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Mechanism of In Vitro Synthesis of Covalently Linked Dimeric RNA Molecules by the Poliovirus Replicase

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Four RNA fragments of approximately 1,000 to 1,200 nucleotides, representing both the 5′ and 3′ termini of poliovirus plus- and minus-strand RNAs, were generated by transcription of poliovirus cDNA by using bacteriophage SP6 RNA polymerase. The copying of these templates by the poliovirus replicase invariably produced RNA products approximately twice the size of the templates. In experiments with templates uniformly labeled with ^{32}P it was shown that some of the apparently double-length products were generated by extension from an internal site of the template. Filter hybridization of the labeled in vitro-synthesized products with various unlabeled templates suggested a second mechanism by which double-length molecules could be synthesized; the results can be best explained by de novo synthesis of the first strand by copying of the template RNA, followed by snap-back of the newly synthesized RNA, generating a template-primer structure for the synthesis of the second strand. Highly purified poliovirus replicase was able to support the synthesis of double-length RNA products in response to these templates. These reactions did not require host factor. In contrast, synthesis of genome-length copies of poliovirion RNA by the same replicase was absolutely dependent on added host factor. The synthesis of double-length RNA products did not require either the 3′-terminal poly(A) of plus RNA or sequences within the 3′ termini of both plus- and minus-strand RNAs.

Replication of the single-stranded, positive-polarity (plus) poliovirus RNA genome proceeds through the formation of a complementary (minus) RNA which is used as the template for the synthesis of progeny viral RNAs (3). In vitro synthesis of genome-length copies of minus-strand RNA from virion RNA template requires a virus-coded RNA-dependent RNA polymerase (replicase) and a host cell protein (host factor) (10). The host factor is believed to be involved in the initiation of RNA synthesis (5, 8, 10) whereas the viral RNA polymerase is thought to elongate already initiated RNA chains (4, 11, 20, 23). We have recently shown that the host factor is a 67,000-dalton phosphoprotein which possesses an autophosphorylation activity (17). The purified protein also phosphorylates the smallest subunit (α) of eucaryotic protein synthesis initiation factor 2 in vitro, suggesting that the normal function of this protein in uninfected cells may be related to regulation of protein synthesis (17). Although it is not clear how host factor catalyzes initiation of viral RNA synthesis, we believe that protein phosphorylation may play a role in the initiation of RNA synthesis. This notion is supported by the fact that hydrolysis of the β-γ bond of ATP is required for initiation of RNA synthesis by the poliovirus replicase (18).

We have recently demonstrated the in vitro synthesis of viral plus strands by the poliovirus replicase from the minus-strand templates generated by transcription of infectious poliovirus cDNA from the SP6 promoter by using bacteriophage SP6 DNA-dependent RNA polymerase (12). The in vitro-synthesized positive-strand RNAs were infectious when transfected into HeLa cells. In vitro synthesis of positive-strand RNA from minus-strand templates required both host factor and the viral RNA polymerase (12).

Initiation of RNA synthesis by the poliovirus replicase is likely to occur at or near the 3′ terminus of template RNA (18). Therefore, small RNA fragments containing intact 3′ termini of both plus and minus strands should, in theory, be able to support the initiation of synthesis of the corresponding strand of RNA. Since functionally competent minus-strand templates can be produced from the SP6 system (12), we reasoned that the generation of small template RNAs corresponding to the 3′ termini of poliovirus plus and minus strands would be useful to study initiation of RNA synthesis in the in vitro system. We therefore generated RNA templates approximately 1,000 to 1,200 nucleotides long, representing the 3′ termini of poliovirus plus- and minus-strand RNAs, by transcription of appropriate poliovirus cDNA constructs with SP6 RNA polymerase. Our assumption was that these 3′-terminal RNA fragments would contain all the necessary information for the proper initiation of RNA synthesis by the poliovirus replicase. Here, we show that copying of these templates by the viral replicase produces RNA products approximately twice the length of the template RNA as opposed to the expected single-standed, template-sized complementary RNA products found previously to be synthesized from poliovirion RNA template (6, 10, 16). Results from template extension experiments with uniformly labeled plus- and minus-strand templates and those from dot blot hybridization studies suggest two mechanisms by which these dimeric RNAs are synthesized. In the first mechanism, the template RNA is extended from an internal site that is probably generated by a nucleolytic cleavage of the template and not by the 3′ OH of the ultimate 3′-terminal nucleotide of the template. A second possible mechanism is the de novo synthesis of the first strand by copying of the template followed by snapback of the resulting first strand, generating a template-primer for the synthesis of the second strand (see Fig. 8B). Results of experiments with SP6-derived plus- and minus-strand templates containing various deletions indicated that neither the 3′-terminal poly(A) tail of the plus strand nor the sequences within the

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3'-termini of plus and minus strands were required for the synthesis of dimeric RNA products.

MATERIALS AND METHODS

All chemicals, unless specifically stated, were purchased from Sigma Chemical Co., St. Louis, Mo. Unlabeled nucleotides were obtained from Calbiochem-Behring, La Jolla, Calif. Poly(U) Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Phosphocellulose was purchased from Whatman, Inc., Clifton, N.J. All radioisotopes were purchased from New England Nuclear Corp., Boston, Mass.

Purification of poliovirus replicase, host factor and poliovirion RNA. Poliovirus replicase was purified by phosphocellulose chromatography (fraction II) as previously described (9). The fraction II replicase was further purified through poly(U) Sepharose 4B either by step elution with 0.25 M KCl (fraction III) or by 50 to 400 mM KCl gradient elution (fraction IV) (10). Further purification of fraction IV replicase involved two passages through poly(U) Sepharose 4B, followed by elution of the enzyme with a 50 to 300 mM KCl gradient from the first column and elution with a 50 to 250 mM KCl gradient from the second column. Peak fractions containing poly(U) polymerase activity were pooled and concentrated with solid sucrose. The concentrated enzyme was dialyzed overnight and stored at −70°C (fraction V) in small portions. The purification of host factor (fraction VII) was described previously (8, 17). Unlabeled poliovirion RNA was prepared by the method of Spector and Baltimore (21).

