
Measurement of poliovirus RNA polymerase binding to poliovirion and nonviral RNAs using a filter-binding assay

M. Steven Oberste and James B. Flanagan

Department of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, FL 32610, USA

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ABSTRACT

The binding of the purified poliovirus RNA-dependent RNA polymerase to viral and nonviral RNAs was studied using a protein-RNA nitrocellulose filter binding assay. A cellular poly(A)-binding protein was found in viral polymerase preparations, but was easily separated from the polymerase by chromatography on poly(A) Sepharose. Optimal conditions for the binding of purified polymerase (fraction 5-PAS) to ³²P-labeled poliovirion RNA were determined. The binding of purified polymerase to ³²P-labeled ribohomopolymeric RNAs was examined, and the order of binding observed was poly(G) >>> poly(U) > poly(C) > poly(A). In competitive binding studies, the polymerase bound with equal efficiency to virion RNA and to a subgenomic transcript which contained the 3' end of the genome. The polymerase bound to 18S ribosomal RNA and to globin mRNA equally well, but with a five-fold lower affinity than to virus-specific RNAs. The results suggest that the polymerase exhibits sequence specificity in binding and that polymerase binding sites in poliovirus RNA may contain (G- and/or U)-rich sequences.

INTRODUCTION

The poliovirus genome is a single strand of positive sense RNA which replicates in the cytoplasm of infected cells (1). Virion RNA contains a poly(A) tract of 75 to 100 residues at its 3' end (2,3,4), most or all of which is coded by a poly(U) sequence in the minus strand RNA (5,6,7,8). A small virus-specific protein, VPg (protein 3B), is linked to the 5' end of virion RNA and to the (+)- and (-)-strands of dsRNA and replicative intermediate RNA (9,10,11). Poliovirus RNA replication requires a virus-specific RNA-dependent RNA polymerase (replicase). A single virus-specific protein (3D^{Pol}) copurifies with polymerase activity isolated from cytoplasmic extracts of infected cells (12). The purified polymerase copies a number of homopolymeric and heteropolymeric RNA templates and requires an oligonucleotide primer to initiate RNA synthesis *in vitro* (13,14). Several different "host factor" preparations have been reported which stimulate the initiation of RNA synthesis by the polymerase in the absence of added primer (15,16,17,18). The precise mechanism of action and the role of the different

host factor activities in viral replication in vivo have not yet been determined, nor has the exact nature of the interactions between the polymerase and its templates, primers, and host factor proteins.

The best studied RNA-dependent RNA polymerase is that encoded by bacteriophage Q β (reviewed in references 19 and 20). The active replicase is a complex of one viral protein and three cellular proteins. An additional cellular protein, known as host factor, is required for in vitro replication of Q β RNA. The Q β replicase binds to Q β RNA about tenfold more tightly than to nonhomologous RNAs. The replicase interacts with two internal binding sites and with a C-rich sequence at the 3' end of Q β RNA, apparently relying more upon RNA structure than sequence for recognition. The Q β replicase-host factor complex exhibits a high degree of specificity for Q β RNA templates. Under certain conditions, however, the template specificity of the replicase is significantly reduced. In the presence of an oligonucleotide primer, the specificity of initiation is entirely bypassed and the replicase will efficiently copy nonhomologous RNAs. This is similar to the elongation activity observed with the poliovirus polymerase on different templates in the presence of oligonucleotide primers.

Only limited information is available on sequence recognition signals for RNA-dependent RNA polymerases of eucaryotic viruses. A 3'-terminal 134-nucleotide sequence with a tRNA-like structure in brome mosaic virus RNA was recently shown to have a very important role in template recognition by its viral polymerase (21). Genetic studies with poliovirus suggest that 3'-terminal sequences in both the (+)- and (-)-strand RNAs are required for RNA replication (22,23). To initiate RNA synthesis, the poliovirus polymerase must bind to the template RNA, perhaps at very specific sites. Thus, it is important to determine whether specific sequence signals exist in poliovirus RNA which are required for polymerase binding and replication. This paper reports the initial results from our studies on polymerase binding to virion and ribohomopolymeric RNAs. A specific filter binding assay and the relative association constants for polymerase binding to ribohomopolymers are described.

