. Afterwards, NaCl, the omitted components, and ^{32}P-UTP were added to the preincubated mixtures in sequential order and the mixtures were incubated at 32 °C for 1 h. The ^{32}P-labeled dsRNA products in the reaction mixtures were resolved by electrophoresis on an SDS-polyacrylamide gel, detected by autoradiography, and quantified by phosphorimaging. The amount of dsRNA synthesized in the presence of all three components (open cores, gene 8 RNA, and NTPs) in the preincubation mixture was considered to be 100%. **B:** Individual NTPs were incubated in the preincubation mixture along with open cores and gene 8 (+) strand RNA at 32 °C for 1 h. NaCl, the omitted NTPs, and ^{32}P-UTP were then sequentially added to the preincubated mixtures, and the reaction mixtures were incubated at 32 °C for 1 h. The ^{32}P-labeled dsRNA products were resolved by electrophoresis on an SDS-polyacrylamide gel, detected by autoradiography, and quantified by phosphorimaging. The amount of dsRNA synthesized in the reaction mixture preincubated with GTP was considered to be 100%.

Replication assays performed with the mutant RNAs showed that UTP in the preincubation mixture failed to promote (−) strand synthesis in the presence of NaCl when anything other than an A residue was present at the −3 position of RNA template (Fig. 4, lanes 9, 14, and 19). In contrast, ATP and CTP in the preincubation mixture allowed (−) strand synthesis to occur with the mutant RNAs, G8–3U and G8–3G, respectively in the presence of NaCl (Fig. 4, lanes 13 and 20). Furthermore, no NTP other than GTP was able to promote the

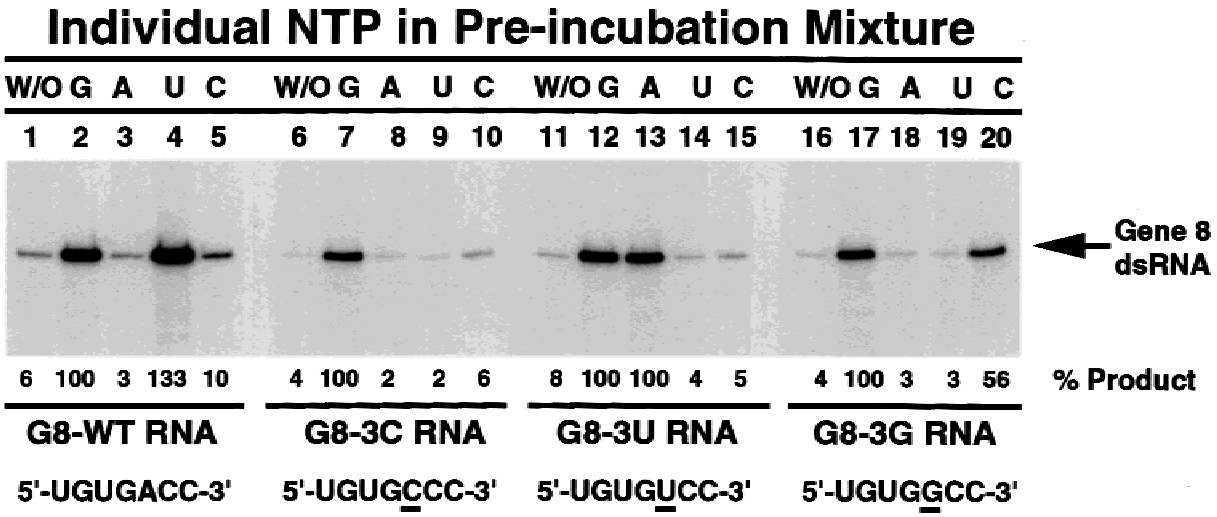

FIGURE 4. Synthesis of (−) strand RNA directed by gene 8 mutant RNAs in the presence of NaCl. The A residue at the third position from the 3′ terminus of wild-type (WT) gene 8 (+) strand RNAs was mutated to C (G8–3C), U (G8–3U), or G (G8–3G). The wild-type and mutant gene 8 RNAs were preincubated with open cores in the presence of an individual NTP at 32 °C for 1 h. NaCl, the omitted NTPs, and ^{32}P-UTP were then sequentially added to the preincubated mixtures. After incubation for 1 h at 32 °C, the reaction mixtures were analyzed by electrophoresis on an SDS-polyacrylamide gel. The ^{32}P-labeled dsRNA products were detected by autoradiography and quantified with a phosphorimager. The amount of dsRNA synthesized in the reaction mixtures preincubated with GTP was considered to be 100%. The sequences of the last 7 nt at the 3′ terminus of the wild-type and mutant gene 8 (+) RNAs are shown, and the mutated nucleotide is underlined.

(−) strand synthesis with the mutant RNA, G8–3C, in the presence of salt (Fig. 4, lanes 6–10). These results demonstrated that the identity of the third residue at the 3′ end of (+) strand RNA dictated which NTP other than GTP was able to support (−) strand synthesis when added to the preincubation mixture.

Synthesis of pGpG and ppGpG by open cores during preincubation

The data raise the possibility that during preincubation of reaction mixtures with GTP, an initiation complex is formed, and subsequently by elongation, the RdRP catalyzes the formation of the first phosphodiester bond of the (−) strand RNA, producing pppGpG. To examine whether the pppGpG was produced during preincubation, open cores were incubated with an individual [α-^{32}P]-NTP and gene 8 (+) strand RNA at 32 °C for 1 h. The reaction mixtures were then analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. Radiolabeled products (bands I–V) were only detected in the mixture containing [α-^{32}P]-GTP (Fig. 5A, lane 1). Although the relative amount of each product varied from experiment to experiment, it usually followed the order of band V > II ≥ III > I ≥ IV. Because GTP was the only NTP in the preincubation mixture, all these products were expected to be derivatives of GTP. To identify these products, the material in bands I–V was gel purified (Fig. 5B), digested with calf intestinal phosphatase (CIP), tobacco acid pyrophosphatase (TAP), or nuclease P1, and then resolved by thin layer chromatography (TLC). CIP removes terminal phosphate groups from nucleic acids and nucleoside phosphates; TAP hydrolyzes pyrophosphate bonds such as those in cap structures and di- or tri-phosphate terminated RNAs, and nuclease P1 cleaves 5′-3′ linked phosphodiester bonds. As shown in Figure 6, band I was resistant to CIP digestion (Fig. 6, lane 8), indicating that there was no terminal phosphate groups in the product. Digestion of band I with TAP resulted in two products which comigrated with pGpG and GMP (Fig. 6, comparing lane 9 with lanes 6 and 4). Nuclease P1 cleaved band I into two products, which comigrated with GpppG and GMP (Fig. 6, comparing lane 10 with lanes 2 and 4).