In vitro synthesis of wild-type and mutant RNA templates with SP6 RNA polymerase. Plasmids used for in vitro transcription with SP6 RNA polymerase were constructed with vectors pSP64 and pSP65 (15) and fragments of poliovirus type I cDNA (19). SP6 transcripts were synthesized as described previously (12). For production of minus- and plus-strand templates representing the 5' end of the poliovirus genome, a *Pst*I-*Nru*I DNA fragment representing nucleotides 1 through 1172 was cloned into the *Pst*I-*Sma*I sites of pSP65 and pSP64, respectively, yielding pSP65(5'Mut) and pSP64(5'Mut). For these studies, a cDNA clone representing the 5'-terminal sequences of the poliovirus genome was used which did not contain oligo(dG)-oligo(dC) tails (G. Kaplan and V. R. Racaniello, manuscript in preparation). The RNAs produced by in vitro transcription of pSP65(5'Mut) and pSP64(5'Mut) are called *Pst*I and *Sst*I templates, respectively, after the enzymes used to linearize the plasmids. For production of minus- and plus-strand templates representing the 3' end of the poliovirus genome, a *Hin*dIII-*Xba*I DNA fragment representing nucleotides from 6516 through the 3' poly(A) of the poliovirus genome was cloned into the *Xba*I-*Hin*dIII sites of pSP65 and pSP64, respectively, yielding pSP65(3' 17) and pSP64 (3' 17). For these constructions, a cDNA clone representing the 3'-terminal sequences of the poliovirus genome was used which did not contain oligo(dG)-oligo(dC) tails (Kaplan and Racaniello, in preparation). The RNAs produced by in vitro transcription of pSP65(3' 17) and pSP64 (3' 17) are called *Hin*dIII and *Xba*I templates, respectively, after the enzymes used to linearize the plasmids. The exact structures of the in vitro-generated templates are as follows: the *Pst*I and *Sst*I templates contain 5'-terminal 24 nucleotides derived from SP6 followed by 1,172 nucleotides of polio-specific sequence. The 3' ends of *Pst*I and *Sst*I templates contain 8 and 15 nucleotides, respectively, of extra nonviral sequences derived from SP6. The *Xba*I template contains 6 nucleotides derived from SP6 at the 5' end, followed by 924 nucleotides of polio-specific sequence joined to 31 adenylyl residues, followed by the 4 3'-terminal nucleotides derived from SP6. The *Hin*dIII template contains 42 nucleotides at the 5' end derived from SP6, followed by 31 uridylyl residues joined to 924 nucleotides of polio-specific sequence. The *Hin*dIII template does not contain any SP6-derived sequence at its 3' end.

Mutated template RNAs were produced from altered versions of the plasmids described above. Plus-strand RNAs representing the 3' terminus of the viral genome and containing different amounts of poly(A) (*dl*A17, *dl*A4, *dl*A0) were produced by synthesizing cDNA from viral RNA with oligonucleotide primers. The primers were constructed to incorporate different amounts of adenylate in the synthesized cDNA, which was made double stranded and substituted for the appropriate fragment in pSP64 (3' 17). This plasmid was also mutagenized by using nuclease BAL 31 to generate RNA templates with internal deletions (*dl*I, *dl*II, *dl*III); in addition, one derivative was constructed by deleting sequences between two restriction sites (*dl*IV). Minus-strand RNAs representing the 5' terminus of the viral genome and containing various deletions were produced from altered versions of pSP65(5'mut). Deletions were introduced into this plasmid by removing DNA sequences with nuclease BAL 31 (*dl*0-20, *dl*0-50, *dl*0-67), or by removing a restriction enzyme fragment (*dl*67-149).

In vitro RNA synthesis with replicase and methylmercuric hydroxide gel electrophoresis. The standard reaction mixture for template-dependent replicase activity contained in 50 μl: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0); 5 mM magnesium acetate; 4 mM dithiothreitol; 10 μg of actinomycin D per ml; 30 mM KCl; 20 μM zinc chloride; 0.2 mM each of ATP, GTP and CTP; 1 to 2 μCi of [α-^{32}P]UTP, 5 μM unlabeled UTP, 1,600 U of RNasin per ml; 200 ng of template RNA; and 2 μg of fraction III replicase. In some experiments, fraction III replicase was replaced by 0.5 μg of fraction V replicase plus 0.03 μg of fraction VII host factor. Incubation was for 1 h at 30°C. The RNA products were precipitated with trichloroacetic acid, collected on membrane filters, and counted in 5 ml of Brays solution.

For analysis of the in vitro product, the phenol-extracted, ethanol-precipitated, labeled RNA was suspended in diethylpyrocarbonate-treated water, denatured with 15 mM methylmercuric hydroxide, and analyzed on a 1.5% agarose gel containing 15 mM methylmercuric hydroxide as described by Bailey and Davidson (2).

S1 nuclease digestion of dimeric products and RNA filter hybridization. Portions of RNA samples were digested in 50-μl reaction mixtures containing 0.28 M NaCl, 0.05 M sodium acetate (pH 5.1), 4.5 mM zinc sulfate, 20 μg of *Escherichia coli* ribosomal RNA per ml, and 250 U of S1 nuclease per ml. Incubation was for 30 min at 30°C, and the reactions were terminated by adding 50 μl of cold 0.3 M sodium acetate (pH 5.1)–20 mM EDTA–0.1% diethylpyrocarbonate–yeast tRNA (25 μg/ml). Samples were then phenol extracted, ethanol precipitated, and analyzed on methylmercuric hydroxide gels. RNA filter hybridization experiments were performed as described by Zinn et al. (25).

RESULTS

Synthesis of double-length RNA molecules by the poliovirus replicase. We have previously shown that in vitro copying of SP6-derived viral minus strands by the poliovirus replicase

results in synthesis of infectious, viral plus strands (12). Since the 5′ and 3′ ends of minus strands derived from the SP6 system contain extra nonviral sequences, this result implies that poliovirus replicase must be able to interact with viral sequences within the template RNA to initiate RNA synthesis. This assumption has led us to examine the copying of small RNA templates (1,000 to 1,200 nucleotides) representing the 3′ termini of poliovirus plus and minus strands. We hoped that these 3′-terminal RNA fragments would be useful in studying the initiation of both plus- and minus-strand RNA synthesis by the poliovirus replicase. Figure 1A illustrates the positions of the 3′-terminal plus-strand (*Xba*I) and minus-strand (*Pst*I) RNA fragments with respect to their locations on the full-length viral plus- and minus-strand RNAs, respectively. The 5′-terminal fragments representing either plus RNA (*Sst*I) or minus RNA (*Hin*dIII) were also synthesized to be used as control RNAs in the replication reaction. It should be noted that most of the SP6 transcripts generated from pSP(5′Mut) cleaved with *Sst*I terminated approximately 800 nucleotides, rather than 1,211 nucleotides, as reported previously (12), from the 5′ end of the DNA. The purified RNAs were used as templates in reaction mixtures containing poliovirus replicase and all other ingredients necessary for RNA replication. The in vitro-synthesized, labeled products were analyzed, after denaturation with methylmercuric hydroxide, on agarose gels containing 15 mM methylmercuric hydroxide. Product RNAs from all four templates were approximately twice the size of the template (Fig. 1B, lanes 1, 3, 5, and 7). Labeled products synthesized from the *Pst*I template also contained a band migrating between single- and double-length RNAs. In addition to the major product of approximately 1,400 nucleotides generated by copying of the *Sst*I template, minor products ranging up to 2,400 nucleotides were detected (Fig. 1B, lane 7). These large fragments most probably resulted from copying of the small amount of full-length, 1,211-nucleotide RNA present in the *Sst*I preparation. The mobility of these dimeric RNA products was unaffected by phenol extraction (Fig. 1) or by proteinase K digestion (data not shown), indicating that these dimeric molecules might have resulted from a covalent linkage between two complementary strands of RNA. The covalent linkage of two complementary strands of RNA would result in the formation of a small single-stranded hairpin loop at the junction between the strands. Treatment with single-strand-specific S1 nuclease should nick the linkage between the strands, generating template-length products (24). Digestion of all four dimeric molecules with S1 nuclease generated approximately template-sized molecules (Fig. 1B, lanes 2, 4, 6, and 8), indicating that the double-length RNA molecules were probably generated by a snap-back mechanism. However, it was not clear from this experiment whether the template snapped back to prime the synthesis of the complementary RNA (cRNA) or the complementary RNA was initiated de novo and the product RNA (cRNA) snapped back on itself to synthesize a second strand. Very little, if any, discrete single-stranded molecules were synthesized from all four templates (Fig. 1B). However, in other experiments, both single- and double-length products were detected (see Fig. 5, lane 5, and Fig. 6). It was surprising to see synthesis of double-length molecules from *Hin*dIII and *Sst*I templates since these 5′-terminal fragments should not conceivably be involved in replication of viral plus- and minus-strand RNAs.