MATERIALS AND METHODS

Enzymes and reagents

T4 RNA ligase, T4 polynucleotide kinase, bacterial alkaline phosphatase, Klenow fragment of *E. coli* DNA polymerase I, SP6 RNA polymerase, and rabbit globin mRNA were purchased from Bethesda Research Laboratories,

Gaithersburg, Md. Ribonucleic acid homopolymers were from Pharmacia P-L Biochemicals, Inc., Piscataway, N.J. The ribohomopolymers were heterogeneous in size as determined by polyacrylamide gel electrophoresis and therefore, concentrations were expressed as $\mu\text{g/ml}$. All ^{32}P - and ^3H -labeled ribonucleoside triphosphates were purchased from Amersham Corp., Arlington Heights, Il.

Cell culture

HeLa cells were maintained in suspension culture and infected with poliovirus type 1 (Mahoney strain) as previously described (24).

Purification and labeling of poliovirion RNA

Poliovirion RNA was isolated from infected cells as described (25). Virion RNA was labeled at the 3' end using T4 RNA ligase and [^{32}P]pCp as described (26) except that a 30 μl reaction contained 8 μg virion RNA, 40 μCi [^{32}P]pCp, and 8 μg ligase. The specific activity of labeled virion RNA was about 1×10^6 cpm/ μg (2.5×10^6 cpm/pmole).

Synthesis of a poliovirus-specific 3'-terminal RNA transcript

A poliovirus-specific cDNA clone was constructed which contained viral nucleotides 6012-7440 plus an 83-nucleotide poly(A) tract in the transcription vector pGEM-1 (27). Full-length cDNA clone pOF2612 was digested with Asu II and Sal I, the overhanging ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I, and the resulting blunt ends were ligated to yield pOF1209 (27). Poliovirus-specific RNA was synthesized by transcription of Eco RI-digested pOF1209 with SP6 RNA polymerase (28). The resulting transcript contained 1514 poliovirus-specific nucleotides with 26 vector-derived nucleotides at the 5' end and five vector-derived nucleotides at the 3' end.

Labeling of ribohomopolymers

Ribohomopolymers were dephosphorylated with bacterial alkaline phosphatase and 5' end labeled with T4 polynucleotide kinase and [γ - ^{32}P]ATP, as described (29). Specific activity of labeled homopolymers was in the range 1×10^6 to 1×10^7 cpm/ μg .

Purification of poliovirus RNA polymerase

Fraction 4-HA polymerase was purified from poliovirus-infected cells as described (25). Polymerase used in some experiments was further purified by chromatography on a 1.8 x 1.0 cm column of poly(A)-Sephacrose (AGPOLY(A)TM type 6, Pharmacia Fine Chemicals, Piscataway, N.J.). The sodium phosphate content of pooled fraction 4-HA polymerase was diluted to 35 mM with poly(A)-Sephacrose (PAS) binding buffer (50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol,

10 $\mu\text{g/ml}$ ovalbumin, 20% glycerol). The polymerase was loaded onto a poly(A)-Sephacrose column equilibrated with PAS binding buffer, washed with 20 ml PAS binding buffer, and eluted with a 60 ml linear 0-0.5 M KCl gradient in 1 ml fractions. KCl concentration of selected fractions was calculated from the conductivity measured with a Radiometer conductivity meter. Polymerase elongation activity was assayed using a poly(A) template and oligo(U) primer as described (30). RNA binding activity was measured as described below, and fractions containing both binding and elongation activities were pooled and stored at -70°C . This polymerase preparation was designated fraction 5-PAS and was stable for at least six months at -70°C .

A control extract was isolated from uninfected cells following the same protocol used to isolate the polymerase. A mock-infected HeLa cell suspension culture was prepared by treating uninfected HeLa cells with 5 $\mu\text{g/ml}$ actinomycin D (Sigma Chemical Co., St. Louis, Mo.) and incubating at 37°C for six hours. A cytoplasmic extract was prepared and carried through the fraction 4-HA step as previously described for the purification of poliovirus polymerase from infected cells (25). Fraction 4-HA control extract was chromatographed as above on a separate poly(A)-Sephacrose column. RNA binding activity was measured as described below. Since RNA-dependent RNA polymerase activity was not present in the uninfected extracts, the fractions which normally contain polymerase activity were selected at each purification step for the isolation of the control extract.