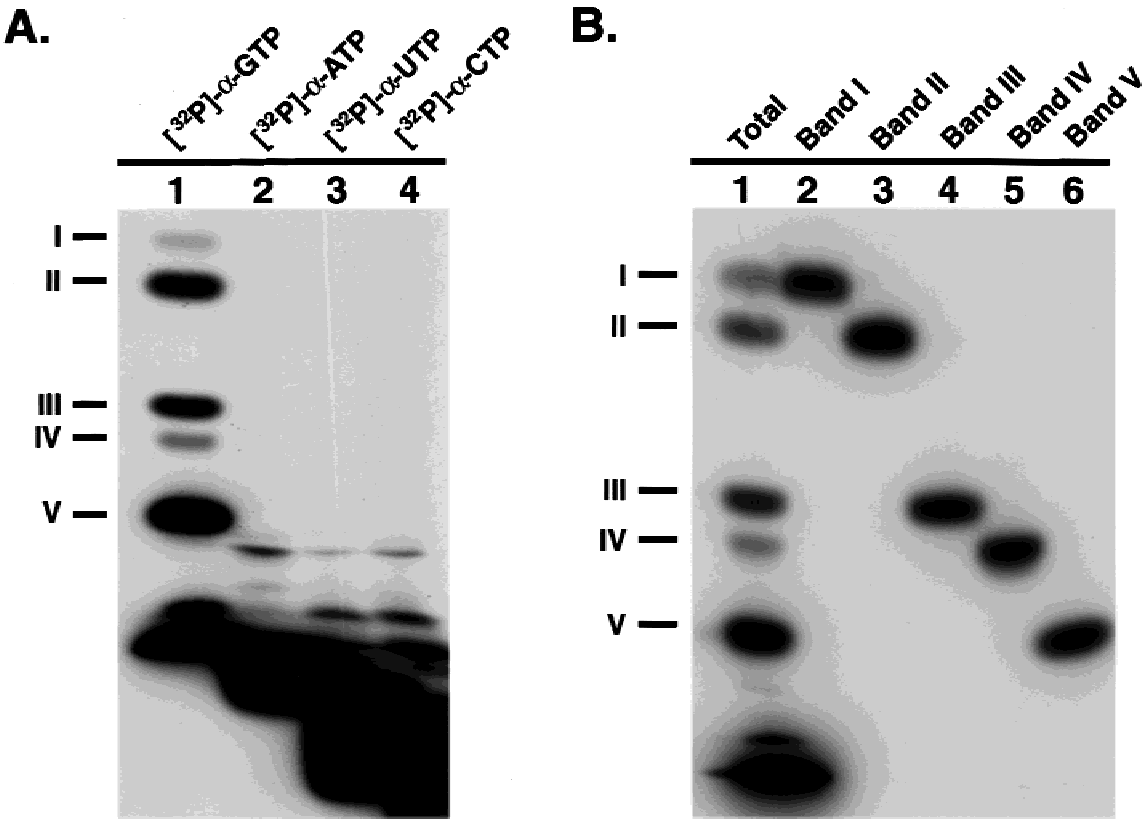

FIGURE 5. Products synthesized during preincubation. **A:** Preincubation mixtures (100 μL) that contained open cores (2.0 μg), gene 8 (+) strand RNA (1.0 μg), an individual NTP (200 μM) and its cognate [α-^{32}P]-NTP (100 μCi) were incubated at 32 °C for 1 h. Following phenol extraction and ethanol precipitation, products were resolved by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. Radiolabeled bands were detected by autoradiography. **B:** ^{32}P-labeled products were eluted by soaking gel slices corresponding to bands I–V (lane 1 of **A**) in RNase-free water overnight at 37 °C, and were recovered by ethanol precipitation. The gel-purified products (~1,000 cpm) were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. The radioactive bands were detected by autoradiography.

