THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 12, Issue of March 20, pp. 6724–6730, 1998
Printed in U.S.A.
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Site Size of Cooperative Single-stranded RNA Binding by Poliovirus RNA-dependent RNA Polymerase*

(Received for publication, August 12, 1997, and in revised form, December 23, 1997)

Mary T. L. Beckman‡ and Karla Kirkegaard§¶

From the ‡Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309 and the §Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305

The poliovirus RNA-dependent RNA polymerase binds cooperatively to single-stranded RNA. We have determined the minimal RNA-binding site size of the poliovirus polymerase using binding titration with oligonucleotides of increasing length. A dramatic increase in affinity was observed when the length of the oligo(U) increased from 8 to 10 nucleotides (nt), arguing that the minimal size of RNA for polymerase binding is 10 nt. Another increase in affinity seen as the oligo(U) reached 24 nt suggests that a 24-nucleotide RNA can be occupied by two polymerase molecules. Direct binding of wild-type polymerase to oligo(U)$_{12}$ and oligo(U)$_{24}$ RNAs showed differences in affinity and cooperativity consistent with this model. The increase in binding affinity seen for oligo(U)$_{10}$ suggests either that the RNA-binding determinants are widely spaced on the polymerase structure or that a substantial conformational change in the polymerase occurs upon the filling of its RNA-binding site.

The poliovirus RNA-dependent RNA polymerase is responsible for phosphodiester bond formation during templated RNA polymerization. A 52-kDa protein, the poliovirus polymerase is encoded by the 3′-most coding sequences of the poliovirus genome and is generated by cleavage from the viral polyprotein by virally encoded proteases. Studies of the mechanism of the poliovirus polymerase have been greatly facilitated by its cloning (1) and expression both in *Escherichia coli* (2–5) and in baculovirus-infected insect cells (6). Polymerase purified from these sources was identical in elongation rate, template specificity, and K_m for nucleotides to polymerase purified from infected cells (6).

The poliovirus polymerase displays a low affinity for RNA, which has led to reports that highly purified polymerase is not an RNA-binding protein (7, 8). However, recent *in vitro* studies using poliovirus polymerase purified from *E. coli* demonstrated that at high concentration, the polymerase is not only capable of binding RNA, but exhibits efficient template utilization (9). Surprisingly, interactions between poliovirus polymerase molecules themselves critically affect elongation activity *in vitro*. Both RNA elongation and RNA binding were shown to be cooperative with respect to polymerase concentration (9). Data in support of polymerase-polymerase interactions have also come from observed interactions in the yeast two-hybrid system (10), from glutaraldehyde cross-linking (9), and from the three-dimensional structure of the polymerase (11). An unusual feature of the poliovirus polymerase x-ray crystallographic structure is an extensive interface between polymerase molecules, termed Interface I (11). More than 23 amino acids are involved in salt bridges, hydrogen bonds, and hydrophobic interactions across Interface I. The packing of polymerase molecules at this interface is head-to-tail, such that long fibers of polymerase extend through the crystal lattice. To characterize further the RNA-binding properties of poliovirus polymerase, we used competition with homopolymeric RNAs of various lengths to determine the minimal site size for polymerase binding to RNA.

EXPERIMENTAL PROCEDURES

Polymerase and Gene 32 Protein—Wild-type poliovirus Mahoney type 1 RNA-dependent RNA polymerase 3D proteins were expressed from plasmids in *E. coli* BL21(DE3)pLysS under the control of a T7 promoter and purified as described elsewhere (11), except that immediately after elution from the final chromatography column, the polymerase preparation was brought to 62% glycerol. The polymerase stocks, in 12.5 mM Tris-HCl (pH 8.0), 0.01% NaN_3, 0.05 mM EDTA, 0.25% β-octyl glucopyranoside, 2 mM dithiothreitol, 120 mM NaCl, and 60% glycerol, were stored at −80 °C. Wild-type polymerase was expressed from the plasmid pT5T-3D constructed by Thale Jarvis (Ribozyme Products, Inc., Boulder, Colorado) and contained the 3D coding region preceded by an initiator methionine.

Purified gene 32 protein, expressed in *E. coli* from a recombinant plasmid (12), was a generous gift from Dr. Y. Shamoo (Yale University). The gene 32 protein stock (89.4 μM) was supplied in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol, and 10% glycerol and was stored at −80 °C.