Poliovirus polymerase-RNA binding reactions

Protein-RNA filter binding assays were performed using a modification of the procedure of Zimmern and Butler (31). Standard binding reactions contained 200 fmole ^{32}P -labeled virion RNA or a varying amount of a ^{32}P -labeled ribohomopolymer, and fraction 4-HA or fraction 5-PAS polymerase in 50 mM Hepes (N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid), pH 8.0, 7 mM MgCl_2 in a total volume of 100 μl . After incubation at 30°C for 60 min, reaction solutions were diluted to 1 ml with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE) and filtered through 0.45 μm nitrocellulose filter disks (Schleicher and Schuell BA 85) which were previously boiled for 30 min in TE. The filters were washed with 6 ml of TE and counted in Aquasure (Beckman Instruments) in a Beckman liquid scintillation counter. Binding was expressed as fmoles of labeled RNA specifically bound, calculated from the specific activity of the labeled RNA. Binding in the absence of added protein or in the presence of a nonspecific protein (eg. bovine serum albumin or ovalbumin) was typically 0.5-2.0% of the total added radioactivity and was subtracted as background from the experimental values.

RESULTS**Binding of polymerase to poliovirion RNA**

The protein-mediated binding of labeled RNA to nitrocellulose filters in low ionic strength buffer is a rapid and sensitive assay for the binding of proteins to RNA. In our initial experiments, we found that our fraction 3 and fraction 4-HA polymerase preparations (25) bound ^{32}P -labeled poliovirion RNA in the filter binding assay. To determine if the observed binding activity was virus specific, a control extract (fraction 4-HA) was prepared from uninfected HeLa cells using the same purification protocol. Unexpectedly, the control extract also bound virion RNA, although at a reduced level. To determine if there were any differences in the specificities of the RNA binding activities in the polymerase and control extract preparations, we used ribohomopolymers as competitive inhibitors. Increasing amounts of unlabeled poly(A), poly(C), poly(G), or poly(U) were added with 200 fmole of ^{32}P -labeled virion RNA during the 60 min binding reaction, and the resulting amount of protein-bound RNA was determined using the filter binding assay. With the viral polymerase, the relative order of inhibition observed was poly(G) >>> poly(U), poly(A) > poly(C) (Fig. 1).

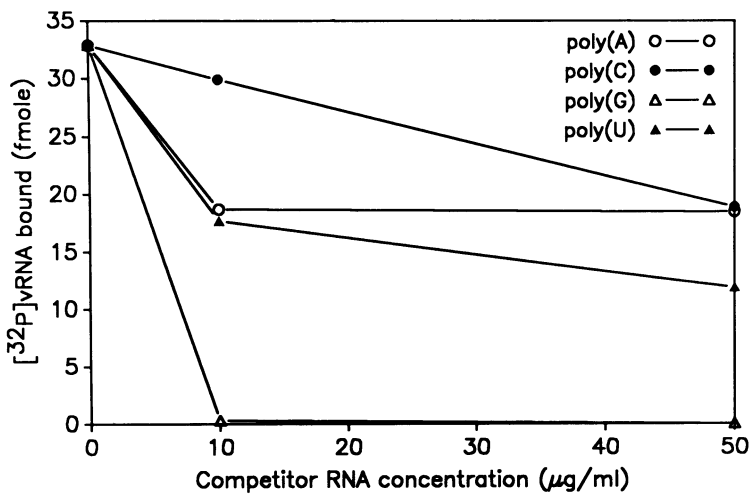


Figure 1. Inhibition of poliovirus polymerase (fraction 4-HA) binding to poliovirion RNA by ribohomopolymers. The filter binding assay was used to measure polymerase binding to ^{32}P -labeled poliovirion RNA in the presence of the indicated concentration of unlabeled ribohomopolymer. The conditions were as described in Materials and Methods except MgCl_2 was not added to the binding reaction.