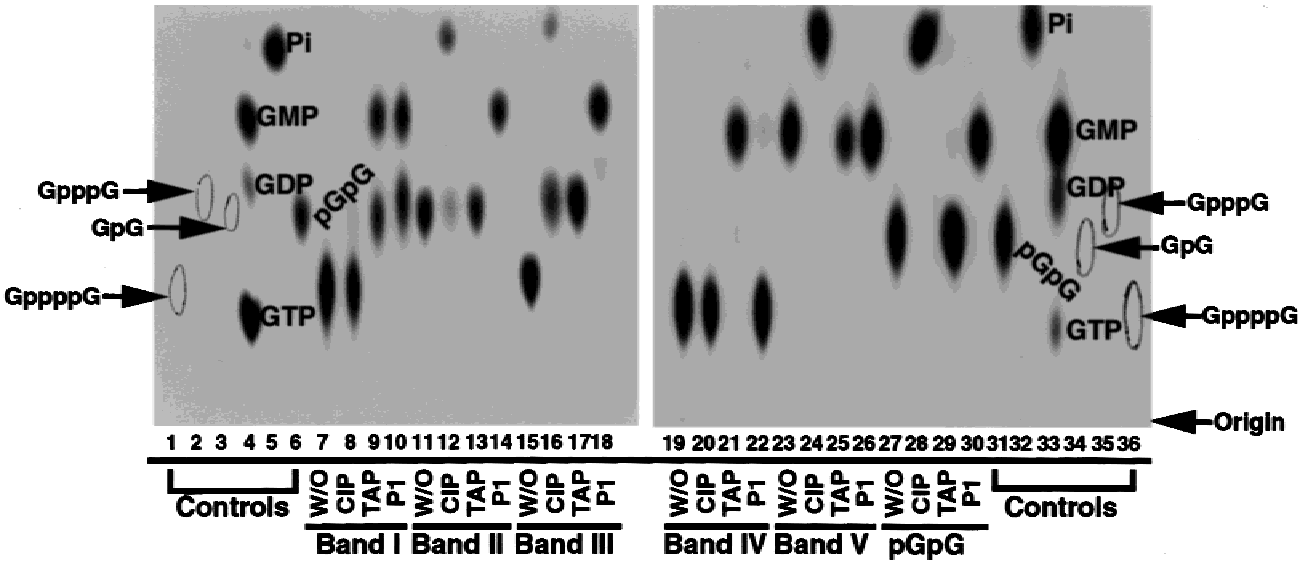

FIGURE 6. Biochemical characterization of bands I–V. Gel purified bands I–V (~2,000 cpm each) along with end-labeled ^{32}pGpG were mock-treated (W/O) or treated with CIP, TAP, or nuclease P1. Following phenol-chloroform extraction, the digested samples were analyzed by TLC using PEI-cellulose sheets, and the radiolabeled products were detected by autoradiography. The positions of the nonradiolabeled controls GppppG, GpppG, and GpG were identified using 254 nm UV light.

When combined, these results indicated that band I had the structure of GpppGpG. By similar enzymatic analysis, bands II, III, IV, and V were identified as pGpG, ppGpG, GppppG, and GMP, respectively (Fig. 6, and Table 1) and the primary polymerization products made during preincubation with GTP were shown to be pGpG (band II) and ppGpG (band III) (Fig. 5 and Table 1).

To determine (1) whether the synthesis of pGpG and ppGpG was template dependent and (2) whether the synthesis of these dinucleotides was salt sensitive, ^{32}P-GTP and open cores were incubated in the presence and absence of gene 8 (+) strand RNA and of 200 mM NaCl. The products of the reaction mixtures were then analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. As shown in Figure 7, both ppGpG and pGpG were made in reaction mixtures lacking gene 8 (+) RNA (comparing lanes 1 and 2 with lanes 3 and 4), suggesting that these dinucleotides were synthesized by a template-independent mechanism. Although the presence of NaCl did not affect the synthesis of ppGpG, it did cause a 50% reduction in the production of pGpG and a 50% increase in the production of GpppGpG (Fig. 7, comparing lanes 1 and 3 with lanes 2 and 4).

Specific priming of (–) strand synthesis by the dinucleotide pGpG

The data from above indicate that the inhibition of (−) strand synthesis is not due to the failure of open cores to make pGpG and ppGpG in the presence of NaCl. Instead, a more likely scenario is that the presence of NaCl prevented open cores, gene 8 (+) strand RNA, and pGpG or ppGpG from interacting to form a stable initiation complex. To test the possibility that pGpG can promote the formation of the (−) strand initiation com-

TABLE 1. Radiolabeled products made during preincubation with [^{32}P]-α-GTP

Band	Relative radioactivity	Products of enzymatic digestion[a]			Identity
		CIP	TAP	P1	
I	4	R	S[pG + pGpG]	S[pG + GpppG]	GpppGpG
II	21	S[Pi + GpG]	R	S[pG]	pGpG
III	14	S[Pi + GpG]	S[pGpG]	S[pG + ppG]	ppGpG
IV	4	R	S[pG]	R	GppppG
V	57	S[Pi]	R	R	pG

[a]R and S indicate resistant and sensitive, respectively, to enzymatic digestion. Products of digestion are given in brackets.

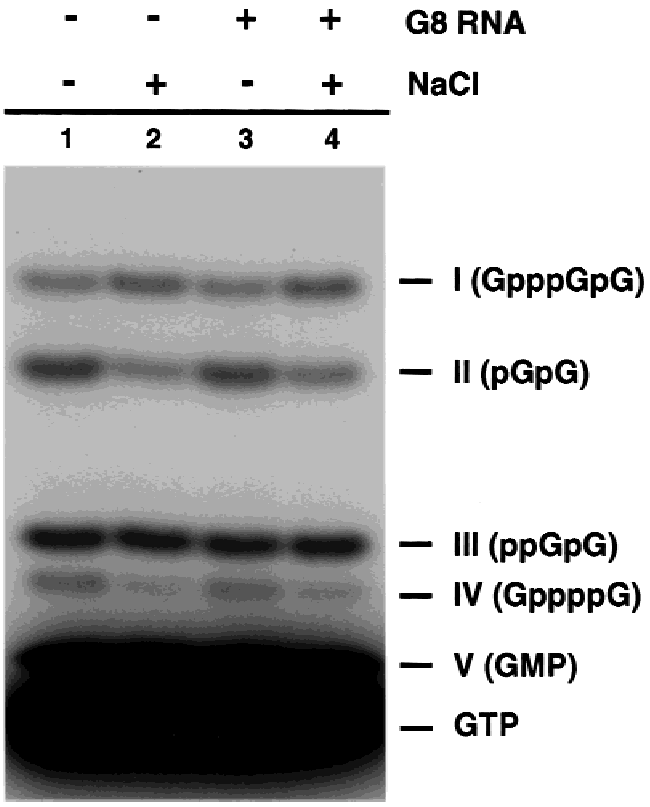

FIGURE 7. Effect of gene 8 (+) strand RNA and salt on the production of the dinucleotide, pGpG. Open cores (0.4 μg) were incubated with GTP (200 mM) and [α-^{32}P]-GTP (10 μCi) in the presence or absence of gene 8 (+) strand RNA (0.2 μg), or 200 mM NaCl, or both at 32 °C for 1 h. Reaction mixtures were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. Products were detected by autoradiography.