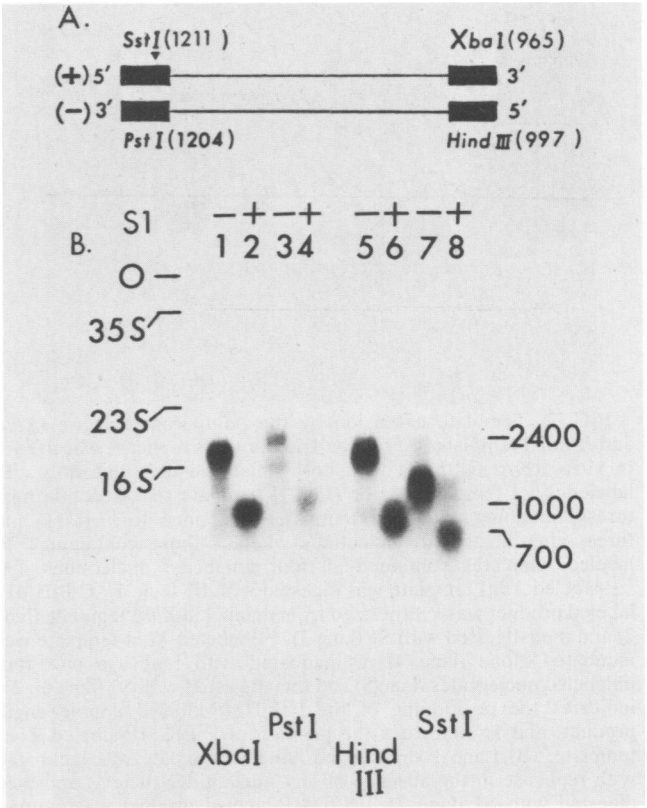

FIG. 1. In vitro synthesis of dimeric RNA by the poliovirus replicase. (A) Locations of the *Xba*I, *Sst*I, *Pst*I and *Hin*dIII RNA templates are shown on poliovirus plus- and minus-strand RNAs. These RNAs were generated by SP6 transcription of pSP64(5′Mut) and pSP65(3′ 17), respectively. Numbers in parentheses indicate the length of the different cDNA fragments used to generate these RNA transcripts from the SP6 promoter with SP6 DNA-dependent RNA polymerase. The approximate position at which transcription of the *Sst*I fragment by SP6 RNA polymerase is terminated is indicated (▼). Thus, the length of most of the *Sst*I RNA is approximately 800 nucleotides. (B) Portions of fraction III poliovirus replicase were incubated with 200 ng each of *Xba*I, *Hin*dIII, and *Sst*I templates and 50 ng of *Pst*I template under the RNA synthesis assay conditions described in Materials and Methods. Labeled products were analyzed on methyl mercury-agarose gels before and after digestion with S1 nuclease as described in Materials and Methods. Lane 1, *Xba*I template, no S1 digestion; lane 2, *Xba*I template, S1 digested; lane 3, *Pst*I template, no S1 digestion; lane 4, *Pst*I template, S1 digested; lane 5, *Hin*dIII template, no S1 digestion; lane 6, *Hin*dIII template, S1 digested; lane 7, *Sst*I template, no S1 digestion; lane 8, *Sst*I template, S1 digested. The migrations of poliovirion RNA (35S) and ribosomal RNAs (23S and 16S) are shown to the left of the gel in panel B. The positions of RNA markers of various sizes (in nucleotides) are shown to the right of the gel.

Template extension by the poliovirus replicase. To examine whether the double-length RNA products synthesized in response to the four RNA templates were due to a snapback followed by extension of the 3′ OH of the templates by the replicase, we generated uniformly ^{32}P-labeled *Xba*I, *Hin*dIII, *Pst*I, and *Sst*I templates by using the SP6 system. These labeled templates were then incubated with the viral replicase in the presence of all four unlabeled nucleotides under the RNA synthesis assay conditions. Parallel reactions with unlabeled RNA templates were run with [α-^{32}P]UTP as the labeled nucleotide and with three unlabeled nucleotides. [^{32}P]UMP-labeled dimeric RNA was synthesized in response to unlabeled *Xba*I template by the replicase (Fig. 2A, lane 2). Digestion of this product with S1

