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Functional oligomerization of poliovirus RNA-dependent RNA polymerase

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ABSTRACT

Using a hairpin primer/template RNA derived from sequences present at the 3′ end of the poliovirus genome, we investigated the RNA-binding and elongation activities of highly purified poliovirus 3D polymerase. We found that surprisingly high polymerase concentrations were required for efficient template utilization. Binding of template RNAs appeared to be the primary determinant of efficient utilization because binding and elongation activities correlated closely. Using a three-filter binding assay, polymerase binding to RNA was found to be highly cooperative with respect to polymerase concentration. At pH 5.5, where binding was most cooperative, a Hill coefficient of 5 was obtained, indicating that several polymerase molecules interact to retain the 110-nt RNA in a filter-bound complex. Chemical crosslinking with glutaraldehyde demonstrated physical polymerase–polymerase interactions, supporting the cooperative binding data. We propose a model in which poliovirus 3D polymerase functions both as a catalytic polymerase and as a cooperative single-stranded RNA-binding protein during RNA-dependent RNA synthesis.

Keywords: RNA replicase; single-stranded RNA-binding protein

INTRODUCTION

Polymerases are the central enzymes in the replication of viral nucleic acids. In the case of poliovirus, this enzyme is an RNA-dependent RNA polymerase and is the 52-kDa protein product of the viral gene 3D (Van Dyke & Flanegan, 1980; Kitamura et al., 1981; Racaniello & Baltimore, 1981). In vitro, this single polypeptide is active in template-dependent RNA elongation when provided with a primer, but is not capable of initiating RNA synthesis de novo (Flanegan & Baltimore, 1977; Van Dyke & Flanegan, 1980). Several host cell proteins that modify the activity of 3D polymerase have been partially purified (Dasgupta et al., 1980; Baron & Baltimore, 1982a, 1982b; Andrews et al., 1985; Young et al., 1985; Andrews & Baltimore, 1986; Hey et al., 1987), but none of these is tightly associated with the 3D polymerase, and their relevance to poliovirus RNA replication has not been demonstrated. In infected cells, the RNA replication complex contains not only the viral RNA-dependent RNA polymerase 3D, but also several viral proteins and their precursors that are known to be required for RNA synthesis: 2B, 2C, 2BC, 3A, 3B, 3AB, and 3CD (Wimmer et al., 1993).

The best-characterized RNA-dependent RNA polymerase activity is that of the RNA replicase from Qβ RNA phage. Qβ replicase is a multisubunit enzyme composed of one virally encoded polypeptide (65 kDa) and three host cell proteins: ribosomal protein S1 (70 kDa), and translation elongation factors EF-Tu (45 kDa) and EF-Ts (35 kDa) (Blumenthal & Carmichael, 1979). The phage-encoded subunit of the replicase presumably contains the polymerase active site because RNA-dependent RNA polymerase activity is isolated only from infected cells (Kamen, 1970; Kondo et al., 1970), and the phage-encoded polypeptide contains extensive sequence similarities with known RNA-dependent RNA polymerases (Kamer & Argos, 1984; Bruenn, 1991). Ribosomal protein S1 is thought to be involved in template recognition and initiation (Blumenthal & Carmichael, 1979). The functions of EF-Tu and EF-Ts in replicase activity are not clear, although they may also be involved in initiation of RNA synthesis (Blumenthal & Carmichael, 1979). The identity and function of an additional host cell protein that weakly associates with the Qβ replicase and greatly stimulates its activity has not been determined, although it is

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known to be a hexamer of identical 12-kDa polypeptides that binds single-stranded RNA (de Fernandez et al., 1968) and is likely to form larger aggregates under physiological salt concentrations (de Haseth & Uhlenbeck, 1980). The multisubunit RNA-dependent RNA polymerases from plant bromoviruses have two virally encoded subunits and several associated host proteins (Hayes & Buck, 1990; Quadt et al., 1993). In the case of brome mosaic virus, a 45-kDa host protein that co-purifies with the viral RNA-dependent RNA polymerase activity appears to be a subunit of eukaryotic translation initiation factor 3 (Quadt et al., 1993).

During the course of poliovirus infection, the poliovirus RNA-dependent RNA polymerase is responsible for producing approximately 50,000 copies of the single infecting viral RNA (Koch & Koch, 1985). This occurs over the course of about eight hours; thus, RNA synthesis by poliovirus 3D polymerase is a very efficient process in infected cells. Unlike the virally encoded polymerase subunits from $Q\beta$ phage and bromoviruses, the single poliovirus-encoded polypeptide, 3D, displays RNA-dependent RNA polymerase activity in vitro. However, although the poliovirus polymerase can utilize primed templates in vitro, curiously, fewer than 1% of template RNA molecules are copied by 3D polymerase in a typical experiment (Van Dyke et al., 1982; our unpublished data). This inefficient template utilization has frustrated attempts to characterize the enzyme's substrate specificity, kinetics, and other biochemical properties. Recently, Lama et al. (1994) and Paul et al. (1994) reported that viral protein 3AB stimulated poliovirus polymerase activity in vitro at low polymerase concentrations, and Cho et al. (1993) reported the utilization of 50% of defined template RNAs after long incubation with a highly purified polymerase preparation. The reasons for these dramatic increases in enzyme activity, template utilization, or both were not clear. We report here that efficient template utilization can be obtained at high polymerase concentrations and that efficient template utilization corresponds well with polymerase oligomerization and cooperative RNA binding. We propose that the poliovirus RNA-dependent RNA polymerase can function as a single-stranded RNA-binding protein and that this single-stranded RNA-binding activity is required for efficient template utilization.

RESULTS

Efficient utilization of a poliovirus-derived primer/template RNA

To identify optimal conditions for utilization of a self-priming RNA template, we assayed polymerase activity as a function of polymerase concentration and other reaction conditions. RNA molecules were synthesized that could serve as both template and primer for RNA-dependent RNA synthesis. Figure 1A diagrams RNA HP1, which contains 85 nt from the most 3′ heteropolymeric sequences of the poliovirus positive strand (Kitamura et al., 1981; Racaniello & Baltimore, 1981) followed by 20 A's and 5 U's. The U's and A's at the 3′ end should form a short duplex to provide a primer for the initiation of RNA synthesis by poliovirus 3D polymerase. The poliovirus genome itself terminates in a variable length poly(A) tail, averaging 75 nt on RNAs isolated from virions and reaching over 150 nt on RNAs isolated from infected cells (Spector & Baltimore, 1975). In vitro, purified poliovirus 3D polymerase is absolutely primer-dependent, using any RNA substrate, including those derived from poliovirus sequences (Flanegan & Baltimore, 1977; Van Dyke & Flanegan, 1980). RNA HP1 is predicted to form a hairpin at its 3′ end, providing the primer for RNA synthesis and ensuring that there will be a 1:1 ratio of template and primer. Because the mechanism for priming negative-strand synthesis during infection is not known, it is difficult to know how closely RNA HP1 resembles a natural template or intermediate in RNA synthesis in infected cells.