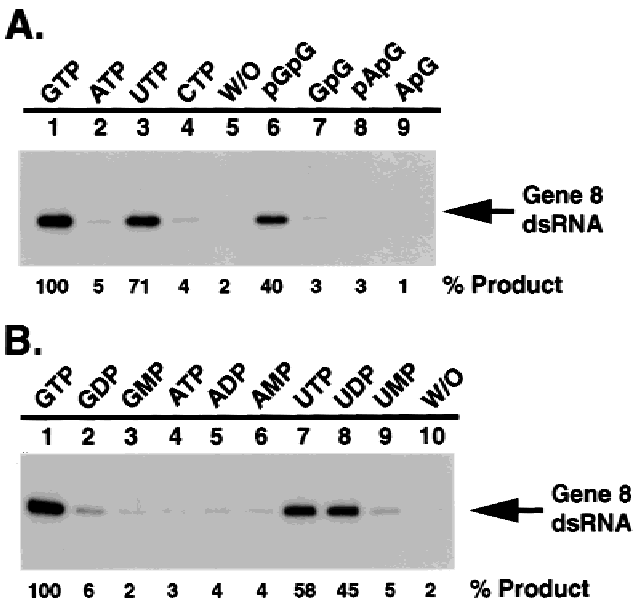

FIGURE 8. Initiation of (−) strand synthesis with pGpG and UDP. **A:** An individual NTP (200 μM) or dinucleotide (200 μM) was incubated with open cores (0.4 μg) and gene 8 (+) strand RNA (0.2 μg) in a final volume of 20 μL at 32 °C for 1 h. Afterwards, NaCl, the four NTPs, and ^{32}P-UTP were added to the preincubated mixtures in sequential order, and the reaction mixtures were then incubated at 32 °C for 1 h. The ^{32}P-labeled dsRNA products were resolved by electrophoresis on an SDS-polyacrylamide gel, detected by autoradiography, and quantified with a phosphorimager. The amount of dsRNA synthesized in the reaction mixture preincubated with GTP was considered to be 100%. **B:** A single NTP, NDP, or NMP (200 μM) was incubated with open cores (0.4 μg) and gene 8 (+) strand RNA (0.2 μg) in a final volume of 20 μL at 32 °C for 1 h. Afterwards, NaCl, the four NTPs, and ^{32}P-UTP were added to the preincubated mixtures in sequential order and the reaction mixtures were incubated at 32 °C for 1 h. The ^{32}P-labeled dsRNA products were resolved by electrophoresis on an SDS-polyacrylamide gel, detected by autoradiography, and quantified with a phosphorimager. The amount of dsRNA synthesized in the reaction mixtures preincubated with GTP was considered to be 100%.

plex, 200 μM pGpG was preincubated with open cores and gene 8 (+) strand RNA in the absence of any individual NTP at 32 °C for 1 h prior to the addition of NaCl, the four NTPs, and [α-^{32}P]-UTP. As shown in Figure 8A, preincubation of pGpG with open cores and gene 8 (+) strand RNA allowed the synthesis of dsRNA to occur in the presence of NaCl (Fig. 8A, lane 6). This result indicates that pGpG supported the formation of a functional initiation complex. In contrast, little dsRNA product was detected in the reaction mixtures that were preincubated with GpG, pApG, or ApG (Fig. 8A, lanes 7, 8, and 9, respectively). The fact that pGpG supported the formation of an initiation complex, but GpG did not, suggests that 5′-terminal phosphate moieties are critical to this event. Because ppGpG is not commercially available and could not be easily produced by ourselves in large quantity, similar experiments were not carried out with this phosphorylated form of the dinucleotide.

When preincubated with open cores and gene 8 (+) strand RNA, GTP and UTP supported (−) strand synthesis in the presence of NaCl, suggesting that (−) strand synthesis can initiate in vitro either at the terminal C (−1) or at the A residue at −3 of the RNA template (Fig. 3B). To gain additional insight into the importance of the 5′-terminal phosphates of guanosine and uridine in promoting (−) strand initiation, reaction mixtures containing open cores, gene 8 (+) strand RNA, and mono-, di-, or tri-phosphorylated nucleosides were preincubated at 32 °C for 1 h. After adding NaCl, the four NTPs, and [α-^{32}P]-UTP, the reaction mixtures were incubated at 32 °C for 1 h and then analyzed for dsRNA products by electrophoresis. The results showed that replacing UTP with UDP in the preincubation mixture had no significant effect on the synthesis of dsRNA in NaCl (Fig. 8B, lanes 7 and 8), indicating that UDP could interact with open core proteins and the RNA template to form a stable initiation complex. Because UDP is not a known substrate for the synthesis of (p)pUpU, these data suggested that a dinucleotide derivative of UTP is probably not involved in the formation of a (−) strand initiation complex. In contrast to the results obtained with UDP, preincubation of open cores and gene 8 (+)

strand RNA with GDP instead of GTP caused a tenfold reduction in dsRNA synthesis (Fig. 8B, lanes 1 and 2). This implied that either the γ-phosphate of GTP is necessary for the nucleotide to become part of the initiation complex or that a derivative product of GTP, such as (p)pGpG, is necessary for the formation of the initiation complex. The former seems unlikely because pGpG, which lacks both β- and γ-phosphate moieties, can promote the formation of initiation complexes. When GMP or UMP was preincubated with open cores and gene 8 (+) strand RNA, little dsRNA was made in these reaction mixtures when NaCl was subsequently added (Fig. 8B, lanes 3 and 9). Taken together, these results provide evidence that pGpG or ppGpG, rather than GTP, interacts with open cores and gene 8 (+) strand RNA to form an initiation complex that starts from the terminal C residue.