Nucleic Acids—Full-length positive-strand poliovirus RNA was transcribed from T7pGEM-polio (13) digested with *Eco*RI. The 116-nt [1] RNA was transcribed from pG4Z-517/670 digested with *Bsa*BI and encodes, from 5′ to 3′ of the T7-transcribed RNA, nt 1–29 derived from the vector followed by nt 517–604 of the poliovirus Mahoney type 1 genome. The plasmid pG4Z-517/670 was constructed by inserting the *Bst*UI-*Bam*HI fragment of T7pGEM-polio into pGEM4Z (Promega, Madison, WI) digested with *Hin*cII-*Bam*HI. Transcription reactions were carried out as described previously (9), except that UTP was included at 0.5 mM.

Homopolymers were purchased from Pharmacia (Uppsala, Sweden) and suspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Uridylyl oligonucleotides 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 nt in length, as well as the 46-nt deoxyoligonucleotide (ATAGTTCTGATCCACTCCGGGCCCTACAGGTCATACTGTAATTGCC), were synthesized by Macromolecular Resources (Fort Collins, CO). The sequence of the 46-nt DNA was derived from the yeast *Saccharomyces cerevisiae* double-stranded RNA virus L-A, nt 4506–4551 (14), and thus should represent a heteropolymeric DNA of irrelevant sequence. Thymidyl deoxyoligonucleotides 3, 4, 5, 6, 7, 8, 9, 10, and 11 nt in length were synthesized by the Protein and Nucleic Acid facility at Stanford University.

For the direct binding of oligo(U)$_{12}$ and oligo(U)$_{24}$ RNAs and the 46-nt DNA, the nucleic acids were end-labeled with 50 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; NEN Life Science Products) with 1 unit/μl

* This work was supported by National Institutes of Health Grant AI-25166 (to K. K.) and by the Colorado Institute for Research in Biotechnology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 650-498-7075; Fax: 650-498-7147; E-mail: karlak@leland.stanford.edu.

[1] The abbreviations used are: nt, nucleotide(s); MES, 4-morpholineethanesulfonic acid.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org at Stanford University on February 4, 2007

T4 polynucleotide kinase (New England Biolabs Inc.) in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM dithiothreitol. Unincorporated [γ-^{32}P]ATP was removed by centrifugation through a Micro-spin G-25 column (Pharmacia) once for the DNA and twice for the RNA. Concentration was determined by UV absorption at 260 nm.

Direct Binding and Competition Reactions—Polymerase at concentrations ranging from 0.20 to 7.0 μM was incubated with the appropriate ^{32}P-labeled nucleic acid in 50-μl reactions with final concentrations of 25 mM MES-NaOH (pH 5.5), 60 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 5 mM dithiothreitol, 0.25 mM ATP, 0.25 mM UTP, 0.25 mM GTP, 0.25 mM CTP, and 24% glycerol. Polymerase binding to RNA and enzymatic activity were previously found to be optimal at pH 5.5 under these conditions (9). Reactions were incubated on ice for 30 min. Full-length poliovirus RNA and the 116-nt RNA were present at final concentrations of 0.01 and 1 nM strands, corresponding to 75 and 116 nM nt, respectively. Oligo$(U)_{12}$ and oligo$(U)_{24}$ were present at 12 and 6 nM strands, respectively, corresponding to 144 nM nt.

For competition experiments, unlabeled competitor RNAs at various concentrations were mixed with the radioactively labeled RNA in binding reaction buffer as described above. Polymerase at either 1.5 μM for the full-length poliovirus RNA or 2.0 μM for the 116-nt RNA was added to a final volume of 50 μl, and the competition reactions were allowed to equilibrate on ice for 30 min.

Gene 32 protein in concentrations ranging from 0.12 to 3.0 μM was incubated with the appropriate ^{32}P-labeled nucleic acid in a 25-μl solution containing 5 mM MES-NaOH (pH 6.5), 2 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 0.35 mM $MgCl_2$ and incubated at room temperature for 30 min. The final pH of this solution was 7.5. The 116-nt RNA and the 46-nt DNA were present at final concentrations of 1 and 2 nM strands, corresponding to 116 and 92 nM nt, respectively.

For competition experiments, unlabeled competitor oligo(dT) molecules at various concentrations were mixed with the radioactively labeled RNA or DNA in binding reaction buffer as described above. Gene 32 protein at either 0.60 μM for the 116-nt RNA or 0.22 μM for the 46-nt DNA was added to 25 μl, and the competition reactions were allowed to equilibrate for 30 min at room temperature.