The utilization of RNA HP1 by 3D polymerase as a function of pH is shown in Figure 1B. Internally labeled RNA was incubated with 4 μM 3D polymerase for 30 min under elongation conditions as a function of pH. When the elongation products were displayed on a denaturing acrylamide gel after boiling in formamide (Fig. 1B, lanes 2–6), much of the RNA incubated at pH 4.5–7.5 stayed in the wells. At all pH values, some RNA migrated slightly below the 225-nt marker RNA, consistent with the expected migration of fully elongated primer/template HP1 RNA. When the elongation products were treated with proteinase K prior to boiling in formamide and loading on the gel, none of the RNA incubated at any pH remained in the wells, migrating instead as fully elongated RNA. Similar results were obtained when the elongation reactions were phenol extracted or treated with 1% SDS prior to loading on the gel (data not shown). At a 3D polymerase concentration of 4 μM, greater than 50% of the primer/template RNA was elongated at every pH except 8.5. At pH 4.5, approximately 75% of the RNA was elongated. These results demonstrate that template utilization by poliovirus polymerase can be very efficient, and that much of the elongated RNA products can be present in protein–RNA complexes that do not readily migrate into the gels.

The elongation of RNA HP1 as a function of 3D polymerase concentration at pH 5.5 is shown in Figure 1C. In this and all subsequent experiments, elongation reactions were treated with proteinase K prior to analysis by gel electrophoresis. A sharp transition in the efficiency of template utilization occurred at a polymerase concentration of approximately 1 μM. Above this concentration, at least 75% of RNA HP1 was elongated,

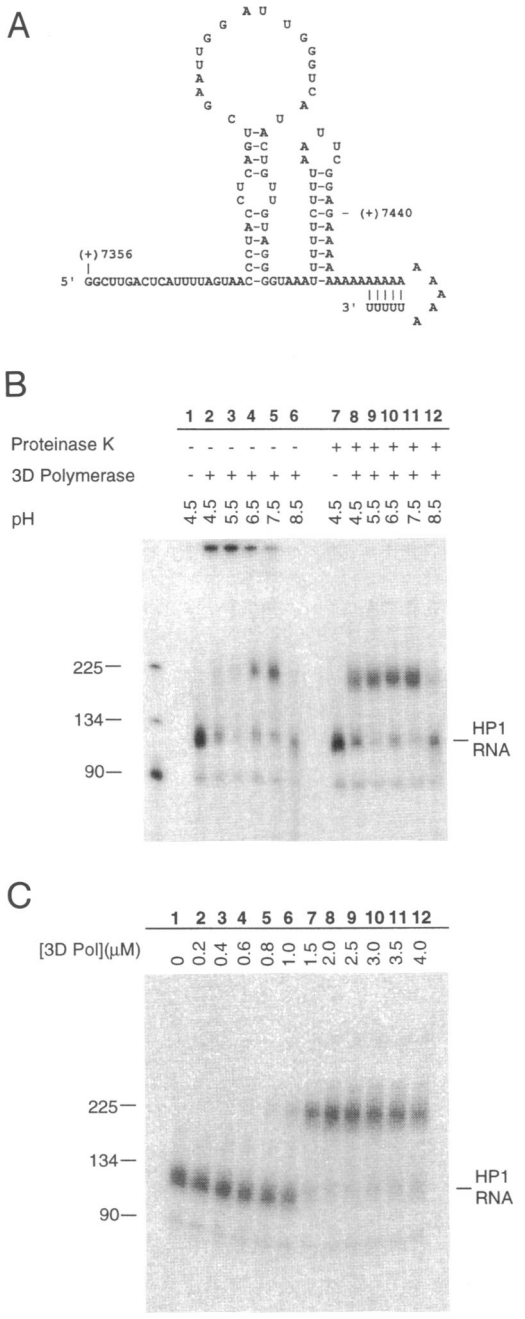

FIGURE 1. A: Sequence and predicted secondary structure of RNA HP1. The 110-nt RNA contains 85 nt from the 3' end of poliovirus RNA, followed by 20 A's and 5 U's; the nucleotide positions in the viral genome are noted. Polymerase extension from the base pairing arrangement of the A's and U's at the 3' end shown would produce a 205-nt full-length product. Internal secondary structures are from Jacobson et al. (1993). **B:** Elongation of RNA HP1 at varying pH. One nanomolar RNA HP1, internally labeled with [α-^{32}P]-UTP, was incubated in the absence (lanes 1, 7) or presence (lanes 2–6, 8–12) of 4 μM 3D polymerase for 30 min at the indicated pH. Labeled RNA markers and their sizes in nucleotides are shown at left, and the position of HP1 RNA is indicated at right. Polymerase reactions were stopped either by the addition of formamide loading buffer (lanes 1–6), or by treatment with proteinase K followed by the addition of formamide loading buffer (lanes 7–12). **C:** Elongation of RNA HP1 at different 3D polymerase concentrations. One nanomolar RNA HP1 was incubated at pH 5.5 with the indicated concentrations of 3D polymerase. Reactions were terminated by treatment with proteinase K followed by addition of formamide loading buffer.

yet at a polymerase concentration of 0.8 μM, only about 10% of the RNA was elongated, and at 0.4 μM, less than 1% of the RNA was elongated. This sharp transition suggests cooperativity of polymerase molecules during template elongation. The dependence of elongation on both polymerase concentration and pH is further documented below.

Correlation between RNA binding and elongation

Because one of the first steps in RNA synthesis must be binding of the primer/template RNA by the polymerase, we investigated the binding of RNA HP1 by poliovirus 3D polymerase. During the course of these studies, it became apparent that much of the RNA bound by polymerase was present in large protein–RNA aggregates. Thus, we modified a standard nitrocellulose filter binding assay by making a three-membrane sandwich to separate RNA bound in large protein complexes, RNA bound in smaller protein complexes, and unbound RNA. The first membrane was polysulfone, which has low affinity for both RNA and protein. With a 0.45-μm pore size, the polysulfone membrane should retain only RNA bound in large complexes. The second membrane was nitrocellulose, to bind smaller protein–RNA complexes. Finally, a positively charged membrane was present to retain all unbound RNA. Figure 2 shows the results of one such binding experiment. In this experiment, RNA HP1 was incubated for 30 min on ice with increasing concentrations of 3D polymerase at pHs 4.5, 5.5, 6.5, 7.5, and 8.5. Aliquots of each reaction were then filtered through the three membranes and the amount of RNA bound to each was visualized by autoradiography (Fig. 2, left panels). The remainder of each binding reaction was then incubated at 30 °C to allow elongation. After 30 min, aliquots were again filtered and visualized by autoradiography (Fig. 2, right panels). From this experiment, it is clear that overall polymerase binding to RNA was tightest at low pH, and that most of the RNA was bound in large complexes that could be retained by the 0.45-μm polysulfone membrane under these conditions. At higher pH, there was less overall RNA binding, but the RNA bound was primarily in smaller complexes that passed through the polysulfone filter and were retained on the nitrocellulose membrane. Interestingly, maximal binding for smaller complexes is at the two pH extremes, pH 4.5 and pH 8.5 (Figs. 2B, 3B).