As a direct test of whether the role of pGpG in the formation of an initiation complex was to serve as a primer for (−) strand synthesis, open cores, gene 8 (+) strand RNA, the four NTPs, and ^{32}P-GpG, ^{32}P-ApG, or [α-^{32}P]-GTP were incubated at 32 °C for 2 h. Half of the dsRNA products recovered from the reaction mixtures was treated with CIP at 37 °C for 1 h and the other half was mock treated. Both the CIP- and mock-treated dsRNA products were then analyzed by gel electrophoresis. As shown in Figure 9, silver staining of the gel revealed that dsRNA products were made in all three reactions (lanes 4, 5, and 6, left panel). Autoradiography of the stained gel showed that ^{32}P-GpG was incorporated into dsRNA products (Fig. 9, lane 6, right panel), whereas ^{32}P-ApG was not (Fig. 9, lane 5, right panel). In addition, the ^{32}P moiety of the ^{32}P-GpG-labeled dsRNA was completely removed by CIP treatment (Fig. 9, lane 3), whereas the ^{32}P moiety of the [α-^{32}P] GTP-labeled dsRNA was largely resistant to CIP treatment (Fig. 9, lane 1). These results demonstrate that the dinucleotide ^{32}P-GpG served as a primer for (−) strand synthesis and was not incorporated internally into the newly synthesized (−) strand RNA.

DISCUSSION

The replicase activity of the open core replication system is significantly inhibited by the presence of even low concentrations of NaCl (Fig. 1A). Similar inhibitory effects were also observed when NaCl was replaced with other monovalent salts such as Na^+, K^+, and Cs^+ of both chloride and acetate forms (data not shown). In an attempt to understand the mechanism of this inhibition, we tested whether it was possible to overcome the effect of salt by preincubating selected components of the replication reaction mixture prior to the addition of NaCl. The results showed that preincubation of open

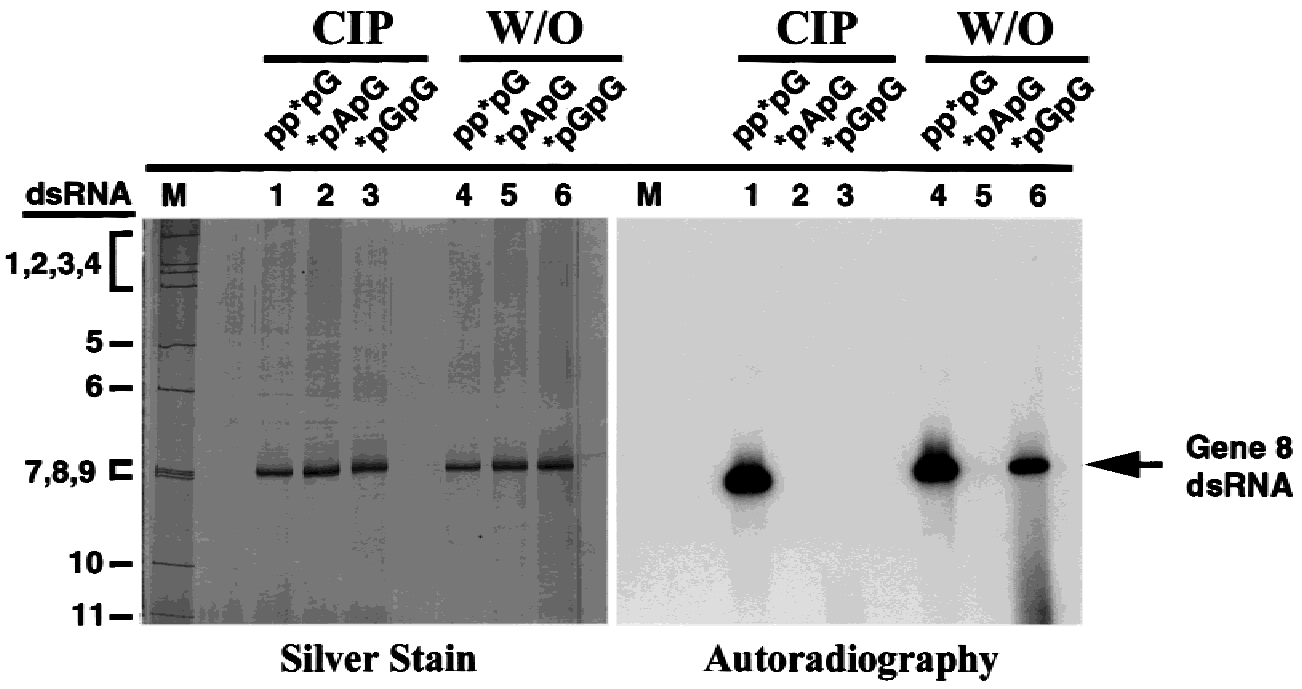

FIGURE 9. Specific priming of (−) strand synthesis by pGpG. Reaction mixtures (100 μL) containing gene 8 (+) strand RNA (1.0 μg), open cores (2.0 μg), the four NTPs (500 μM of each), and [α-^{32}P]-GTP, ^{32}P-ApG, or ^{32}P-GpG were incubated at 32 °C for 2 h. Following phenol-chloroform extraction, the dsRNA products were recovered by ethanol precipitation. Half of the dsRNA products was treated with 30 U of CIP at 37 °C for 1 h, and the other half was mock treated. The samples were then analyzed by electrophoresis on a 12% polyacrylamide gel containing 0.1% SDS, and the dsRNA bands were visualized by silver staining. The radiolabeled dsRNAs were detected by autoradiography. The position of radiolabeled phosphate groups are indicated with *. M: genomic dsRNA markers.

cores, viral (+) strand RNA, and an NTP mixture consisting of ATP, CTP, and GTP allowed the synthesis of (−) strand synthesis to take place in the presence of high concentrations of salt (Fig. 3A). The fact that (−) strand synthesis did not occur in the presence of NaCl when any one of these three basic components were deleted from the preincubation mixture indicated that the formation of a functional ternary initiation complex for (−) strand synthesis is a salt-sensitive process that requires the interaction of the RdRP, the viral (+) strand RNA, and NTPs.