Filter Binding Assay—Complexes present in aliquots of the polymerase-nucleic acid binding reactions were separated by a modified nitrocellulose binding assay described previously (9). For the polymerase binding experiments, the filters were washed with 500 μl/well of a solution containing 50 mM Hepes-NaOH (pH 7.5) and 5 mM $MgCl_2$. When measuring the direct binding of polymerase to ^{32}P-labeled oligo$(U)_{12}$ and the 116-nt RNA in Fig. 5, the filters were washed with 2 ml/well of buffer containing 25 mM MES-NaOH (pH 5.5), 5 mM $MgCl_2$, 0.1 mM $ZnSO_4$, and 5 mM dithiothreitol.

Separation of gene 32 protein-nucleic acid complexes was performed using a similar binding assay with only the nitrocellulose filter, to capture the gene 32 protein-nucleic acid complexes, and the positively charged nylon filter, to capture the unbound nucleic acids. Complexes bound to the membranes were washed with 1 ml/well of a solution containing 50 mM Hepes-NaOH (pH 7.5) and 5 mM $MgCl_2$. Detection and quantitation of the radiolabeled nucleic acids bound to the filters were performed using a Storm PhosphorImaging System with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Data Analysis—All graphing and curve fitting were performed using GraphPAD Prism software (GraphPAD Software for Science, San Diego, CA). Data points for direct binding of nucleic acids to proteins were collected in duplicate or triplicate at each protein concentration. The mean values are given in the figures, with error bars representing S.D.

Direct binding curves that displayed cooperative binding behavior with respect to polymerase concentration were fit to Equation 1, based on the Hill equation.

$$\%\ \text{RNA bound} = 100 \cdot ([\text{polymerase}]^n/(K + [\text{polymerase}]^n)) \quad \text{(Eq. 1)}$$

The exponential term (n) is the Hill coefficient, which can be used as a rough estimate of the extent of cooperativity (15). However, since gene 32 protein binding saturated with <100% of the input nucleic acid bound, the amount of nucleic acid bound at saturation was estimated, and Equation 1 was modified to reflect this (Equation 2).

$$\%\ \text{RNA bound} = 29 \cdot ([\text{gene 32 protein}]^n/(K + [\text{gene 32 protein}]^n)) \quad \text{(Eq. 2)}$$

Direct binding curves that did not display cooperative binding behavior with respect to polymerase concentration were fit to a simple binding curve derived from the law of mass action, where Equation 3 follows,

$$\%\ \text{RNA bound} = B_{max} \cdot ([\text{protein}]/(K_d + [\text{protein}])) \quad \text{(Eq. 3)}$$

and B_{max} is the maximum percent RNA bound, [protein] is the micromolar concentration of the protein, and K_d is the dissociation constant. To determine whether direct binding curves displayed simple or cooperative binding, all curves were fit to both types of equations, and the correlation coefficients (R^2 values) were compared. The binding curves that gave rise to the highest R^2 values were chosen for presentation.

Competition data were fit to Equation 4, which describes competition between bound ligand and competitor for binding to the same site.

$$\%\ \text{Nucleic acid bound} = \text{nonspecific binding} + \frac{(\text{total} - \text{nonspecific binding})}{(1 + [\text{competitor}]/\text{IC}_{50})} \quad \text{(Eq. 4)}$$

Values of IC_{50}, the concentration of competitor at which binding of labeled heteropolymeric RNA was reduced by 50%, were determined by nonlinear regression analysis. Error bars shown for the values of IC_{50} as a function of oligonucleotide competitor length show the 90% confidence interval for each IC_{50} value, calculated from the standard error of the individual competition curves.

RESULTS

Experimental Rationale—These experiments were performed to determine the minimal size of RNA that could be bound by the poliovirus RNA-dependent RNA polymerase. We tested the ability of small RNA oligonucleotides of different lengths to compete for binding of poliovirus polymerase to a 116-nt labeled RNA to which multiple polymerase molecules bind cooperatively (9). The effectiveness of competition is measured as the IC_{50}, the concentration of RNA oligonucleotide (in nucleotides) at which binding to the 116-nt RNA is inhibited by 50%. The IC_{50} values are linearly related to the K_d and therefore reflect the binding energy of the complex between the competing RNA oligonucleotide and polymerase.

Two outcomes are possible, depending on the nature of the polymerase-RNA interaction. If a protein binds to RNA noncooperatively or binds to RNA cooperatively but without an appreciable change in binding energy when its entire RNA-binding site is filled, the outcome shown in Fig. 1*A* will be observed. In this case, the affinity of RNA oligonucleotides smaller than a single binding site and those that can accommodate only one protein molecule will be approximately a function of the number of nucleotides, with each nucleotide contributing equal binding energy. Then, when the RNA oligonucleotide is long enough to accommodate two proteins, an increase in binding affinity that reflects the energy of the protein-protein interaction may or may not be observed.