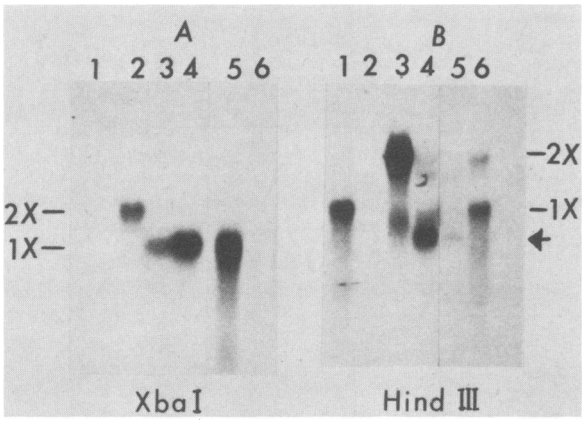

FIG. 2. Template extension by the poliovirus replicase (*Xba*I and *Hin*dIII templates). Fraction III poliovirus replicase was used in in vitro RNA synthesis with both unlabeled and uniformly ^{32}P-labeled *Xba*I template (A) or *Hin*dIII template (B). Reaction mixtures containing unlabeled templates contained [α-^{32}P]UTP and three other unlabeled nucleotides whereas those containing ^{32}P-labeled templates contained all four unlabeled nucleotides. (A) ^{32}P-labeled *Xba*I template was digested with S1 (lane 1). [^{32}P]UMP-labeled product was synthesized from unlabeled *Xba*I template (lane 2) and then digested with S1 (lane 3). ^{32}P-labeled *Xba*I template was incubated alone (lane 4) or incubated with replicase plus four unlabeled nucleotides (lane 5) and then digested with S1 (lane 6). 2× indicates the positioning of the [^{32}P]UMP-labeled double-length product, and 1× indicates the position of [^{32}P]UMP-labeled *Xba*I template. (B) Lane 1, ^{32}P-labeled *Hin*dIII template was incubated with replicase in the absence of any nucleotides (lane 1) and then digested with S1 (lane 2). [^{32}P]UMP-labeled product was synthesized from unlabeled *Hin*dIII template (lane 3) and then digested with S1 (lane 4). [^{32}P]*Hin*dIII template was incubated with replicase plus four unlabeled nucleotides (lane 6), and the product was digested with S1 (lane 5). Arrow indicates the position of the S1-digested [^{32}P]UMP-labeled product.

produced a labeled species (Fig. 2A, lane 3) which comigrated with the ^{32}P-labeled *Xba*I template (lane 4). Incubation of ^{32}P-labeled *Xba*I template with the replicase and unlabeled nucleotides under the RNA synthesis assay conditions did not result in the extension of the labeled template (Fig. 2A, lane 5). Digestion of the labeled *Xba*I template, before or after incubation with the replicase, with S1 completely hydrolyzed all labeled RNAs (Fig. 2A, lanes 1 and 6). These results suggested that under the conditions used for RNA synthesis, the generation of double-length RNA molecules in response to *Xba*I template probably did not result from an extension of the template itself. However, the possibility that only a very small fraction of the templates were being elongated by the replicase, which was beyond detection by this method, could not be ruled out. A significant amount of the ^{32}P-labeled *Hin*dIII template migrated with the double-length RNA product when incubated with poliovirus replicase and unlabeled nucleotides (Fig. 2B, lane 6). Digestion of this material with S1 nuclease generated a labeled species which comigrated with the S1-digested [^{32}P]UMP-labeled product; both of these labeled molecules migrated faster than the untreated ^{32}P-labeled *Hin*dIII template (Fig. 2B, cf. lanes 4 and 5 with lane 1). These results suggested that the majority of double-length RNA products synthesized in response to *Hin*dIII template were indeed a consequence of extension of the template RNA; however, the template must have been extended from an internal site rather than from the 3′ end of the template. In addition, a small fraction of [^{32}P]UMP-labeled product comigrated with the labeled template after digestion with S1 nuclease (Fig. 2B, lane 4), suggesting that another mechanism could be involved in the synthesis of product RNA from *Hin*dIII template.

When *Pst*I (3′-terminal, minus RNA) was used as the template in the replicase reaction (Fig. 3A), the [^{32}P]UMP-labeled product contained double-length molecules in addition to a faster-moving tight band which migrated between single- and double-length RNAs (lane 3). Extension of ^{32}P-labeled *Pst*I template showed that this intermediate molecule was synthesized by extension from an internal sequence within the template since S1 digestion of the extended ^{32}P-labeled *Pst*I template (shown by <) produced labeled species which migrated faster than the labeled template (indicated by arrow). The double-length RNA molecules were probably not synthesized by the same mechanism since no double-length ^{32}P-labeled template molecules could be detected in the reaction mixture (Fig. 3A, lane 6).

The synthesis of double-length RNA product in response to *Sst*I template appeared to be entirely due to extension of the template (Fig. 3B). However, in this particular case, the majority of the labeled template appeared to be degraded first before being extended by the viral replicase (Fig. 3B, cf. lanes 4, 5, and 6). This result was consistent with our previous finding that the size of the S1-digested dimeric RNA synthesized from the *Sst*I template was smaller than the template itself (Fig. 1B, lane 8).

Hybridization of replicase products with different RNAs. The synthesis of exactly dimeric RNA molecules in response to *Xba*I and *Pst*I templates could not be explained by the template extension mechanism (Fig. 2A and 3A). An alternate possibility is that the first RNA strand is synthesized by

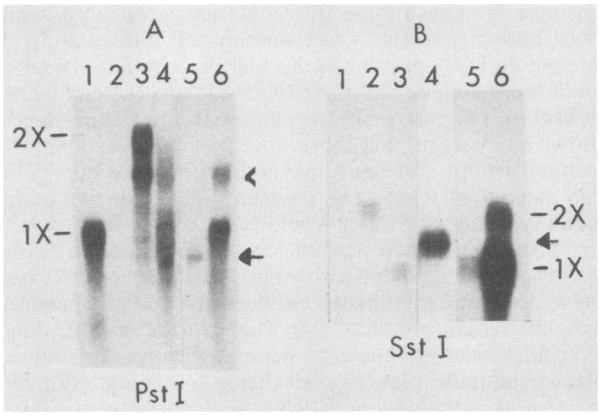

FIG. 3. Template extension by the poliovirus replicase (*Pst*I and *Sst*I templates). RNA synthesis was performed as described in the legend to Fig. 2 except that unlabeled and labeled *Pst*I template (A) or *Sst*I template (B) was used. (A) ^{32}P-labeled *Pst*I template incubated with replicase without any nucleotide (lane 1) and the product was digested with S1 (lane 2). [^{32}P]UMP-labeled product was synthesized from unlabeled *Pst*I template (lane 3) and digested with S1 (lane 4). ^{32}P-labeled *Pst*I template was incubated with replicase plus all four unlabeled nucleotides (lane 6), and the product was digested with S1 (lane 5). (B) ^{32}P-labeled *Sst*I template was digested with S1 (lane 1). [^{32}P]UMP-labeled product was synthesized from unlabeled *Sst*I template (lane 2) and digested with S1. ^{32}P-labeled *Sst*I template was digested with S1 (lane 4). ^{32}P-labeled *Sst*I template was incubated with replicase plus four unlabeled nucleotides (lane 6), and the product was digested with S1 (lane 5). 2× and 1× indicate the positions of the [^{32}P]UMP-labeled product before and after S1 digestion, respectively. Arrow indicates the position of the ^{32}P-labeled template.