There were no dramatic differences in the amount of RNA binding by polymerase before or after elongation. Similar results were obtained when the reactions were incubated at 30 °C in the absence of one of the nucleotides to prevent polymerase elongation (data not shown). Thus, RNA binding by poliovirus RNA-dependent RNA polymerase resulted in the formation of complexes, large and small, that were either not disrupted

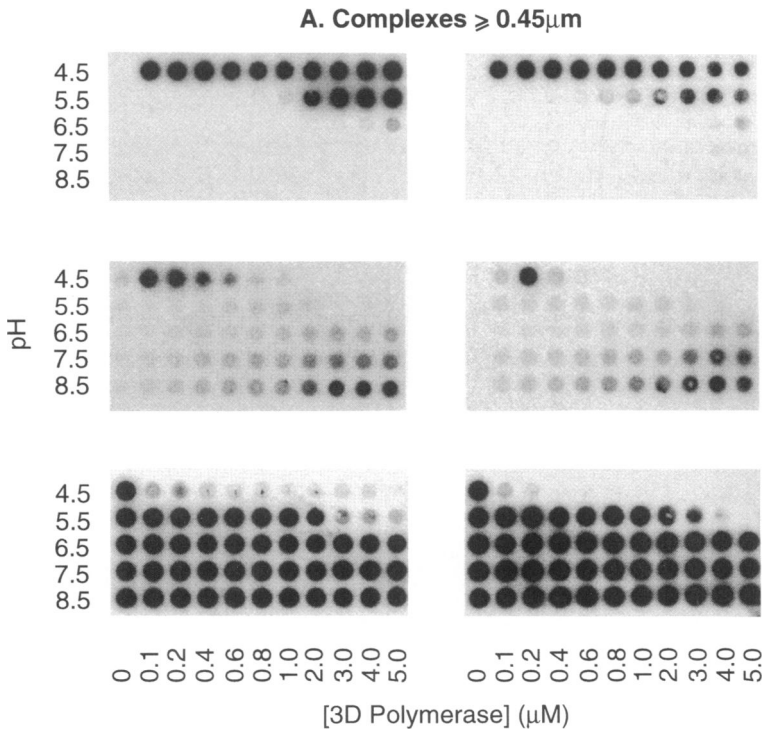

FIGURE 2. Binding of RNA HP1 to 3D polymerase. RNA HP1, present at 1 nM, was incubated at pH 4.5, 5.5, 6.5, 7.5, or 8.5 with increasing concentrations of 3D polymerase as indicated, then filtered through three membranes to separate bound and unbound RNA. **A:** Polysulfone membrane retained RNA-polymerase complexes of sizes 0.45 μm or larger. **B:** Nitrocellulose membrane-retained RNA-polymerase complexes smaller than 0.45 μm. **C:** Positively charged nylon retained uncomplexed RNA. Membranes on left display RNAs after binding for 30 min on ice, and membranes on right display RNAs after elongation for 30 min at 30 °C.

during elongation or were able to reform immediately following elongation.

Quantitation of the amount of RNA in large and small complexes is shown in Figure 3. In an experiment similar to that shown in Figure 2, RNA binding to polymerase was assayed with the three-membrane filter system. After incubation, the same reactions were incubated at 30 °C and elongation was quantified from

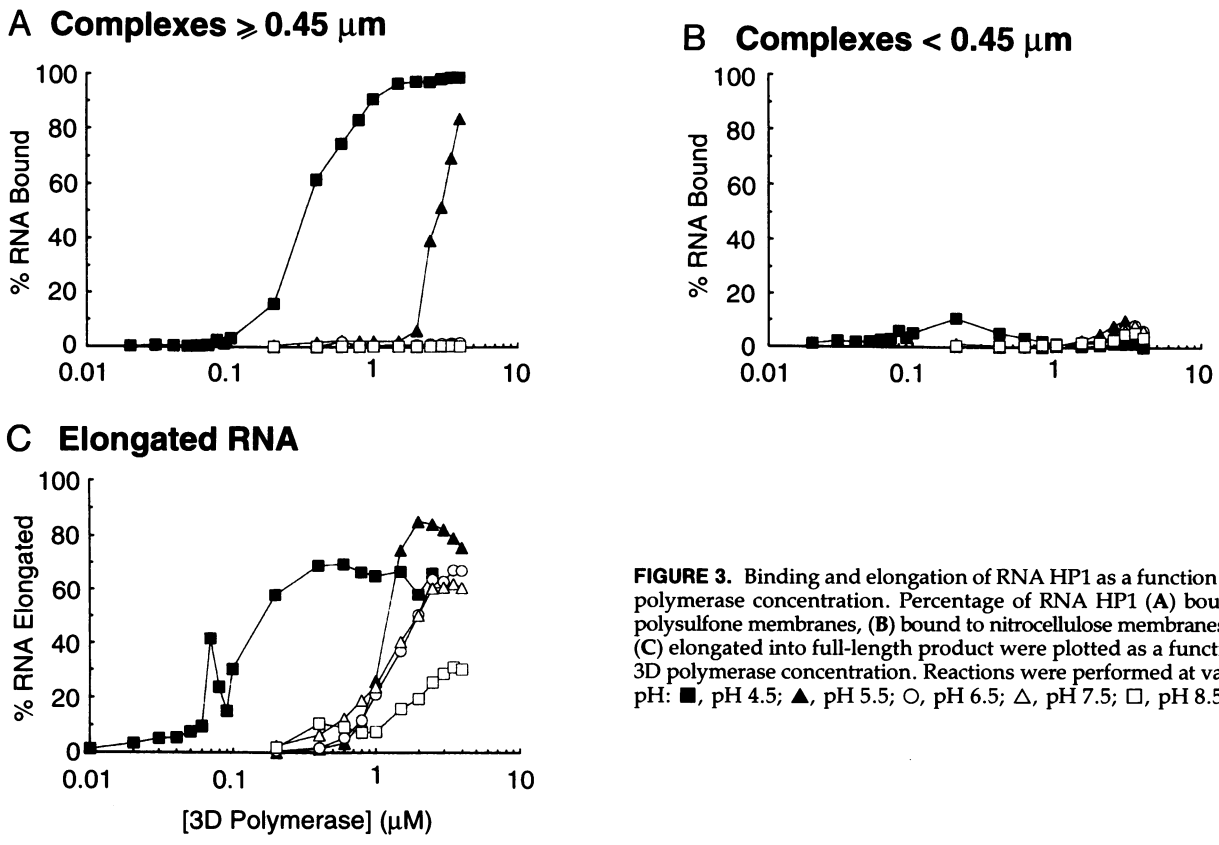

FIGURE 3. Binding and elongation of RNA HP1 as a function of 3D polymerase concentration. Percentage of RNA HP1 (**A**) bound to polysulfone membranes, (**B**) bound to nitrocellulose membranes, and (**C**) elongated into full-length product were plotted as a function of 3D polymerase concentration. Reactions were performed at varying pH: ■, pH 4.5; ▲, pH 5.5; ○, pH 6.5; △, pH 7.5; □, pH 8.5.