Analysis of the replication products made when open cores and viral template RNA were preincubated with individual NTPs revealed that both GTP and UTP were able to support the formation of the initiation complex for (−) strand synthesis, but that ATP and CTP were not. The ability of GTP alone in the preincubation mixture to support this process was expected given that the (+) strand template for dsRNA synthesis ends with the sequence, 5′-UGUGACC-3′, and therefore positions +1 and +2 of the (−) strand product would require G residues. However, it was surprising to find that when UTP was preincubated with open cores and template RNA, the reaction mixture supported (−) strand synthesis in the presence of NaCl. Mutational analysis indicated that in the presence of UTP, the RdRP of open cores initiated (−) strand synthesis from the A residue at the third position (−3) upstream from the 3′ end of the (+) strand RNA. Remarkably, the analysis suggested that the RdRP could initiate (−) strand synthesis regardless of the residue that was present at the −3 position, as long as the nucleotide complementary to that residue was added to the preincubation mixture. That is when the residue at the −3 position was mutated from an A → U or an A → G, the RdRP was able to initiate (−) strand synthesis from the −3 position as long as ATP or CTP, respectively, was included in the preincubation mixture. Because preincubation with open cores, the wild-type template RNA, and either ATP or CTP failed to support dsRNA synthesis in the presence of NaCl, the viral RdRP apparently cannot initiate (−) strand synthesis at positions, for example, −4 and −5, that are upstream of −3 in the template RNA. While it may be possible that the RdRP can initiate (−) synthesis at the −3 position in vitro, sequence analysis has indicated that the 5′ end of dsRNAs purified from rotavirus virions begins with (p)ppGpG and, thus, there is no direct evidence that internal initiation occurs from this site during (−) strand synthesis in vivo.

Analysis of the products made during preincubation with open cores, the RNA template, and individual radiolabeled NTPs showed that the presence of ^{32}P-GTP resulted in the formation of pGpG, ppGpG, GppppG, and GpppGpG, and GMP. No corresponding products were made when radiolabeled ATP, CTP, or UTP were included in the preincubation mixture instead of GTP. These results suggest that a GTP-binding protein, and not a nonspecific NTP-binding protein, is involved in the formation of the GTP-derivative products. Open cores contain VP3, a multifunctional capping enzyme that binds GTP specifically as part of its guanylyltransferase activity. Previous studies have shown that the guanylyltransferase activity of VP3 is functional in the open core replication system and, in the presence of GTP, will add caps in a nonspecific manner to single-stranded RNAs (Chen et al., 1999). Because of its guanylyltransferase activity, the formation of two of the products, GppppG and GpppGpG, likely derives from VP3 transferring a GMP cap to GTP and to ppGpG. While it is reasonable to assume that VP1 is responsible for forming the phosphodiester bonds of pGpG, ppGpG, and GpppGpG, VP3 also contains motifs common to other known RNA polymerases (Mitchell & Both, 1990), raising the possibility that VP3 may instead be responsible for catalyzing formation of the bonds.

We anticipated that the synthesis of pGpG and ppGpG would represent a template-dependent process requiring the addition of viral (+) strand RNA to the preincubation reaction mixture. However, these products were made even when the (+) strand RNA was excluded from the reaction mixture, suggesting that these products were in fact made by a template-independent process. Indeed, an alternative source of the template for the synthesis of pGpG and ppGpG may have been residual dsRNA or RNA fragments that remained in the open core preparation, despite treatment of the preparation with micrococcal nuclease to hydrolyze the endogenous genomic dsRNAs. But it seems unlikely that RNA in the open core preparation served as a template for these products given that open cores derived from empty virus particles, which lacked detectable dsRNA, also efficiently catalyzed the synthesis of pGpG and ppGpG (data not shown). From these data, we conclude that the phosphodiester bonds in the pGpG, ppGpG, and GpppGpG products of preincubation are probably not formed by the same template-dependent process by which RdRPs form such bonds during elongation.

Two lines of evidence indicated that pGpG and, possibly ppGpG, are components of initiation complexes formed to initiate (−) strand synthesis: (1) Preincubation of pGpG, (+) strand RNA, and open cores in the absence of any NTPs allowed (−) strand synthesis to occur in the presence of NaCl. (2) When ^{32}P-labeled pGpG is included in the open core replication system, the dinucleotide was incorporated into the (−) strand RNA product, but only at the 5′ terminus. The role of pGpG in initiation is specific, as substitution of it with pApG during preincubation did not result in the synthesis of (−) strand RNA when NaCl and all the NTPs were subsequently added to the reaction mixture. Notably, inclusion of GMP or GDP in the preincubation reaction mixture instead of GTP or pGpG also did not result in (−) strand synthesis when salt was subsequently added,

suggesting that only when the substrate is present for catalysis of a guanosine dinucleotide (e.g., GTP → pGpG), can assembly of a salt-stable initiation complex take place. Although the presence of pGpG supported the formation of the (−) strand initiation complex, GpG did not, reflecting the apparent importance of the 5′-phosphate moiety in the formation of this complex during rotavirus RNA replication. Interestingly, dinucleotides have also been shown to serve as primers for RNA synthesis in cell-free replication systems that have been developed for influenza virus (Plotch & Krug, 1977), QB bacteriophage (Blumenthal, 1980) and brome mosaic virus (Kao & Sun, 1996). However, in these systems, the presence of a 5′-phosphate moiety on the dinucleotide is not required for priming RNA synthesis. Importantly, in these other systems, there is also no evidence that viral proteins are capable of synthesizing a specific dinucleotide.