de novo copying of the template, and when synthesis is complete, the 3′ end of the newly synthesized RNA snaps back on itself and primes the synthesis of the second strand. In this case, neither of the two newly synthesized RNA strands would be linked to the input template RNA; rather, these complementary RNAs would be covalently linked to each other, generating a dimeric RNA molecule with a small loop at the junction between the two strands. Whereas template extension would predict labeling of only the newly synthesized complementary RNA, in this case, both strands of the dimeric RNA should be labeled during in vitro synthesis in the presence of labeled nucleotides. If, indeed, both newly synthesized RNA strands are labeled, then it should be possible to use the products to detect both unlabeled plus- and minus-strand template RNAs in a dot blot hybridization experiment. In the case of template extension, the labeled product would only hybridize with the complementary unlabeled template RNA. Figure 4A shows an experiment in which double-length RNA products were synthesized in response to unlabeled *Xba*I template in the presence of one labeled and three other unlabeled nucleotides. Labeled RNA product recovered after phenol extraction was digested with S1 nuclease, and its ability to hybridize with unlabeled *Xba*I, unlabeled *Hin*dIII, or unlabeled rRNA was examined by dot blot analysis. Various amounts of unlabeled template RNAs (1 to 100 ng) were spotted onto filter paper, and it was incubated with the S1-digested, labeled RNA products from reaction mixtures containing *Xba*I template. It is evident that the dimeric products synthesized in response to *Xba*I template contained sequences which hybridized with both *Xba*I and *Hin*dIII RNA fragments, indicating that the dimeric product must contain both labeled minus (*Hin*dIII) and plus (*Xba*I) strands (Fig. 4A). A similar result was obtained with the labeled product synthesized in response to *Pst*I template; the product hybridized with both *Pst*I and *Sst*I RNAs (Fig. 4C). The

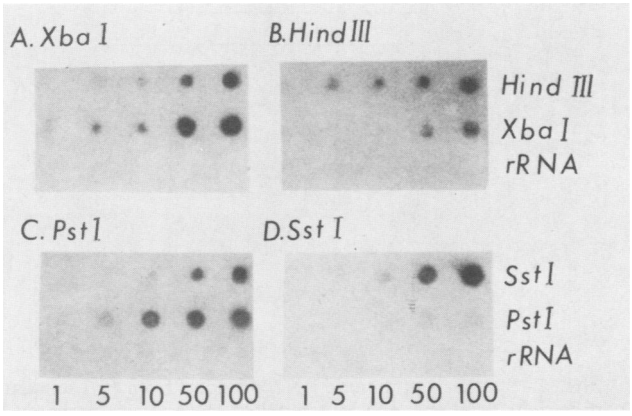

FIG. 4. Dot blot hybridization of labeled replicase products to different unlabeled template RNAs. Different amounts (1, 5, 10, 50, and 100 ng) of unlabeled template RNAs were spotted on filter papers. [^{32}P]UMP-labeled replicase products synthesized in vitro in response to *Xba*I (A), *Hin*dIII (B), *Pst*I (C), and *Sst*I (D) templates were digested with S1 nuclease. The products were diluted with prehybridization buffer and the labeled products in solutions were allowed to hybridize with the spotted unlabeled driver RNAs by incubating the filter papers with the appropriate labeled solutions as described in Materials and Methods. Numbers (1, 5, 10, 50, 100) at the bottom of the figure indicate increasing amounts (nanogram) of unlabeled driver RNAs spotted on the filters. Identities of the unlabeled driver RNAs spotted in each row are indicated on the right.

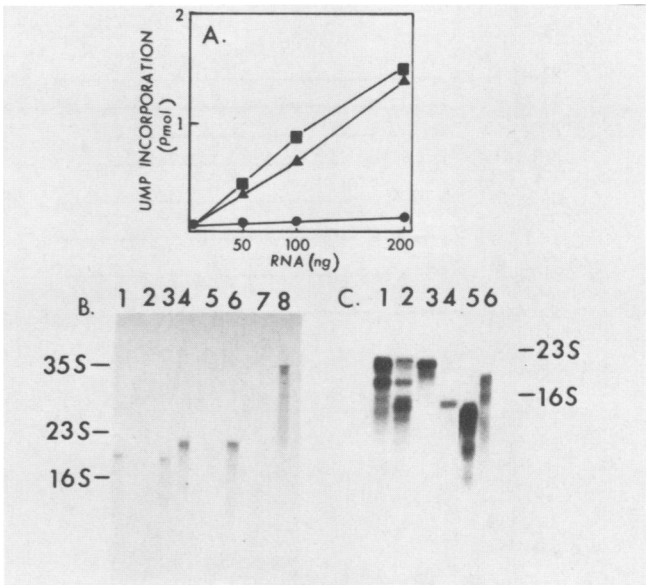

FIG. 5. Effect of purified replicase and host factor on RNA synthesis. (A) Fraction V replicase was incubated with poliovirion RNA (●), *Pst*I RNA (▲), or *Xba*I RNA (■) under RNA synthesis assay conditions. [^{32}P]UMP-labeled products were precipitated with trichloroacetic acid, collected on membrane filters, and counted. (B) [^{32}]UMP-labeled RNA was synthesized in response to *Xba*I, *Pst*I, or poliovirion RNA by fraction V replicase in the absence or presence of fraction VII host factor. Labeled products were analyzed by methylmercuric hydroxide gel electrophoresis as described in Materials and Methods. Lane 1, *Xba*I template, replicase; lane 2, *Xba*I template, host factor; lane 3, *Xba*I template, replicase plus host factor; lane 4, *Pst*I template, replicase; lane 5, *Pst*I template, host factor; lane 6, *Pst*I template, replicase plus host factor; lane 7, poliovirion RNA, replicase; lane 8, poliovirion RNA, replicase plus host factor. (C) S1 digestion of dimeric RNA synthesized by fraction III replicase (lanes 1 and 2) or fraction V replicase (lanes 3 through 6). Shown are RNAs synthesized with a *Pst*I template and fraction III replicase, undigested (lane 1) and digested with S1 (lane 2); RNAs synthesized with a *Pst*I template and fraction V replicase, undigested (lane 3) and digested with S1 (lane 4); RNAs synthesized with a *Xba*I template and fraction V replicase, digested with S1 (lane 5) and undigested (lane 6).

product synthesized from *Hin*dIII template also contained RNAs of both polarities (Fig. 4B). However, when the *Sst*I template was used in the replicase reaction, most if not all of the product was of the opposite polarity to the template, indicating that in this particular case, mostly RNA products complementary to template RNA were made (Fig. 4D). This result was consistent with the outcome of the template extension experiment, in which most of the product RNA synthesized in response to *Sst*I template was due to template extension, thus producing RNAs of only opposite polarity. It is evident that more first strands (i.e., RNAs with the polarity of *Hin*dIII fragment) than second strands were synthesized in vitro from the *Xba*I template (Fig. 4A). Quantitation of the radioactive spots in Fig. 4A indicated that approximately 45% of the total first strands were utilized for the synthesis of second strands. The products of the *Pst*I template contained a higher proportion of first strands compared with those of the *Xba*I template. This was expected since in response to the *Pst*I template, a significant fraction of the first strands were synthesized as a result of template extension in addition to de novo synthesis.