gels such as those shown in Figure 1. The percentage of RNA bound in large complexes to polymerase as a function of polymerase concentration at each pH is shown in Figure 3A, the percentage of RNA bound in smaller complexes is shown is Figure 3B, and the percentage of RNA elongated is shown in Figure 3C. Maximal binding and elongation activity was observed at pH 4.5, followed by pH 5.5. Binding at pH 4.5 was first detected at a polymerase concentration of approximately 0.1 μM; at the higher pH values, 10-fold higher polymerase concentrations (approximately 1 μM) were required to detect binding. At each pH, the formation of smaller complexes reached a maximum at a slightly lower polymerase concentration than the formation of the larger complexes. This is consistent with the idea that, under conditions that favor protein-protein interactions, smaller complexes form first. Elongation of HP1 RNA was first detectable at approximately the same or at slightly lower polymerase concentrations than the formation of the smaller complexes. Because multiple turnovers could be observed at the 30-min timepoint, and because elongation is not an equilibrium process, RNA template utilization is a slightly more sensitive assay than RNA binding. Given these considerations, inspection of Figure 3 reveals that there is a good correlation between conditions that favor the formation of polymerase-RNA complexes, large and small, and polymerase elongation.

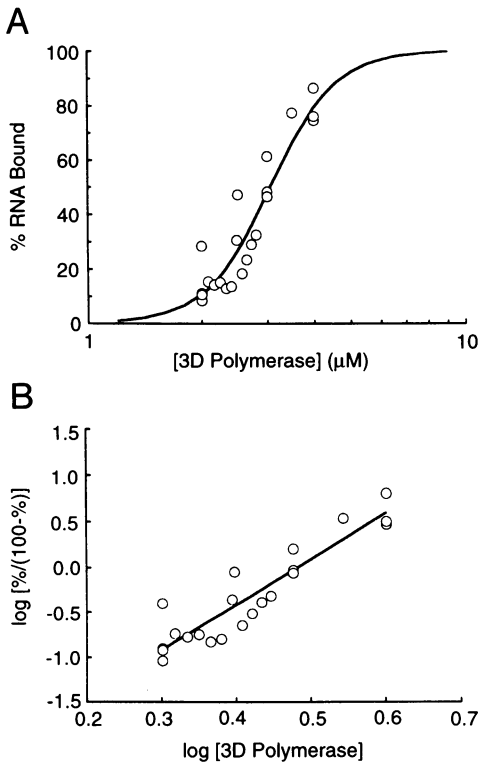

FIGURE 4. **A:** Percentage of RNA HP1 bound to 3D polymerase at pH 5.5, as measured by RNA retention on both polysulfone and nitrocellulose filters, as a function of 3D polymerase concentration. Data points were compiled from binding reactions performed using different preparations of polymerase and RNA; only those points in which 10-90% of the RNA was bound were included. In all experiments, RNA HP1 was present at a concentration of 1 nM. The line is a theoretical curve described by the equation: %RNA bound = $100 \times [\text{3D polymerase}]^n/(K + [\text{3D polymerase}]^n)$, with $n = 5$ and $K = 270\ \mu M^5$, as determined from a Hill plot of the data shown. **B:** Hill plot of the binding data shown in A. The line is the best fit to the data, as determined by least-squares analysis, and is described by the equation:

$$\log(\%\text{RNA bound}/(100 - \%\text{RNA bound})) = n \log[\text{3D polymerase}] - \log K$$

where, n is the Hill coefficient and K is the apparent dissociation constant for the binding interaction. The best fit line gives a Hill coefficient of 5.0 and an apparent dissociation constant of 270 μM^5, and has a correlation coefficient of 0.92.

Poliovirus polymerase can bind RNA in a highly cooperative manner

As seen in Figure 3A, polymerase binding to RNA increases very sharply with increasing polymerase concentration, indicating that it is highly cooperative. Indeed, Hill plot analysis for binding at pH 5.5 (Fig. 4) shows that total RNA binding by the polymerase gives a Hill coefficient of 5.0, with an apparent dissociation constant of 270 μM^5. Thus, several polymerase molecules must interact to retain the RNA in filter-bound complexes.

Physical interactions between polymerase molecules are promoted at low pH

The results described above suggested the presence of strong interactions between polymerase molecules. To observe such interactions directly, in the absence of RNA, we purified 3D polymerase labeled with [^{35}S]-methionine and followed its behavior in the three-membrane filter system. As shown in Figure 5, the amount of 3D polymerase present in large complexes increases both with increasing polymerase concentration and with decreasing pH, corresponding with the conditions that promote RNA binding and efficient template utilization.

Although at low pH it is clear that polymerase-polymerase interactions accompanied RNA elongation activity, at higher pHs, RNA elongation was observed in the absence of formation of large aggregates. To detect possible physical interactions between 3D polymerase molecules at higher pH, we tested the ability of polymerase molecules to be crosslinked at low concentrations of glutaraldehyde. Highly purified 3D polymerase, present at several different concentrations, was treated with 0.005% glutaraldehyde and analyzed by SDS/PAGE. The resulting gel (Fig. 6) shows that the crosslinking reaction produced multiple slower-migrating species. The most prominent bands that appeared after glutaraldehyde treatment migrated with apparent molecular weights of 114, 131, and 141 kDa. Also present were groups of crosslinked species that migrated with apparent molecular weights of 200 kDa and

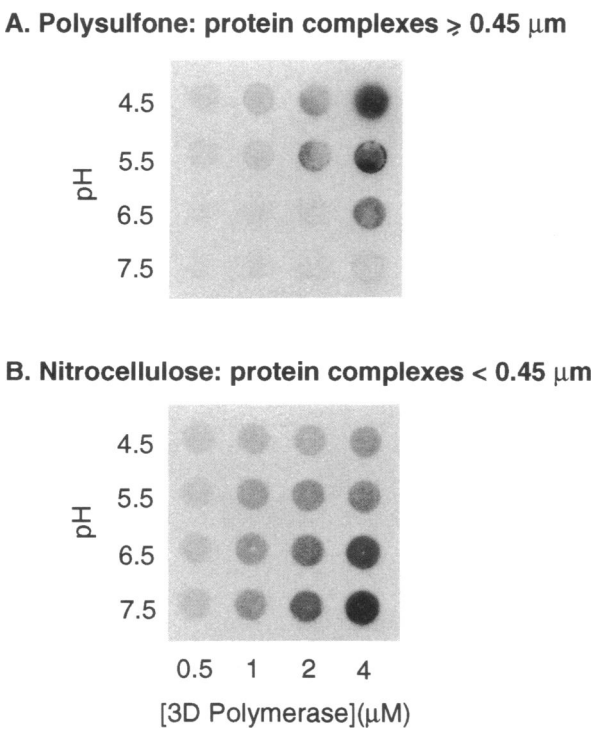

FIGURE 5. Dependence of polymerase–polymerase complex formation on pH. 3D polymerase, labeled with [^{35}S]-methionine and present at 0.5, 1, 2, or 4 μM, was incubated at pH 4.5, 5.5, 6.5, or 7.5 in the absence of RNA for 30 min on ice. Reactions were then filtered through three membranes. **A:** Polysulfone membrane retained polymerase complexes of sizes 0.45 μm or larger. **B:** Nitrocellulose membrane retained smaller polymerase complexes and uncomplexed polymerase. No detectable polymerase was retained on the positively charged nylon membrane (not shown).