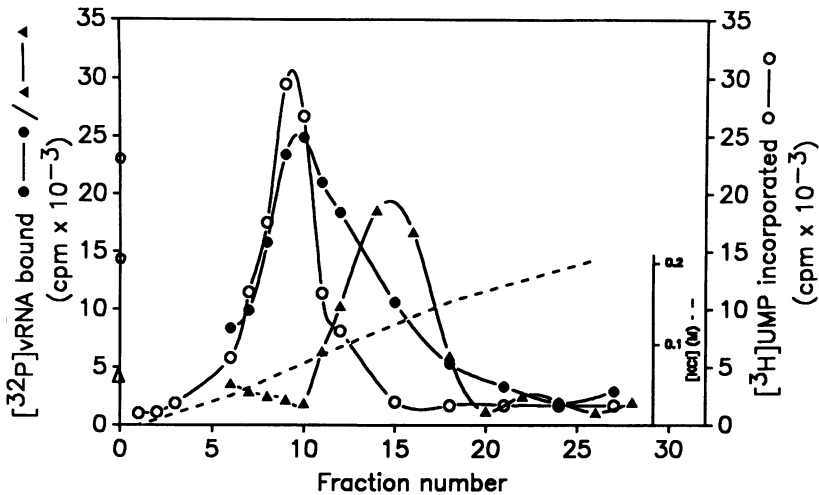


Figure 2. Purification of poliovirus polymerase on poly(A) Sepharose column. Fraction 4-HA polymerase and control extract were isolated and chromatographed on separate poly(A) Sepharose columns using a linear KCl gradient (dashed lines) as described in Materials and Methods. The separate elution profiles were superimposed for clarity. The polymerase fractions were assayed for both elongation activity (open circles) and binding activity (closed circles). The control extract fractions were assayed for binding activity (closed triangles) only. The binding reactions contained ³²P-labeled virion RNA as described in Materials and Methods. The activities recovered in the flow-through are indicated in fraction 0.

With the control extract, however, poly(A) was a very strong inhibitor, and the other ribohomopolymers had little or no effect (data not shown). Thus, the cellular binding activity was very specific for poly(A) and was distinct from the binding activity observed with the viral polymerase.

Based on the above results, we reasoned that it should be possible to separate the viral polymerase from a contaminating cellular poly(A)-binding protein by chromatography on a poly(A)-Sepharose column. The polymerase and control extract preparations (fraction 4-HA) were chromatographed on separate columns. The peak of binding activity in the control extract eluted from the column with 120 mM KCl (Fig. 2). In contrast, the peak of RNA binding activity in the polymerase eluted from the column at 70 mM KCl along with the peak of polymerase elongation activity (Fig. 2). The trailing shoulder of RNA binding activity in the polymerase appeared to represent the contaminating cellular binding activity. The peak column fractions (i.e., fractions 8-10) which contained both polymerase elongation activity and RNA

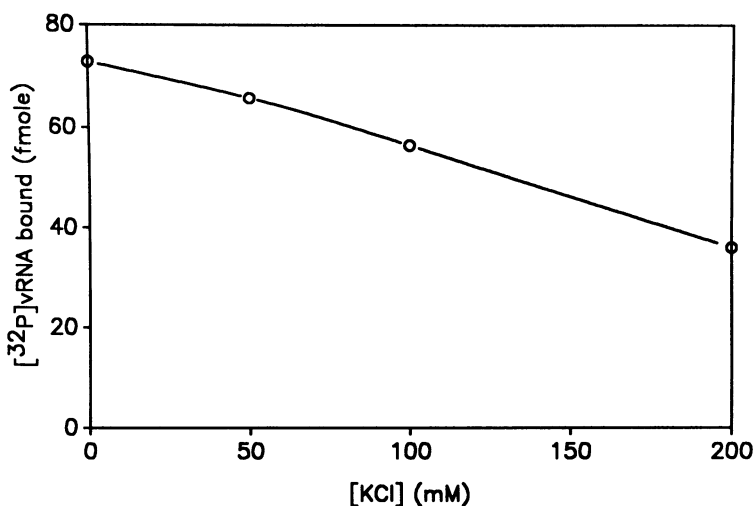


Figure 3. Effect of KCl concentration on poliovirus polymerase binding to virion RNA. The binding of purified polymerase (fraction 5-PAS) to labeled virion RNA was measured using the filter binding assay. KCl was added at the indicated concentration during the 60 min binding reaction.

binding activity and which did not overlap with the fractions containing cellular RNA binding activity were pooled for further studies. This added step of purification resulted in a polymerase preparation (fraction 5-PAS) which exhibited only virus-specific binding to poliovirion RNA.