Previous studies have indicated that complementary sequences at the 5′ and 3′ ends of rotavirus (+) strand RNA template interact to form a panhandle-type structure. Deletion of these complementary sequences significantly inhibits the ability of the RNA template to serve as a template for (−) strand synthesis in the open core replication system. Because the complementary sequences differ for each of the 11 viral (+) strand RNAs, it must be the structure formed by the complementary sequences and not their primary sequence that contributes to optimal (−) strand synthesis. The 3′-consensus sequence, 5′-UGUGACC-3′, of the (+) strand RNAs extends as a short un-base paired tail from the 5′-3′ panhandle structure. If the RNA is mutated such that the 3′-consensus sequence is fully base paired to the 5′ terminus, the RNA no longer serves as a template for (−) strand synthesis in vitro, suggesting a defect in initiation (Chen & Patton, 1998). Furthermore, if the terminal CC residues of the RNA template are deleted or are mutated, the synthesis of the (−) strand RNA becomes inefficient (Patton et al., 1996). Based on mutagenesis experiments and comparison of the sequences of the 11 viral (+) strand RNAs, the only sequence-specific requirement for initiation of rotavirus (−) strand synthesis appears to be the presence of 3′-terminal CC residues. The contribution of the 5′-3′ panhandle and other secondary structures within the (+) strands RNA is probably to ensure that the initiation site is sterically accessible and is placed in appropriate structural context opposite the 5′ end of the RNA template. This latter point may be critical for efficient initiation from the terminal CC residues, particularly, if VP3, a capping enzyme that can interact with the 5′ end of an RNA, is involved with (−) strand initiation. The N-terminus of VP2 has affinity for single-stranded RNA, and VP1 and VP3 interact with VP2 and colocalize at channels situated at the five-fold axes of the VP2 core (Labbe et al., 1994; Prasad et al., 1996; Lawton et al., 1997). Thus, VP2 may also be involved in the formation of initiation complexes by forming a platform on which the necessary protein and RNA components of the complex can collect and interact. In fact, such a function of VP2 may explain why the protein is essential for dsRNA synthesis in vitro and is a component of all replication intermediates with replicase activity in vivo (Mansell & Patton, 1990; Patton et al., 1997).

The mechanism by which salt prevents the formation of a functional (−) strand initiation complex in the open core replication system is not known. One possibility is that salt increases the stability of the 5′-3′ panhandle or causes other structural changes in the (+) strand RNA template, making it more difficult for the viral single-stranded RNA-binding proteins to interact to form a ternary initiation complex. Indeed, neither VP1 nor VP3 has affinity for dsRNA and the affinity of VP2 for dsRNA is less than that for single-stranded RNA (Patton & Chen, 1999); thus any changes in the stability or the extent of secondary structure in the template RNA could significantly impact the ability of these proteins to become part of an initiation complex. In contrast to what is observed in vitro, salt may not have any real impact on the formation of initiation complexes in vivo because the nonstructural proteins, NSP2 and NSP5, are components of replication intermediates with replicase activity in the infected cells (Gallegos & Patton, 1989). In particular, NSP2 is an NTPase with helix destabilizing activity and the protein may assist in the formation of initiation complexes by disrupting base pairing that impedes the necessary binding of VP1, VP2, and/or VP3 to the (+) RNA template (Taraporewala et al., 1999; Taraporewala & Patton, unpubl. results).

MATERIALS AND METHODS

Preparation of open cores

The VP7 segment of reassortant virus RRVxDS1 is derived from human rotavirus DS1 and the rest of the segments are from rhesus rotavirus RRV (Midthun et al., 1985). RRVxDS1 was grown in MA104 cells and purified by two rounds of CsCl gradient centrifugation. Open cores were prepared from purified virions, and the endogenous genomic dsRNA were removed by micrococcal nuclease as previously reported (Chen & Patton, 1998).

Preparation of DNA templates for T7 transcription

PCR was used to prepare the wild-type and mutant gene 8 DNA templates for T7 transcription. The plasmid SP65g8R (Patton et al., 1996), which contains a full-length gene 8 cDNA of simian rotavirus SA11, was digested with *Bam*HI and *Hin*dIII to release the cDNA insert. The released gene 8 cDNA fragment was gel purified with a Qiaex II kit (Qiagen) and used as the template for PCR amplification following the exact protocol described earlier (Chen & Patton, 1998). To generate the wild-type gene 8 DNA template, the plus sense primer T7G8/+

(5′-agataatacgactcactataGGCTTTTAAAGCGTCTC-3′) and the minus sense primer G8/− (5′-GGTCACATAAGCGCTTTCTATTCTTGC-3′) were used in the PCR amplification. Viral-specific sequences are indicated in upper case and the T7 promoter sequence in the primer T7G8/+ is underlined. To produce the DNA templates for mutant RNAs G8–3U, G8–3C, and G8–3G, the primer pairs T7G8/+ and G8/3a (5′-GGaCACATAAGCGCTTTCTATTCTTGC-3′), T7G8/+ and G8/3g (5′-GGgCACATAAGCGCTTTCTATTCTTGC-3′), and T7G8/+ and G8/3c (5′-GGcCACATAAGCGCTTTCTATTCTTGC-3′), respectively, were used in PCR amplification. The single nucleotide substitution at the third position upstream from the 3′ end of gene 8 (+) strand RNA is indicated by the lower case letter in the primers G8/3a, G8/3g, and G8/3c. The amplified products were purified by phenol-chloroform (1:1, pH 8.0) extraction and ethanol precipitation. The unincorporated primers and free dNTPs were removed by two passages of the samples through Sephadex G50 spin columns.

Preparation of T7 transcripts

The wild-type and mutant gene 8 (+) strand RNAs were synthesized using a MEGAscript kit (Ambion) according to the protocol recommended by the manufacturer. To minimize the carry-over of unincorporated NTPs in the final products, the RNA samples were passed three times through Sephadex G-50 columns with isopropanol precipitations included between passages. The quality of the RNA transcripts was assessed by electrophoresis on 1% agarose gels containing 2% formaldehyde (Sambrook et al., 1989). RNA concentrations were determined spectrophotometrically.