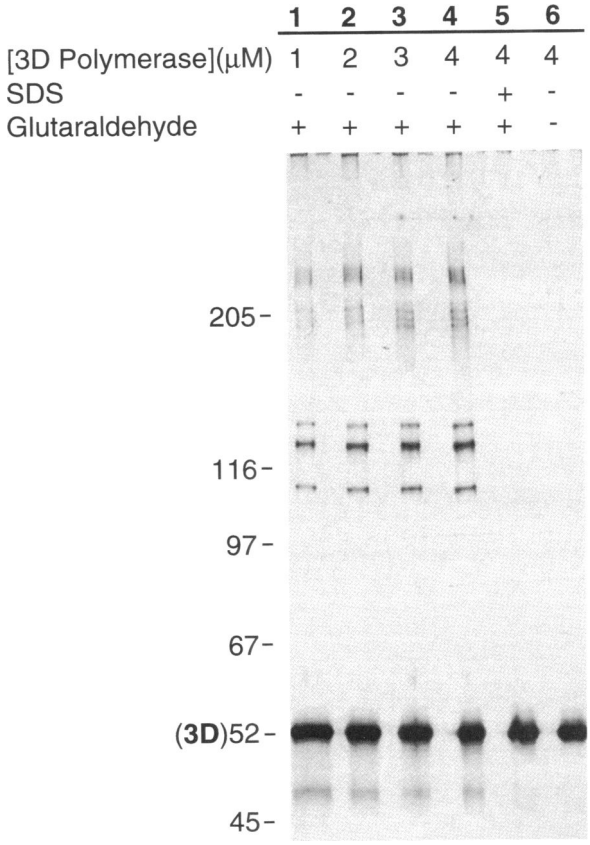

FIGURE 6. Glutaraldehyde crosslinking of 3D RNA-dependent RNA polymerase. 3D polymerase, in the absence of RNA and in the concentrations indicated, was incubated with 0.005% glutaraldehyde for 5 min at room temperature. Equivalent amounts of protein from each sample were analyzed by SDS/5% PAGE and visualized by silver staining. Lanes 1–4, 3D polymerase present at 1, 2, 3, or 4 μM, respectively, and incubated with 0.005% glutaraldehyde. Lane 5, 0.4% SDS was added to 4 μM 3D polymerase prior to crosslinking with 0.005% glutaraldehyde. Lane 6, 4 μM 3D polymerase incubated in the absence of glutaraldehyde. The mobilities of protein standards of known molecular weights are shown at left. The species that migrates at approximately 45 kDa is likely to result from intramolecular crosslinking of full-length polymerase molecules.

above. Although the precise identities of these higher-order complexes are not known, it seems likely that the nominally 114-kDa species represents a dimer of the 52-kDa polymerase with a simple crosslinked topology, and the more slowly migrating species correspond to complexes containing more than two polymerase molecules, more complex topologies, or both.

The amount of crosslinked product increased when the concentration of 3D polymerase was varied from 1 to 4 μM. This range of concentrations corresponds to the polymerase concentrations required for efficient elongation activity at pH 7.5 (Fig. 3C). When 0.4% SDS was present in a reaction containing 4 μM 3D polymerase and glutaraldehyde, no crosslinking was observed. Thus, the crosslinking of 3D polymerase molecules is sensitive to denaturation of the protein and reflects protein–protein interactions that occur between molecules in their native state.

Cooperative binding of poliovirus positive and negative strand RNAs

To test the relevance of cooperative binding of polymerase to less artificial substrates than RNA HP1, full-length positive and negative strand poliovirus RNAs were synthesized in vitro. As shown in Figure 7, polymerase binding to these 7,500-nt RNAs was highly cooperative, closely paralleling the binding to the 110-nt HP1 RNA at pH 5.5. Interestingly, polymerase binding to these longer RNAs was very similar at both pH 5.5 and pH 7.5, unlike the situation with the shorter HP1 RNA. Thus, at the higher pH, either there is a more pronounced RNA length dependence of cooperative binding, or the poliovirus RNAs contain high-affinity sites that serve to nucleate cooperative binding. In any case, cooperative RNA binding was observed with RNAs and pH conditions that presumably resemble those within an infected cell.

Full-length poliovirus RNAs that were bound to 3D polymerase were found predominantly in large complexes with polymerase, even at pH 7.5 (Fig. 7). However, only a small fraction of the polymerase population