Host factor is not required for the synthesis of double-length

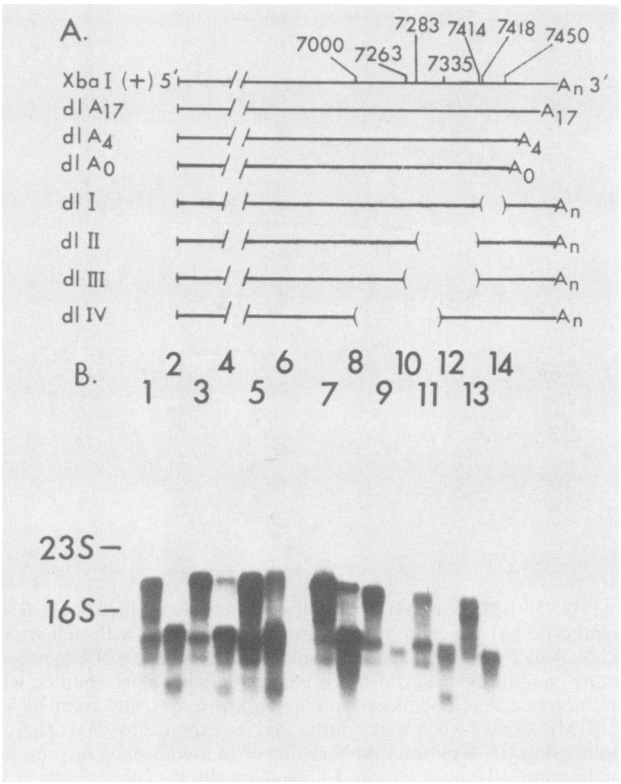

FIG. 6. Synthesis of dimeric RNA products from *Xba*I templates with various deletions. (A) Mutated *Xba*I RNAs were prepared by transcription of pSP64(3′ 17) containing different deletions (see Materials and Methods). *Xba*I templates designated *dl*A_{17}, *dl*A_4, and *dl*A_0 represent *Xba*I RNAs containing 17, 4, and no adenylate residues at the 3′ end, respectively. The RNAs designated *dl*I, *dl*II, *dl*III, and *dl*IV represent *Xba*I, templates with the following sequences deleted: *dl*I, 7418 through 7450; *dl*II, 7283 through 7414; *dl*III, 7263 through 7414; and *dl*IV, 7000 through 7335. All four deleted RNAs contain 3′-terminal poly(A). Numbers refer to the nucleotide position in the type 1 (Mahoney) sequence (19). (B) Fraction III poliovirus replicase was incubated with various deleted templates under the RNA synthesis assay conditions. The labeled products, before or after digestion with S1 nuclease, were analyzed by methyl mercury-agarose gel electrophoresis. Lane 1, *dl*A_{17}, no S1; lane 2, *dl*A_{17}, S1 digested; lane 3, *dl*A_4, no S1; lane 4, *dl*A_4, S1 digested; lane 5, *dl*A_0, no S1; lane 6, *dl*A_0, S1 digested; lane 7, *dl*I, no S1; lane 8, *dl*I, S1-digested; lane 9, *dl*II, no S1; lane 10, *dl*II, S1 digested; lane 11, *dl*III, no S1; lane 12, *dl*III, S1 digested; lane 13, *dl*IV, no S1; lane 14, *dl*IV, S1-digested.

RNAs. In vitro synthesis of poliovirus complementary (minus) RNA from plus RNA template requires both poliovirus replicase and host factor (6, 8, 16). Since the experiments described in the previous section were performed with a partially purified replicase preparation, it was not clear whether both viral replicase (P63) and host factor were required for the synthesis of dimeric RNA products. We therefore purified viral replicase by gradient elution through poly(U) Sepharose, a step which is known to separate the bulk of host factor activity from the viral polymerase (5, 10). However, we found that such preparations still contained limiting amounts of host factor. The enzyme was further purified by two additional passages through poly(U) Sepharose, and each time the enzyme was eluted with a shallower salt gradient. This purified replicase preparation did not contain host factor as judged by gel electrophoresis followed by staining with Coomassie blue; however, it contained other cellular proteins (data not shown). This replicase preparation did not copy poliovirion RNA in the absence of host factor (Fig. 5A). However, the enzyme was able to copy both *Xba*I and *Pst*I templates, as measured by acid-insoluble incorporation of labeled UMP. Figure 5B shows the effect of purified host factor (67,000-molecular-weight protein [67K protein]) on the synthesis of product RNAs in response to *Xba*I, *Pst*I, and poliovirion RNA templates. The purified replicase alone (in the absence of host factor) was able to synthesize double-length RNA molecules when *Xba*I and *Pst*I RNAs were used as templates (Fig. 5B, lanes 1 and 4); host factor, by itself, had no detectable activity (lanes 2 and 5). When replicase and host factor were added together, no significant increase in the synthesis of double-length RNA products was observed compared with that obtained with replicase alone (Fig. 5B, lanes 3 and 6). However, synthesis of poliovirus minus-strand RNA by the purified replicase was completely dependent on added host factor (Fig. 5B, lanes 7 and 8), a result consistent with our previous finding (8, 16). The products synthesized by the purified replicase in response to *Xba*I and *Pst*I templates were indeed dimeric molecules since S1 digestion of these RNAs generated template-length molecules (Fig. 5C, lanes 3 through 6). Note that the intermediate product (the faster-moving band in Fig. 5C, lane 1), synthesized by the partially purified replicase in response to *Pst*I template, was not present in the reaction mixture containing purified replicase (lane 3). This product was synthesized by extension of an internal 3′ OH probably generated by an endonucleolytic cleavage of the template (Fig. 3A, lanes 3, 5, and 6). The lack of synthesis of this product by purified replicase can be explained by the ab-