A second possible outcome would be observed if the energy/nucleotide of the protein-RNA interaction were to increase when the RNA-binding site was filled (Fig. 1*B*). One possible reason for this effect would be a conformational change in the protein upon binding to a complete site. Large apparent conformational changes upon DNA binding have been reported for human immunodeficiency virus type 1 reverse transcriptase and RecA (16–18). Another example of large increases in binding affinity with only small increases in oligonucleotide length was seen in the binding of *E. coli* cyclic AMP-binding protein to small DNA molecules (19). In this case, DNA-binding determinants are located on opposite sides of single cyclic AMP-binding proteins. Once a DNA molecule is large enough to contact both sites simultaneously, its binding affinity for cyclic AMP-binding protein increases dramatically (19). If either of these effects were to contribute additional energy to the binding of small homopolymeric RNAs long enough to fill the binding site of poliovirus polymerase, comparing the effectiveness of competition of different length oligonucleotides would be a useful technique to measure the binding site size. This is the approach taken in the experiments described below.

Downloaded from www.jbc.org at Stanford University on February 4, 2007

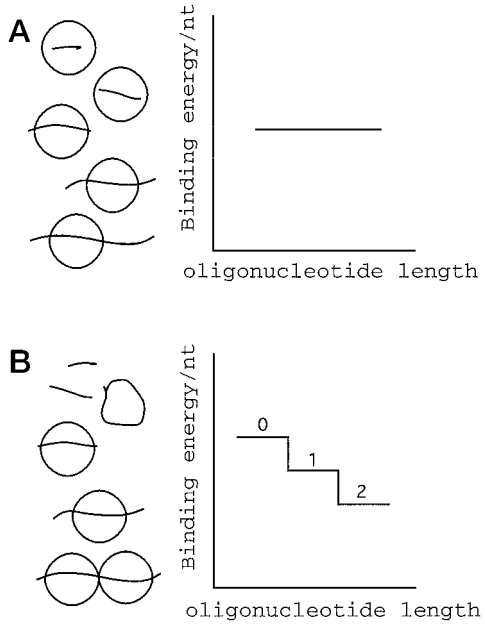

FIG. 1. **Predicted outcomes of oligonucleotide competition experiments to determine the minimal binding site size of a single-stranded nucleic acid-binding protein.** *A*, effect of oligonucleotide length on binding energy/nucleotide of protein-RNA complex if each nucleotide contributes equal binding energy and no significant contribution to binding energy is derived from the filling of the RNA-binding site. *B*, effect of oligonucleotide length on binding energy/nucleotide of protein-RNA complex if changes in overall affinity of the protein-RNA complex occur upon the filling of the RNA-binding site. *0*, no bound polymerase; *1*, one bound polymerase molecule; *2*, two bound polymerase molecules. *Circles*, bound polymerase; *irregularly shaped circle*, unbound polymerase; ——, RNA.

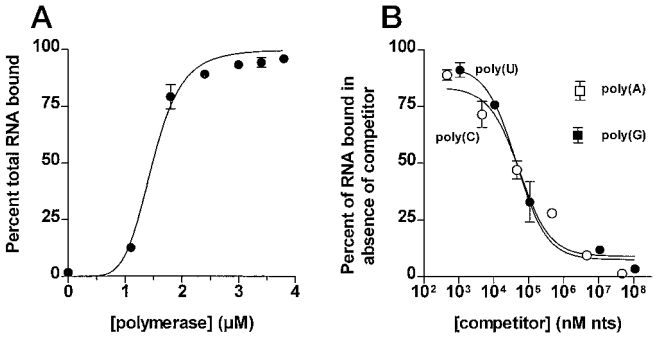

FIG. 2. **Cooperative binding of polymerase to poliovirus RNA and competition by long homopolymeric RNAs.** *A*, percentage of total poliovirus RNA bound as a function of increasing polymerase concentration. The theoretical curve (——) for cooperative binding (see "Experimental Procedures," Equation 1) gives values of $n = 5.7$, $K = 9.6$ $\mu M^{5.7}$, and $R^2 = 0.98$. *B*, competition of polymerase binding to poliovirus RNA by homopolymeric RNAs. Only one point for poly(A) and one point for poly(G) are shown for clarity. Theoretical curves assume competition between bound ligand and competitor for binding to the same site (see "Experimental Procedures," Equation 2). ●, poly(U), $R^2 = 0.98$; ○, poly(C), $R^2 = 0.95$; ■, poly(G); □, poly(A).