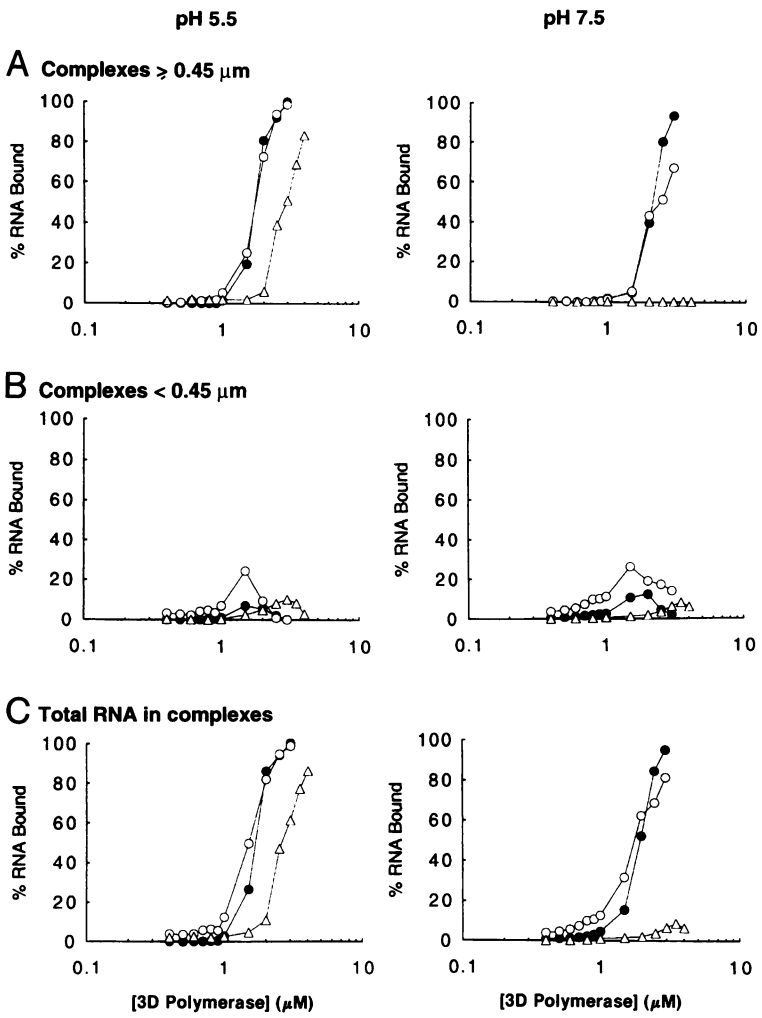

FIGURE 7. Binding of poliovirus positive-strand and negative-strand RNA transcripts as a function of 3D polymerase concentration. Percentages of RNA bound to (**A**) polysulfone membrane, (**B**) nitrocellulose membrane, and (**C**) both membranes together were plotted as a function of 3D polymerase concentration at pH 5.5 (left panels) and pH 7.5 (right panels). ●, poliovirus positive-strand RNA; ○, poliovirus negative-strand RNA. Data for 3D polymerase binding to HP1 RNA (△) were replotted from Figure 3 for comparison.

was present in such large complexes at pH 7.5 (Fig. 5). Thus, the 7,500-nt polioviruses RNAs either promote polymerase aggregation or preferentially bind to polymerase molecules that are in preformed complexes.

DISCUSSION

Oligomerization of poliovirus RNA-dependent RNA polymerase

The observations that RNA binding by poliovirus polymerase was highly cooperative, and that approximately 1 μM polymerase concentrations were required to detect binding at most pHs, were surprising based on previous studies of poliovirus polymerase isolated from infected HeLa cells (Oberste, 1988; Oberste & Flanegan, 1988). In those experiments, polymerase binding to all RNAs tested, including the full-length poliovirus genome, subgenomic transcripts, and homopolymers, fit simple binding curves with dissociation constants in the nanomolar range. It is possible that the polymerase used in the present work, which was produced in *Escherichia coli* cells, behaves differently than polymerase purified from poliovirus-infected HeLa cells. However, it seems unlikely that this explains the difference in the observed RNA-binding activities because highly purified polymerase from *E. coli* and baculovirus expression systems has been shown to behave identically to polymerase purified from infected cells with regard to the kinetics of primer elongation on both poly(A) and poliovirus RNA templates and by the efficiency of UTP incorporation (Neufeld et al., 1991). An alternative explanation is that one of the other differences between the binding reactions, besides the polymerase itself, causes a dramatic difference in the RNA-binding properties of the polymerase. For example, because the binding of RNA by poliovirus polymerase itself is so weak, a contaminant that could bind RNA even moderately tightly would overwhelm the binding by the polymerase.

Poliovirus 3D polymerase molecules can interact with each other to form large complexes. These large complexes, as well as smaller complexes, are clearly active in the elongation of template RNAs. If the larger

complexes were not active, the percentage of RNA elongated would decrease at high polymerase concentrations when all the RNA was bound in large complexes (see Fig. 3, especially the data for pH 4.5). Instead, it appears that the conditions that promote polymerase aggregation also promote the polymerase–polymerase interactions required for efficient template utilization.

Another unexpected result from these experiments is the low pH optima for both RNA binding and elongation of the hairpin primer/template RNA. At pH 4.5, approximately 10-fold lower polymerase concentrations were required to achieve 50% template binding and elongation than at any of the higher pHs. This is likely to be due to the increased polymerase–polymerase interactions at low pH. It is interesting to note that the binding of poliovirus subviral particles to RNA is also optimal at pH 4.5 (Nugent & Kirkegaard, 1995). The pH in the local environment of the membranous replication complexes in which poliovirus RNA synthesis and RNA packaging occur is not known. When polymerase binding of full-length positive and negative strand poliovirus RNAs was studied, polymerase binding was highly cooperative and no longer showed a pH dependence. This could result from the presence of higher-affinity binding sites in the poliovirus RNAs or a more pronounced length dependence of RNA binding at higher pHs. In either case, cooperative RNA binding by poliovirus polymerase occurs on the biologically relevant RNAs under pH conditions predicted to be physiologic.

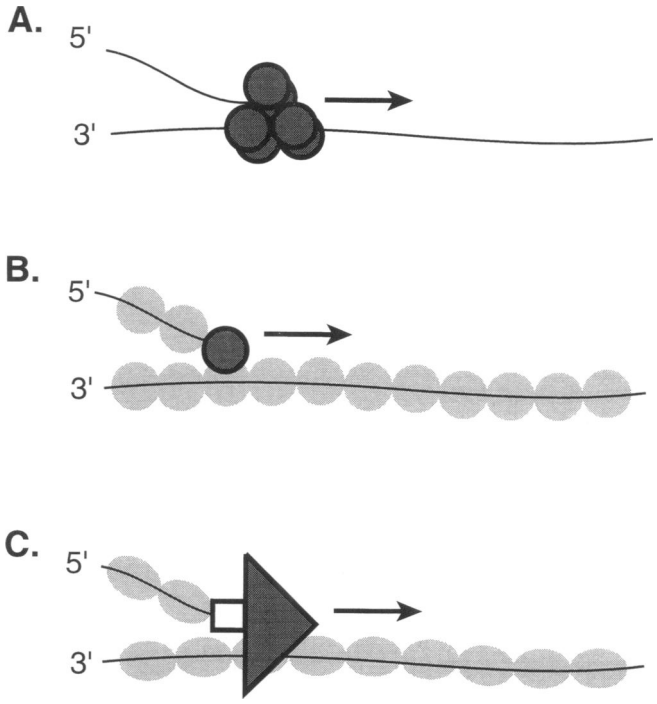

FIGURE 8. Models for RNA production by poliovirus. **A:** A multimeric 3D polymerase catalyzes RNA synthesis. **B:** Viral RNAs serve as templates for 3D polymerase when coated with 3D polymerase. **C:** Schematic drawing of RNA production by negative-strand RNA viruses, as exemplified by VSV. Negative-strand RNAs coated with viral protein N serve as templates for the viral polymerase complex containing proteins L and NS.

RNA synthesis by poliovirus RNA-dependent RNA polymerase in infected cells

A straightforward interpretation of cooperative RNA binding by poliovirus 3D polymerase is that the polymerase functions as a multisubunit enzyme (Fig. 8A). That polymerases can function as oligomers is not without precedent. For example, HIV reverse transcriptase functions as an asymmetric dimer in which one of the subunits is proteolytically processed (di Marzo Veronese et al., 1986; Le Grice et al., 1991; Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993). This type of oligomerization is fundamentally different from that described here, however. The cooperative binding and parallel template utilization by poliovirus 3D polymerase indicate that the functionally important polymerase–polymerase interactions occur during the course of the in vitro experiments. The binding data indicate that several polymerase molecules would be present in the hypothetical multisubunit enzyme. It seems unlikely to us that several loosely associated subunits would be required for activity.