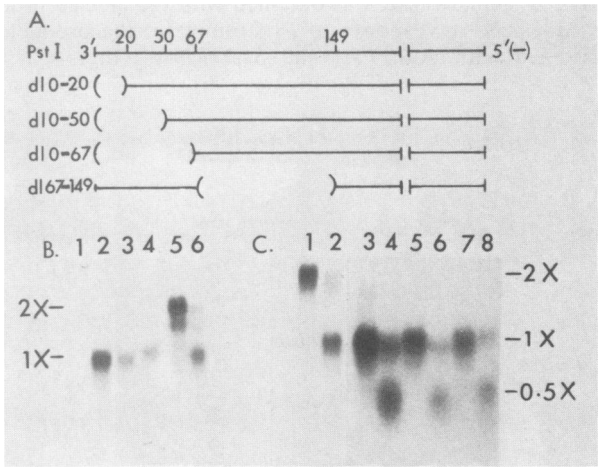

FIG. 7. Synthesis of dimeric RNA products from *Pst*I templates with various deletions. (A) Mutated *Pst*I RNAs were prepared by transcription of pSP65(5′Mut) containing different deletions (see Materials and Methods). The *Pst*I templates designated *dl*0-20, *dl*0-50, and *dl*0-67 carry deletions of the first 20, 50, and 67 nucleotides, respectively, from the 3′ end of the original *Pst*I RNA. The template designated *dl*67-149 contains a deletion of nucleotides 67 to 149. (B) In vitro RNA synthesis was performed with fraction III replicase and the following deleted templates, as described in the legend to Fig. 6B: lane 1, no template; lane 2, *dl*0-67; lane 3, *dl*0-50; lane 4, *dl*0-20; lane 5, *dl*67-149; lane 6, same as lane 5, but product was digested with S1. (C) RNAs synthesized with replicase and the following templates were either digested with S1 or left undigested: *Pst*I, undigested (lane 1) and S1 digested (lane 2); *dl*0-67, undigested (lane 3) and S1 digested (lane 4); *dl*0-50, undigested (lane 5) and S1 digested (lane 6); *dl*0-20, undigested (lane 7) and S1 digested (lane 8).

sence of an enzyme responsible for nicking the template in the purified replicase. Indeed, an endonuclease activity was removed from fraction III replicase during further purification of this enzyme by repeated passage through poly(U) Sepharose 4B (data not shown).

3'-Terminal, poly(A), and heterologous sequences are not required for synthesis of dimeric products. Our previous studies have shown that the 3'-terminal poly(A) tail of poliovirion RNA was necessary for initiation of copying of virion RNA by the poliovirus replicase (10). To determine whether 3'-terminal poly(A) and some heterologous sequences of *Xba*I template would be necessary for the synthesis of double-length RNA molecules, we constructed appropriate deletions in the poliovirus cDNA clone and generated template RNAs by transcription of these altered cDNAs off of the SP6 promoter. *Xba*I templates containing 17, 4, or no 3'-terminal adenylate residues were able to support synthesis of double-length RNA molecules (Fig. 6B, lanes 1 through 6). Also, deletions of some of the heterologous sequences adjacent or close to 3'-terminal poly(A) did not affect the synthesis of double-length products (Fig. 6B, lanes 7 through 14).

We also analyzed *Pst*I templates with various deletions at the 3' termini for their ability to support synthesis of double-length RNA molecules. Three *Pst*I templates, from which 20, 50, or 67 3'-terminal nucleotides were deleted, were incubated with replicase, and the product RNAs were analyzed on a methyl mercury gel. All three product RNAs comigrated with unit-length template (Fig. 7B, lanes 2, 3, and 4). A fourth *Pst*I template, in which nucleotides 67 through 149 were deleted, however, supported synthesis of double-length RNA product (lane 5) which could be digested with S1 to generate a template-length product (lane 6). To determine whether the apparently unit-length products synthesized in response to *dl*0-20, *dl*0-50, and *dl*0-67 templates were truly single stranded, the products were analyzed, after digestion with S1, by denaturing gel electrophoresis. If these molecules were truly single stranded, then S1 would completely digest the labeled RNA. Extensive digestion with S1 generated labeled molecules which migrated exactly half the size of the unit length products (Fig. 7C). This result suggested that the apparent template-length products synthesized in response to the three *Pst*I templates containing various 3'-terminal deletions were actually dimeric RNA molecules and that these dimeric RNA molecules were probably synthesized from internal sites which were close to the middle of the deleted templates. Hybridization of these dimeric molecules with *Pst*I and *Sst*I RNAs after S1 digestion indicated that these molecules consisted of RNAs of both polarities (data not shown).

DISCUSSION

The poliovirus replicase is capable of synthesizing dimeric RNA molecules in vitro in which two strands of RNA appear to be covalently attached to each other (24). Studies presented here were directed to understand the mechanism underlying this unusual property of the viral replicase. Based on the results presented here, we propose two mechanisms by which covalently linked dimeric molecules can be produced in vitro by the poliovirus replicase.

(i) After nicking at a hairpin loop on the template which generates a snap-back structure at the 3' end of the nicked template, the viral replicase adds nucleotides to the newly generated 3' OH group and copies the template to produce a dimeric RNA (Fig. 8A). This model would be consistent with the known ability of poliovirus replicase to elongate an

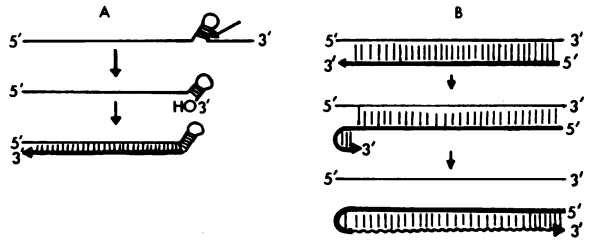

FIG. 8. Models for in vitro synthesis of dimeric RNA products by the poliovirus replicase. (A) Template extension. Arrow indicates the position of cleavage on an RNA template near a putative stem-loop structure generating a 3' OH group. Thin and thick lines represent RNAs of opposite polarities. (B) De novo synthesis and snap-back. Thick line represents RNA complementary (first strand) to template RNA (thin line). Wavy line represents RNA complementary (second strand) to first strand.

oligo(U) primer hydrogen bonded to the 3' poly(A) tail of viral RNA. This model is supported by the results indicating that viral replicase can elongate uniformly labeled *Hin*dIII,*Pst*I, and *Sst*I templates and that digestion of these elongated products with S1 produces labeled molecules which migrate faster than the template RNAs (Fig. 2 and 3). The results suggest that the template RNA is elongated from an internal site rather than from the exact 3' end of the template (Fig. 8A). If the templates were extended from the exact 3' end, then one would expect to observe template-length molecules after digestion with S1. This mechanism seems to be the only way by which complementary RNA is synthesized in response to *Sst*I template. It is important to note that in this particular case, only RNA molecules complementary to the template are produced (Fig. 4D). A similar mechanism also appears to be responsible for the synthesis of a significant fraction of the total RNA products from the *Pst*I and *Hin*dIII templates (Fig. 3A and 2B, respectively).