Optimal conditions for RNA binding

The conditions for RNA binding by the purified polymerase (fraction 5-PAS) were optimized with respect to time, temperature, pH, KCl and $MgCl_2$ concentration. Under standard reaction conditions (see Materials and Methods), maximum binding to virion RNA was observed in less than 5 min. Thus, 60 min. binding reactions were adequate to reach binding equilibrium. Changes in the temperature from 10-42°C and changes in pH from 6.5 to 8.5 had little or no effect on polymerase binding. Increasing the KCl concentration from 0-200 mM was found to inhibit polymerase binding activity (Fig. 3). At 200 mM KCl, the binding activity was reduced by about 50% (Fig. 3). This concentration of KCl, however, will totally inhibit polymerase elongation activity on virion RNA (13). This suggests that the inhibition of polymerase activity by added KCl can be explained only in part by decreased template binding. The requirement for Mg^{+2} in the RNA binding assay was determined by increasing the $MgCl_2$ concentration from 0-16 mM (Fig. 4). Significant

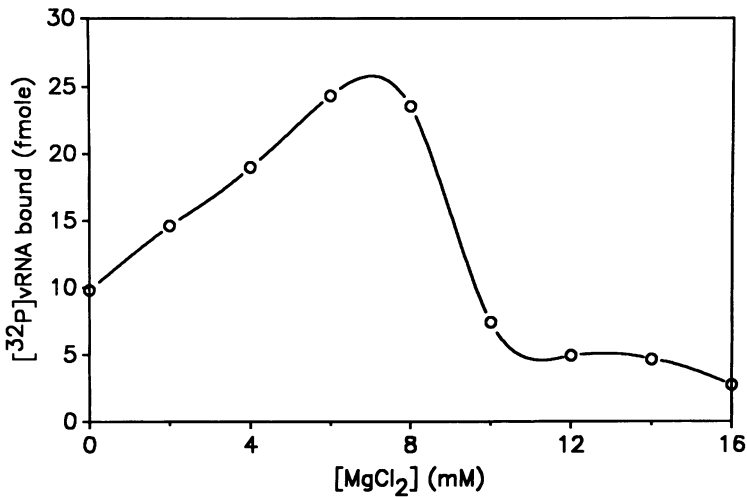


Figure 4. Effect of MgCl₂ concentration on polymerase binding to virion RNA. The filter binding assay was used to measure the binding of fraction 5-PAS polymerase to labeled virion RNA with the indicated concentration of MgCl₂ added during the 60 min binding reaction.

binding activity was observed in the absence of any added MgCl₂, but optimal binding was achieved at 7 mM MgCl₂. This concentration of Mg⁺² also gave maximum elongation rates on virion RNA *in vitro* (32). Addition of EDTA to 1 mM in the absence of added Mg⁺² had no effect on binding (data not shown). The addition of 10 mM dithiothreitol to a standard binding reaction had no effect, and as expected the addition of 0.1% SDS blocked all binding.

Specificity of polymerase binding to ribohomopolymers

The binding specificity of fraction 5-PAS polymerase to labeled ribohomopolymers was determined using the filter binding assay. Increasing amounts of each labeled ribohomopolymer were added to the polymerase under the optimal binding conditions determined above. The polymerase showed a high degree of specificity for binding poly(G) (Fig. 5A). A reduced but significant amount of binding was also observed with poly(U) and poly (C) (Fig 5B). No detectable binding was observed with poly(A) at the concentrations tested (Fig 5B). Thus, the overall order of binding was poly(G) >>> poly(U) > poly(C) > poly(A). The lack of binding with poly(A) indicated that essentially all of the contaminating cellular poly(A)-binding protein was removed in the purification of the fraction 5-PAS polymerase.