An alternative hypothesis is that the poliovirus 3D polymerase functions both as a catalytic RNA-dependent RNA polymerase and as a cooperative single-stranded RNA-binding protein (Fig. 8B). Many viruses produce a single-stranded nucleic acid binding protein that is required for genome replication. For example, vesicular stomatitis virus (VSV), a negative-strand RNA virus, produces a nucleocapsid protein (N) that binds relatively nonspecifically to RNA. The formation of a complex between VSV RNA and multiple copies of the N protein is required for utilization of the RNA as a template by the VSV polymerase (Fig. 8C; Emerson & Yu, 1975; De & Banerjee, 1984; Wertz et al., 1987; Peluso & Moyer, 1988; Pattnaik & Wertz, 1990). A similar situation exists for influenza virus and many other negative-strand RNA viruses (reviewed in Fields et al., 1990). In the case of poliovirus RNA synthesis, however, we suggest that the single-stranded RNA-binding protein and the polymerase could be the same polypeptide.

The highly cooperative nature of RNA binding by poliovirus polymerase is reminiscent of the nucleic acid binding properties of known single-stranded DNA-binding proteins. For example, bacteriophage T4 gene 32 protein binding to single-stranded nucleic acids is sigmoidal and occurs between 1 and 10 μM gene 32 protein concentrations (Kowalczykowski et al., 1981). For poliovirus, a cooperative single-stranded RNA-binding protein could provide a mechanism for regu-

lating the utilization of RNA templates for either translation or replication by inhibiting translation and promoting RNA replication. If the polymerase does indeed function as a cooperative single-stranded binding protein, mutations in the polymerase that disrupt cooperative RNA binding would be expected to increase translation of viral proteins by increasing the availability of viral RNAs. Two temperature-sensitive mutations in poliovirus polymerase (Diamond & Kirkegaard, 1994) have been characterized that decrease RNA synthesis but increase translation after shift to the nonpermissive temperature. Experiments are in progress to determine if these mutant polymerases exhibit decreased abilities to bind RNA cooperatively in vitro.

Thus far, the involvement of single-stranded nucleic acid binding proteins in the replication of positive strand RNA viruses has not been determined, though it has been clearly demonstrated that such proteins are important in the replication of DNA viruses and negative-strand RNA viruses. Psoralen crosslinking of poliovirus replication complexes in infected cells revealed that the RNAs contained little or no duplex structure (Richards et al., 1984). Thus, we expect that single-stranded RNA-binding proteins, host or viral, are required during RNA synthesis in the infected cell.

What are the conditions that favor polymerase–polymerase interactions during viral infection? The poliovirus RNA replication complex assembly and function may require protein constituents from the host cell. Candidate host factors for RNA replication include a 36-kDa protein that, with viral protein 3CD, forms a ribonucleoprotein complex at the 5′ end of the viral genome (Andino et al., 1990, 1993). This 36-kDa protein may be a proteolytic fragment of EF-1α (Harris et al., 1994). Other candidate host factors include a 36-kDa protein that binds to the 3′ end of the viral negative strand (Roehl & Semler, 1995), factors that restore RNA replication to in vitro extracts prepared in the presence of guanidine (Barton & Flanegan, 1995), and a 68-kDa protein that interacts with 3D polymerase in the yeast two-hybrid system (A. McBride, A. Schlegal, & K. Kirkegaard, manuscript in prep.). Any of these proteins, or host factors that are not yet identified, could function to facilitate RNA recognition, initiation, or elongation by the poliovirus polymerase.

The expression strategy of poliovirus makes it possible to put any of the viral polypeptides to a structural use. Because poliovirus proteins are translated from a single long open reading frame, the same number of "nonstructural" proteins are synthesized as capsid proteins. Thus, over the course of a poliovirus infection, approximately 3×10^7 copies of each protein are produced (Koch & Koch, 1985), yielding a final concentration of each protein of approximately 25 μM by the end of an infectious cycle, if processing were complete. From similar calculations, the final concentration of viral RNA is approximately 0.5 μM (Koch & Koch, 1985). Given the ability of 3D polymerase to bind RNA in a highly cooperative manner, we suggest that it could play a structural role as a single-stranded binding protein as well as a catalytic polymerase.

The intrinsic activities of 3D polymerase in isolation may then be promoted or facilitated by the action of other viral or cellular proteins. 3AB, for example, a membrane-associated protein that is the precursor for the genome-linked 22-amino acid protein 3B, is known to interact with 3D polymerase: micromolar concentrations of 3AB stimulate RNA-dependent RNA polymerization by nanomolar concentrations of 3D polymerase (Lama et al., 1994; Paul et al., 1994) and the presence of 3AB stimulates the specific proteolytic processing of 3CD, the precursor of 3D polymerase (Molla et al., 1994). In addition, 3AB may itself have RNA-binding properties, certainly forming large aggregates in which RNA is present (Paul et al., 1994; Harris et al., 1994). It is possible that the known 3AB–3D interactions facilitate the polymerase–polymerase interactions required for efficient RNA-dependent RNA synthesis documented here, that both proteins bind to poliovirus RNAs, or that alternative complexes with different viral and perhaps host proteins function during different steps of viral RNA synthesis.

MATERIALS AND METHODS

Polymerase

Poliovirus 3D polymerase was purified using methods to be described elsewhere (J. Hansen, A. Long, & S. Schultz, manuscript in prep.). Briefly, polymerase was expressed in BL21(DE3)pLysS *E. coli* cells from a plasmid, pT5T-3D, constructed by Thale Jarvis (Ribozyme Products, Inc., Boulder, Colorado), that contained the 3D-coding sequence preceded by an initiator methionine codon. Polymerase was precipitated from a cell lysate by the addition of ammonium sulfate to 40% saturation. The precipitate was resuspended and polymerase was further purified by chromatography over a Hi-Load S 16/10 column (Pharmacia, Uppsala, Sweden) followed by chromatography over a Hi-Load Q 16/10 column (Pharmacia). The polymerase was used directly after elution from the Q column, and was present in a solution containing 25 mM Hepes, pH 8.5, 15% glycerol, 0.25% β-octylglucoside, 0.02% NaN_3, 0.1 mM EDTA, and 300 mM NaCl. The polymerase was greater than 95% pure, as estimated from a Coomassie-stained protein gel. The concentration of polymerase was determined by UV absorption using a calculated extinction coefficient of $7.243 \times 10^4\ M^{-1}cm^{-1}$ (Gill & von Hippel, 1989). Concentration of purified polymerase ranged from 0.2 to 2.5 mg/mL (approximately 4–50 μM). Polymerase was stored at 4 °C. Under this storage condition, elongation activity and cooperative RNA-binding activity were found to be stable for one month.

3D polymerase labeled with [^{35}S]-methionine was purified as above except that 5 mCi L-[^{35}S]-methionine (1,110 Ci/mmol, NEG-009L, NEN, Boston, Massachusetts) was added to 1.5 L of cell culture at the time of induction, and

gravity flow S-sepharose and Q-sepharose columns were used for the chromatography.