(ii) A second possible mechanism involves de novo synthesis of the first strand by copying of the template and snap-back of the newly synthesized first strand to generate a template-primer structure which eventually is elongated to form the second strand (Fig. 8B). Unlike the first mechanism, the product RNAs synthesized by the second mechanism are not linked to the input template RNA; rather, both strands are covalently linked to each other. This model is supported by the following results. First, all of the exactly dimeric RNA products synthesized in response to *Xba*I and *Pst*I templates did not appear to result from an extension of the corresponding templates since labeled *Xba*I and *Pst*I templates could not be extended to molecules having twice the size of the template (Fig. 2A and 3A), yet all of the dimeric RNAs synthesized in response to *Xba*I could be converted to template-length molecules by S1 digestion (Fig. 2A). In the case of *Pst*I template, some of the dimeric molecules comigrated with template RNA after S1 digestion (Fig. 3A). Second, dot blot hybridization studies suggested that significant amounts of labeled second strand (which is complementary to the first strand) were synthesized in response to both *Xba*I and *Pst*I templates. Our preliminary result obtained with ^{32}P-labeled *Xba*I template and [^{3}H]nucleotides suggests that the ^{3}H-labeled dimeric product can be separated from the ^{32}P-labeled template in a velocity gradient. Nevertheless, it is possible that a small fraction of the template is elongated from the 3' end to generate the first strand (complementary to template) which then snaps back on itself to prime the synthesis of the second strand. Such a

model would explain the result of the hybridization experiment presented in Fig. 4A. This explanation, however, seems unlikely since no products larger than dimeric RNA could be detected in the reaction; if this model were correct, one would expect synthesis of at least some trimeric products. Although the results of the hybridization experiment can be best explained by the model presented in Fig. 8B, this model should be considered tentative until the reaction is further characterized.

We previously showed that the removal of the 3′ poly(A) tail from the virion RNA drastically reduced the ability of the deadenylated RNA to be copied by the replicase (10). The poly(A) tail also appears to be indispensible for the infectivity of poliovirion RNA (21). Furthermore, a model proposed by Andrews et al. for the in vitro synthesis of minus-strand RNA by the replicase would require the 3′-terminal poly(A) tail of poliovirion RNA (1). It seems surprising, however, that the synthesis of dimeric products from the *Xba*I template did not depend on the presence of the poly(A) on this template. No difference was found in the extent of copying of *Xba*I templates containing 3′ poly(A) compared with that lacking poly(A) tail (Fig. 6). It is also expected that the heterologous noncoding sequences near the 3′ terminus of both plus- and minus-strand RNAs should be important for initiation of RNA synthesis. However, our results suggest that at least some of the sequences near the 3′ end of both *Xba*I and *Pst*I RNA templates are not necessary for the synthesis of dimeric products. Four *Xba*I templates containing various deletions (*dl*I 7418 through 7850; *dl*II, 7283 through 7414; *dl*III, 7263 through 7414; *dl*IV, 7000 through 7335) were all active in directing the synthesis of dimeric RNAs (Fig. 6). The *Pst*I template containing a deletion from nucleotide 67 through nucleotide 149 still synthesized dimeric RNA. An unexpected result was the synthesis of apparently template-length molecules from the *Pst*I templates with 3′ deletions spanning 0 through 20, 0 through 50, and 0 through 67 nucleotides (Fig. 7B). Further analysis, however, showed that these products with apparent molecular weights of the templates were not truly single-stranded molecules. In fact, the template-sized product fell to half the length of the original *Pst*I template after S1 digestion. Therefore, these molecules must have been synthesized from an internal site probably close to the middle of the template. Dot blot hybridization showed that these products contained RNAs of both polarities. The 3′ terminus of *Pst*I (minus-strand) template is known to contain a stem-loop structure (nucleotides 9 through 37) (13) which may interact with the viral replicase to initiate RNA synthesis de novo; dimeric products are produced by snap-back of the newly synthesized plus strand followed by elongation of the snapped-back template-primer. Deletion of 3′-terminal nucleotides 0 through 20 (or 0 through 50 or 0 through 67) destabilizes or eliminates the stem-loop structure; however, the replicase initiates approximately in the middle of the template and eventually synthesizes a dimeric molecule whose size appears to be the same as that of the template. This model is consistent with the finding that a deletion encompassing nucleotides 67 to 149 does not interfere with the synthesis of dimeric molecules.

The results presented in Fig. 5 suggest that the 67K host factor is not necessary for the synthesis of dimeric RNA products by using *Xba*I and *Pst*I templates. Young et al. reported that both poliovirus replicase and host factor were necessary for the synthesis of dimeric RNA molecules in response to poliovirion RNA template (24). However, they used a partially purified preparation of host factor which is likely to contain other proteins in addition to the 67K polypeptide (host factor) (17). The possibility that a different host cell protein is required along with replicase for the synthesis of dimeric molecules cannot be ruled out since our host factor-dependent replicase used in these experiments contains a few host cell proteins other than the 67K host factor (unpublished data). Further purification of the replicase used in these reconstitution studies (Fig. 5) would be required to identify the host cell protein necessary for synthesis of dimeric RNAs.

It is surprising that the purified viral replicase synthesized dimeric RNA molecules from smaller templates, but the product synthesized in response to poliovirion RNA by the same replicase in the presence of purified host factor consisted of RNAs of up to 35S. It is possible that sequences well upstream from the 3′ end of the viral RNA may be necessary for proper initiation on the intact viral RNA template and that host factor may confer this specificity upon replicase. The well-studied replicase of the Qβ bacteriophage is believed to specifically interact with sequences from three different regions of Qβ-RNA which are widely separated from each other. The idea that template recognition might involve binding of Qβ replicase to internal regions of Qβ RNA was suggested by the failure of fragmented Qβ RNA to serve as a template in vitro (7). In addition to the 3′ end of Qβ RNA, two other internal sites, one approximately 70% from the 3′ end of the RNA and the other approximately 50% from the 3′ end were necessary for proper binding and initiation by the Qβ replicase (7). Unlike intact poliovirion RNA, the *Xba*I and *Pst*I templates may lack sequences necessary for replicase binding which may help to position the enzyme in such a way that the 3′ end of the RNA is near the initiation site of the enzyme for proper initiation to occur. In the absence of these putative upstream sequences, the replicase may bind nonspecifically to the template RNA and copy the RNA, eventually producing dimeric RNA molecules by one of two proposed mechanisms. This scenario is analogous to one in which DNA-dependent RNA polymerase II can nonspecifically initiate at nicks or at the ends of a DNA template (14). The physiological significance of the mechanisms responsible for dimeric RNA synthesis by the poliovirus replicase is not known. It is highly unlikely that synthesis of dimeric RNA by the template-extension mechanism as illustrated in Fig. 8A would actually be responsible for the replication of the poliovirus RNA genome. The relevance of dimeric RNA synthesis by the second mechanism, i.e., de novo synthesis of the first strand that snaps back and becomes a template for synthesis of a second strand of RNA, is not known at present. Whether poliovirus RNA synthesis in vivo initiates de novo or with a protein (VPg) primer, the ability of poliovirus replicase to catalyze synthesis of dimeric RNA by the second mechanism may explain the existence of dimeric RNAs found in small quantities in infected cells (24).

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