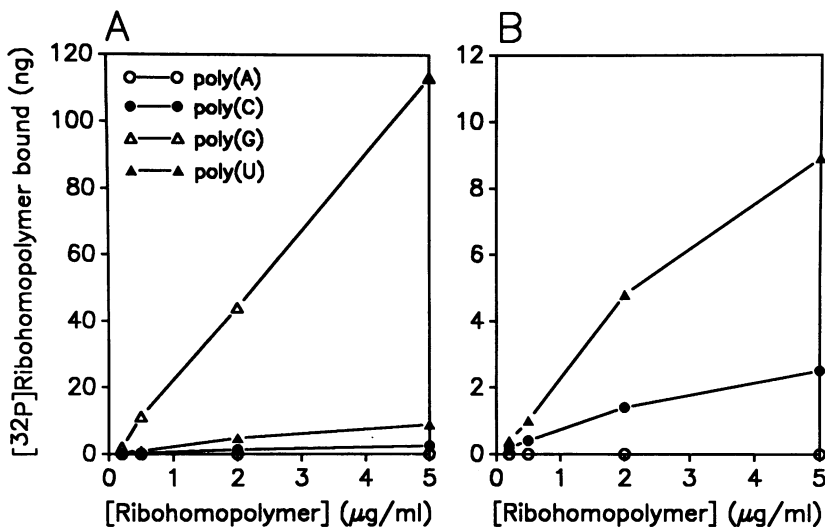


Figure 5. Binding of polymerase to ³²P-labeled ribohomopolymers. (A)- The filter binding assay was used under optimal RNA binding conditions as described in Materials and Methods to determine the amount of labeled ribohomopolymer bound to fraction 5-PAS polymerase in reactions containing the indicated concentration of each ribohomopolymer. (B)- Data for poly(U), poly(C), and poly(A) from panel A plotted on an expanded scale.

Competitive binding curves using ribohomopolymers

Ribohomopolymers were used as competitor RNAs for the binding of purified polymerase to ³²P-labeled virion RNA. Competitive binding data were analyzed using equation (5) described by Lin and Riggs (33), in which the parameter θ is defined as the ratio of the amount of labeled nucleic acid bound to the filter in the presence of competitor to the amount bound in the absence of competitor, i.e.,

$$\theta = \frac{\text{cpm } [^{32}\text{P}]\text{vRNA bound plus competitor RNA}}{\text{cpm } [^{32}\text{P}]\text{vRNA bound}} \quad (1)$$

This term is useful because of the automatic correction for any changes in the retention efficiency or specific activity of the labeled RNA. In addition, the concentration of competitor RNA which reduces θ to 0.5 (i.e., C_4), is inversely proportional to K_a , the apparent equilibrium association constant for polymerase binding to competitor RNA.

Using poly(A), poly(C), poly(G), and poly(U) as competitor RNAs, we

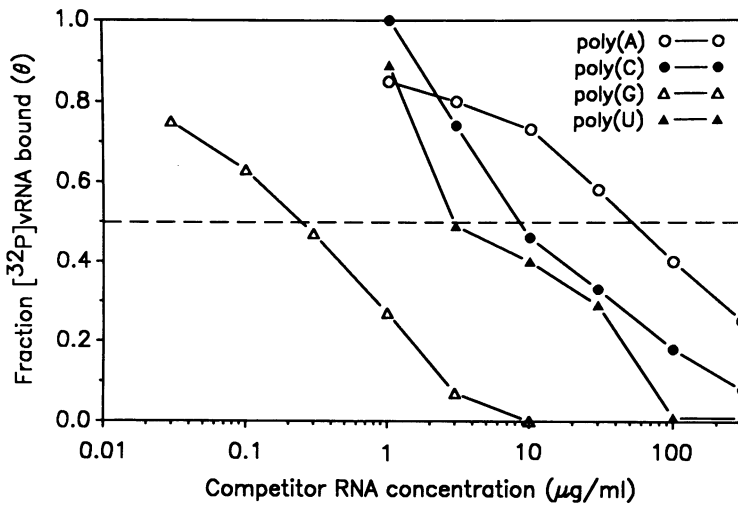


Figure 6. Inhibition of polymerase binding to virion RNA by ribohomopolymeric competitor RNAs. The binding of fraction 5-PAS polymerase to ³²P-labeled virion RNA was measured in the presence of the indicated concentration of each unlabeled ribohomopolymer using the filter binding assay under optimal RNA binding conditions as described in Materials and Methods. The data are presented as the fraction of labeled RNA bound using the term θ as defined in equation (1) in the text. A logarithmic scale was used for the competitor RNA concentrations. The horizontal dashed line indicates $\theta = 0.5$. The values of $C_{1/2}$ in Table 1 were determined at $\theta = 0.5$ for each ribohomopolymer.