Oligonucleotides

T7P7356A (5′-TAATACGACTCACTATAGGCTTGACTCATT TTAGTAA-3′) and PN7440B (5′-AAAAATTTTTTTTTTTTTT TTTTTCTCCGAATTA-3′) were synthesized by Macromolecular Resources (Fort Collins, Colorado).

RNA

T7 7356-7440H, the DNA template for HP1 RNA, was constructed by PCR using 400 pmol each T7P7356A and PN7440B deoxyoligonucleotides and 1.4 μg plasmid pBSII T7 7440-7053, a derivative of pBluescript II KS+ (Stratagene, La Jolla, California) containing poliovirus sequences 7440–7053 downstream of a T7 RNA polymerase promoter. Taq polymerase reaction conditions were: 50 mM potassium glutamate, 20 mM Hepes-KOH, pH 8.4, 5.5 mM $MgCl_2$, 1 mM each dATP, dCTP, dGTP, dTTP (Pharmacia), and 0.05 U/μL Amplitaq (Perkin-Elmer Cetus, Norwalk, Connecticut) (Jarvis & Kirkegaard, 1992). Reactions were performed in 100 μL total volume for 10–12 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. PCR products were separated on 3% Nuseive/1% Seakem/0.5× TBE agarose gels (FMC Bioproducts, Rockland, Maine) containing 0.5 μg/mL ethidium bromide and were purified with DEAE membranes (Sambrook et al., 1989).

Full-length poliovirus positive and negative strand RNAs were synthesized, respectively, from plasmid DNAs T7pGEM-polio (Sarnow, 1989) digested with *Eco*R I, and T7oilop (Nugent & Kirkegaard, 1995) digested with *Sma* I.

Transcription reactions contained: 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each ATP, CTP, GTP (Pharmacia), approximately 0.1 μM PCR DNA template or approximately 1 μg plasmid DNA template, 4 U/μL T7 RNA polymerase (Promega, Madison, Wisconsin), 0.05 mM UTP (Pharmacia), and 0.5 μCi/μL [α-^{32}P]-UTP (3,000 Ci/mmol, NEN). After incubations at 37 °C for 2 h, RNase-free DNase (Boehringer-Mannheim, Indianapolis, Indiana) was added to 0.2 U/μL and incubation was continued for 15 min at 37 °C. RNA was purified by gel filtration over NICK-spin columns (Pharmacia), to remove unincorporated nucleotides, phenol extraction, and ethanol precipitation from 2.5 M ammonium acetate. RNA from each 100 μL transcription reaction was resuspended in 100 μL of a solution containing 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

Polymerase binding and elongation reactions

HP1 RNA at 1 nM, or poliovirus positive and negative-strand RNA at 0.01 nM, was incubated with 10 nM to 5 μM 3D polymerase in polymerase reaction buffer (25 mM Hepes-NaOH, pH 7.5 or 8.5, or 25 mM MES-NaOH, pH 4.5, 5.5, or 6.5, 5 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 10 μg/mL actinomycin D, 5 mM DTT, and 0.25 mM each ATP, CTP, GTP, and UTP [Pharmacia]). One-fifth of the reaction volume was composed of polymerase or polymerase buffer, such that final reaction also contained 60 mM NaCl. For binding of polymerase to RNA followed by elongation, 50-μL reactions were incubated in polystyrene microtiter dishes for 30 min on ice, 35-μL aliquots were filtered as described below, then the remaining 15-μL reaction was incubated for 30 min at 30 °C. Unless otherwise noted, elongation reactions were terminated by the addition of 3 μL of 5× proteinase K mix (250 μg/mL proteinase K [Sigma, St. Louis, Missouri], 375 mM Hepes-NaOH, pH 8, 0.5% SDS, 25 mM EDTA) and incubation for 10 min at 37 °C, followed by the addition of 30 μL formamide. Extension products were boiled 3 min, then separated by electrophoresis on 8% acrylamide (19:1)/7 M urea/0.5× TBE gels run at approximately 50 °C. Reaction products formed at pH 6.5 with Hepes as a buffer did not differ from those formed at pH 6.5 with MES (data not shown).

Filter-binding assay

Detection of RNA bound to polymerase was performed using a modified nitrocellulose filter-binding assay (Wong & Lohman, 1993). Three filters were used concurrently: the top filter was polysulfone (HT Tuffryn, 0.45-μm pore size, Gelman Sciences, Ann Arbor, Michigan) to retain aggregated RNA-polymerase complexes, the middle filter was nitrocellulose (BA-85, Schleicher and Schuell, Keene, New Hampshire) to retain soluble RNA-polymerase complexes, and the bottom filter was positively charged nylon (Hybond-N+, Amersham, Arlington Heights, Illinois) to capture unbound RNA. Membranes were soaked in wash buffer (50 mM Hepes-NaOH, pH 7.5, 5 mM $MgCl_2$) for at least 1 h prior to use. An unmodified 96-well dot blot apparatus (Schleicher and Schuell) was used for the filtration. The three membranes were placed on top of a pre-wet gel blot paper (GB002, Schleicher and Schuell). Aliquots (35 μL) of each binding reaction were placed in individual wells and all were filtered simultaneously by applying vacuum. Vacuum was then stopped, 500 μL wash buffer was added to all wells, and vacuum was reapplied until all wells were dry. Filters were then separated and the amount of RNA bound to each was measured with a phosphorimager (Molecular Dynamics, Sunnyvale, California). In the absence of protein, less than 1% of the RNA bound to the nitrocellulose membrane and undetectable amounts bound to the polysulfone membrane.

To determine the affect of pH on the aggregation of polymerase, in the absence of RNA, 0.5–4.0 μM [^{35}S]-methionine 3D polymerase, prepared as described above, was incubated in 25 mM MES-NaOH, pH 4.5, 5.5, or 6.5, or 25 mM Hepes-NaOH, pH 7.5, for 30 min on ice. The total reaction volume was 50 μL; one-fifth of the reaction volume was composed of polymerase or polymerase buffer, such that the final reaction also contained 60 mM NaCl. Aliquots (40 μL) of each reaction were filtered as described above. Distribution of the labeled polymerase among the three filter membranes was determined by autoradiography. No polymerase was detected bound to the positively charged nylon membrane.

Chemical crosslinking

3D polymerase, present at 1–4 μM, was incubated for 30 min at 30 °C in 5–20 μL of crosslinking buffer (50 mM Hepes-NaOH, pH 7.5, 5 mM $MgCl_2$, 0.5 mM GTP) with or without 0.4% SDS. Glutaraldehyde, which can form intermolecular or intramolecular crosslinks of a variety of lengths between

primary amines (Wold, 1972; Peters & Richards, 1977), was then added to a final concentration of 0.005% and the incubation was continued for 5 min at room temperature. Reactions were stopped by adding an equal volume of 2× SDS/PAGE sample loading buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 0.01% β-mercaptoethanol) and heating at 95 °C for 5 min. Samples were analyzed by SDS/5% PAGE (Sambrook et al., 1989). Silver staining was performed as described (Blum et al., 1987) except that the developer was used at one-fifth dilution.

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