Open core replication assay

Replication assays were performed as described previously (Chen & Patton, 1998). The standard reaction mixture contains 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 5 mM DTT, 20 U of RNasin (Promega), 200 μM each of the four dNTPs (Life Technologies), 0.2 μg of gene 8 (+) strand RNA, 0.4 μg of open cores, and 10 μCi [α-^{32}P]-UTP (800 Ci/mmol, NEN) in a final volume of 20 μL. Assuming that a core contains 12 functional replicase complexes (1 for each of the 12 vertices of the core), then the calculated ratio of gene 8 (+) strand RNA:replicase complexes in the reaction mixture was 20:1. Thus, the concentration of template RNA was in vast excess over the concentration of replicase complexes in the assay. Reaction mixtures were incubated at 32 °C for 30–120 min. To examine the effect of salt on (−) strand synthesis, NaCl was added to reaction mixtures to a final concentration of 25–1,000 mM. The dsRNA products were analyzed by electrophoresis on a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and were detected by autoradiography. In some cases, a phosphorimager was used to quantify the amount of dsRNA products detected on gel.

In attempting to overcome the inhibitory effect of NaCl on (−) strand synthesis, a preincubation step was introduced into the standard open core replication assays. The components of the preincubation reaction mixtures were the same as the standard reaction mixtures except that one or more NTPs were omitted. Unless otherwise indicated, preincubation reactions were carried out at 32 °C for 1 h. After sequential addition of NaCl, the omitted NTP(s), and [α-^{32}P]-UTP, the reaction mixtures were incubated at 32 °C for an additional 1 h.

Kinetic analysis of (–) strand RNA synthesis

Three identical preincubation mixtures (180 μL each in volume) were assembled on ice, and they contained 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 5 mM DTT, 200 U of RNasin, 200 μM each of the four NTPs, 1.0 μg of gene 8 (+) strand RNA, 2.0 μg of open cores, and 90 μCi [α-^{32}P]-UTP. All three reaction mixtures were then incubated at 32 °C for 3 h. NaCl was added to a final concentration of 200 mM to two of the three reactions at 1 or 2 h after beginning the incubation at 32 °C. No NaCl was added to the remaining reaction mixture. At indicated times, aliquots (10 μL) were taken from each of the three reaction mixtures and combined immediately with an equal volume of 2× SDS sample buffer (Laemmli, 1970). All three sets of samples were analyzed by gel electrophoresis and the level of dsRNA products were quantified with a phosphorimager.

Purification and characterization of the products synthesized during preincubation

To determine if any products were synthesized by open cores in preincubation mixtures supplied with only a single NTP, reaction mixtures were incubated at 32 °C for 60 min. The components of the reaction mixtures included 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 5 mM DTT, 200 U of RNasin, 200 μM of an individual NTP (GTP, ATP, UTP, or CTP), 100 μCi of the cognate [α-^{32}P]-NTP, 1.0 μg of gene 8 (+) strand RNA, and 2.0 μg of open cores. Following phenol extraction and ethanol precipitation, products were resolved by gel electrophoresis on a 20% polyacrylamide gel containing 7 M urea. The products were visualized by autoradiography and recovered by soaking the gel slices in RNase-free water overnight at 37 °C. The gel purified products (~2,000 cpm) were incubated in a final volume of 20 μL with 10 U of CIP in 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, or 10 U of TAP in 50 mM sodium acetate (pH 6.0), 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.01% Triton X-100, or 4 μg of nuclease P1 in 25 mM sodium acetate (pH 6.2), 2.5 mM $MgCl_2$ at 37 °C for 1 h. Following phenol-chloroform extraction, samples (0.5 μL) were spotted directly onto PEI-cellulose F sheets (EM Science) and analyzed by TLC. The sheets were placed vertically in a sealed tank containing 100 mL 1.2 M LiCl and developed at room temperature for 1 h. The positions of digestion products were identified by autoradiography. The cap analogs (Pharmacia), GpppG and Gpppp G, and the dinucleotide GpG (Sigma), were included as markers and their positions on cellulose sheets were determined using UV light (254 nm).

Specific priming of (–) strand RNA synthesis by pGpG

The dinucleotides, GpG and ApG (Sigma), were 5′-end-labeled in 100 μL reaction mixtures that contained 70 mM

Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol, 20 U of T4 kinase (Life Technologies), 250 μM ATP, 250 μM of GpG or ApG, and 200 μCi [γ-^{32}P]-ATP (3,000 Ci/mmol, NEN). The reaction mixtures were incubated at 37 °C for 1 h followed by phenol-chloroform (1:1, pH 4.7) extraction and ethanol precipitation at −70 °C for 2 h. Phosphorylated pGpG and pApG were recovered by centrifugation at 14,000 rpm for 30 min at 4 °C and pellets were dissolved in 40 μL RNase-free water. Residual ATP was not removed from the products.

Radiolabeled ^{32}P-GpG or ^{32}P-ApG (final concentration approximately 150 μM) was added to 100 μL replication mixtures containing 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 5 mM DTT, 100 U of RNasin, 500 μM each of the four dNTPs, 1.0 μg of gene 8 (+) strand RNA, and 2.0 μg of open cores. The reaction mixtures, along with a control reaction that contained 50 μCi [α-^{32}P]-GTP (800 Ci/mmol, NEN) instead of ^{32}P-GpG or ^{32}P-ApG, were incubated at 32 °C for 2 h. Following phenol-chloroform extraction and ethanol precipitation, the samples were passed twice through Sephadex G-50 columns to remove unincorporated ^{32}P-GpG, ^{32}P-ApG, or [α-^{32}P]-GTP. Each sample was then split into two equal portions. One set of samples was digested with 30 U of CIP (NEB) at 37 °C for 1 h, and the other set was mock digested. After phenol-chloroform extraction and ethanol precipitation, the samples were analyzed on a 12.5% polyacrylamide gel containing 0.1% SDS. The dsRNA were visualized by silver staining, and the radiolabeled bands were detected by autoradiography.

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