determined θ as a function of competitor RNA concentration in the binding reaction (Fig. 6). Values of $C_{1/2}$ were determined for each competitor RNA at $\theta = 0.5$ in Fig. 6 (Table 1). A relative K_a for each ribohomopolymer was

Table 1 - Relative association constants for polymerase binding to ribohomopolymers.

Ribohomopolymer	$C_{1/2}$ ($\mu\text{g/ml}$) ^a	Relative K_a ^b
poly(G)	0.25	1.0
poly(U)	3.0	8.3×10^{-2}
poly(C)	9.0	2.8×10^{-2}
poly(A)	50.0	5×10^{-3}

^a Concentration of ribohomopolymeric competitor RNA required to reduce the fraction of labeled virion RNA bound to 0.5 in Fig. 6.

^b The relative association constants (K_a) are inversely proportional to the values of $C_{1/2}$ and were calculated assuming a value of 1 for poly(G).

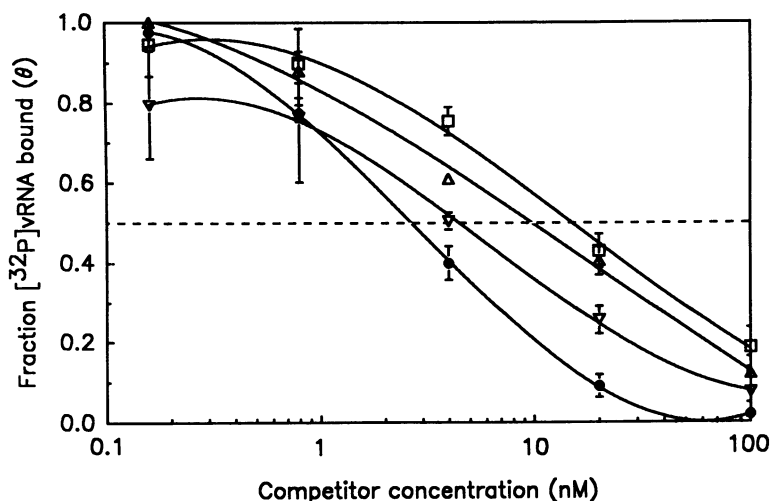


Figure 7. Inhibition of polymerase binding to virion RNA by viral and nonviral competitor RNAs. The binding of fraction 5-PAS polymerase to ^{32}P -labeled virion RNA was measured in the presence of the indicated concentration of unlabeled competitor RNA using the filter binding assay under optimal RNA binding conditions as described in Materials and Methods. The RNAs used were: poliovirion RNA (closed circles), HeLa 18S ribosomal RNA (open triangles), rabbit globin mRNA (open squares), and poliovirus-specific RNA transcribed from pOF1209 (open diamonds). The data are presented as the fraction of labeled RNA bound using the term θ as defined in equation (1) in the text. A logarithmic scale was used for the competitor RNA concentrations. The horizontal dashed line indicates $\theta = 0.5$.

calculated assuming a value of 1 for poly(G) (Table 1). Relative K_a values varied as much as 200-fold, depending on the competing ribohomopolymer, and were consistent with the relative order of binding observed with the individual ribohomopolymers (Fig. 5).

Competitive binding curves using viral and nonviral RNAs

The binding of polymerase to poliovirion RNA, a subgenomic poliovirus-specific transcript RNA, and to nonviral RNAs (HeLa 18S ribosomal RNA and rabbit globin mRNA) was measured using the competitive binding assay (figure 7). In a separate study, the K_a for virion RNA was determined to be $1 \times 10^9 \text{ M}^{-1}$ (27). Based on this value, the K_a values for the competitors were determined relative to virion RNA and were: transcript RNA, $6.0 \times 10^8 \text{ M}^{-1}$; 18S RNA, $2.5 \times 10^8 \text{ M}^{-1}$; and globin mRNA, $1.7 \times 10^8 \text{ M}^{-1}$. The values for virion RNA and the transcript are within a factor of two of one another, which is probably within experimental error. The values for 18S RNA and

globin mRNA were four- to six-fold less than that of virion RNA and this appears to be a significant difference.

DISCUSSION

We have developed a filter-binding assay for quantitating the binding of purified poliovirus RNA polymerase to viral and nonviral RNAs and ribohomopolymers. The binding of labeled RNA to nitrocellulose filters was dependent upon the presence of purified polymerase and was inhibited by conditions which also inhibit RNA synthesis, such as high salt concentration and ionic detergent. The optimum reaction conditions for polymerase binding to virion RNA were similar to those which give maximum elongation rates and yield increased amounts of full-length product RNA *in vitro*. RNA binding activity copurified with the elongation activity of the polymerase, and only virus-specific RNA binding activity was observed after the final step of purification on a poly(A) Sepharose column (fraction 5-PAS).

We were surprised to find a cellular poly(A) binding protein associated with the fraction 4-HA polymerase. We have not yet determined if this protein directly interacts with the viral polymerase or if the addition of this protein stimulates polymerase activity on virion RNA in the oligo(U)-primed or host factor-dependent reactions. This is an interesting possibility since the results of this study showed that the polymerase had a very low binding affinity for poly(A). Since negative strand RNA synthesis initiates at the 3'-end of the poly(A) sequence in virion RNA, one can hypothesize that the polymerase forms a complex with the poly(A) binding protein and that this mediates its binding to virion RNA. Direct binding of the polymerase to a short 3' terminal oligo(U) primer synthesized by a terminal uridylyl transferase host factor (16) or a VPg-pUpU primer (34,35) are other possibilities.

In the binding studies with ribohomopolymers, the polymerase exhibited a high degree of specificity for binding poly(G) relative to poly(C) and for binding poly(U) relative to poly(A). These results were consistent with the data from competitive binding experiments with labeled virion RNA and unlabeled ribohomopolymers. The value of the association constant, K_a , for polymerase binding to poly(G) was 36 times the value for binding to poly(C). Likewise, the K_a for binding poly(U) was 17 times the value for binding to poly(A). In our past studies where polymerase activity was measured using ribohomopolymeric template:primers, we found high levels of activity with poly(A):oligo(U) and poly(C):oligo(G), a five-fold lower level of activity

with poly(U):oligo(A) and essentially no activity with poly(G):oligo(C) (14,36). Taken together, our data suggest that preferential binding of the polymerase to the primer correlates with high levels of RNA synthesis, whereas tight binding to the template correlates with very low levels of synthesis. A high binding affinity between the polymerase and the template may slow the movement of the polymerase on the template and inhibit RNA synthesis. A similar idea was previously proposed for AMV reverse transcriptase based on a correlation between low levels of DNA synthesis and high binding affinities for template:primers (37).

The polymerase binds with about equal efficiency to virion RNA and to a subgenomic transcript which contains the 3' end. The polymerase binds to 18S RNA and to globin mRNA equally well, but with a five-fold lower affinity than to virus-specific RNAs. Thus, there appears to be a significant difference between polymerase binding to poliovirion RNA and to these two nonviral RNAs. The fact that polymerase binds to an RNA representing the 3' 1.5 kb of the genome with about the same affinity as to the full-length RNA suggests the presence of a binding determinant near the 3' end of the genome. Based on our results with the ribohomopolymers, it is tempting to speculate that polymerase binding sites in viral RNA may contain G- and/or U-rich sequences. In a preliminary computer search of the viral genome, we found several very (G/U)-rich sequences in the 3'-terminal region. One could envision that the polymerase might first bind to a specific site near the 3' end and then translocate to the end of the poly(A) tract to initiate negative strand RNA synthesis. The results of this study demonstrate the importance of expanded studies on the binding of purified polymerase to poliovirus RNA. Detailed kinetic parameters for RNA binding can now be determined and factors such as sequence, structure, and the presence of other proteins can be investigated for their effect on polymerase binding.

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