Competition of Polymerase Binding to Poliovirus RNA by Long Homopolymeric RNAs—Poliovirus polymerase has been shown to bind to heteropolymeric RNA with little sequence specificity and relatively low affinity, but high cooperativity (Ref. 9 and data not shown). Fig. 2*A* shows the highly cooperative binding of purified polymerase to full-length transcripts of poliovirus RNA. The lack of sequence specificity of polymerase binding makes it reasonable to use homopolymeric RNAs to compete with heteropolymeric RNAs for polymerase binding. Fig. 2*B* shows that poly(C) and poly(U) proved to be good competitors, whereas neither poly(G) nor poly(A) competed effectively with heteropolymeric RNA for polymerase binding under these conditions, chosen to optimize the cooperativity of polymerase binding (9). The poor competition observed with polypurines could be due to alternative structures formed by polypurines. Competition experiments with oligonucleotides of different lengths, described below, employed uridylyl oligonucleotides of varying size.

Competition of Polymerase Binding to 116-nt Heteropolymeric RNA by Oligo(U) of Varying Lengths—To test the effect of oligonucleotide length on its affinity for poliovirus polymerase, oligo(U) molecules of varying lengths thought to span a range of sizes smaller than one polymerase-binding site, sufficient to bind one polymerase molecule, and perhaps long enough to bind two or multiple polymerase molecules were synthesized. The recently determined structure of poliovirus polymerase has revealed that the direct distance between corresponding residues in the active sites of adjacent polymerase molecules in the crystal is ~46 Å (11). An observed correlation between cooperative RNA binding by polymerase and polymerase activity has led us to hypothesize that the template for poliovirus polymerase is single-stranded RNA complexed with cooperatively bound polymerase molecules (9). If polymerase molecules can bind head-to-tail in a fiber along regions of an RNA molecule, the bound single-stranded RNA is expected to traverse the length of each polymerase molecule. Depending on the conformation of the bound RNA, the length of RNA bound to a site that is at least 46 Å in length could range from 16 or more nucleotides for an A-form conformation to as few as 8 nt for an extended single-stranded conformation. These values are based on observed internucleotide distances of ~6.0 Å/nt for the highly extended single-stranded RNAs bound to U1A spliceosomal protein (20), of ~5.0 Å/nt for the single-stranded DNA bound to bacteriophage T4 gene 32 protein (21), and of 2.8 Å/base pairs for A-form RNA (22).

Fig. 3*A* shows the cooperative binding of poliovirus polymerase to a 116-nt RNA derived from the poliovirus genome. This RNA, considerably smaller than the full-length viral RNA used in Fig. 2, was used to avoid masking the effect of competitor oligonucleotides by the high degree of cooperativity seen with very long RNAs. Half-maximal binding of polymerase to the 116-nt RNA occurred at ~3 μM polymerase 3D. Competition experiments were then performed using a lower concentration of polymerase (2 μM) to maximize sensitivity of the competition assay. Oligo(U) molecules 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 nt in length were tested for their affinities for poliovirus polymerase in competition experiments against the 116-nt RNA. Fig. 3*B* shows individual competition curves of oligo$(U)_{6-24}$. The individual data points were fit to an equation that describes competition for binding to one site. The cross-hairs in each graph denote the IC_{50} in nanomolar nucleotides. A lower IC_{50} value indicates greater affinity for the polymerase.

For lengths smaller than 8 nt, the RNAs did not compete effectively (Fig. 3*B*). In fact, conditions under which competitor oligo$(U)_4$ (data not shown) and oligo$(U)_6$ displayed any competition, and therefore any measurable binding to polymerase, were not achieved. When the length of the oligo(U) reached 10 nt, however, substantial competition for binding to the 116-nt labeled RNA was seen. Little further increase in the ability to compete was observed until the longest RNA oligonucleotide, oligo$(U)_{24}$, was tested.

RNA-binding Site Size—When the IC_{50} values determined in Fig. 3*B* are plotted as a function of oligonucleotide length, it is clear that appreciable binding to polymerase was not observed until the RNA reached a length of 10 nt (Fig. 4). The drop in IC_{50} at 10 nt is substantial: the addition of 2 nt to an RNA oligonucleotide of 8 nt decreased the IC_{50} by ~20-fold, and the

Downloaded from www.jbc.org at Stanford University on February 4, 2007

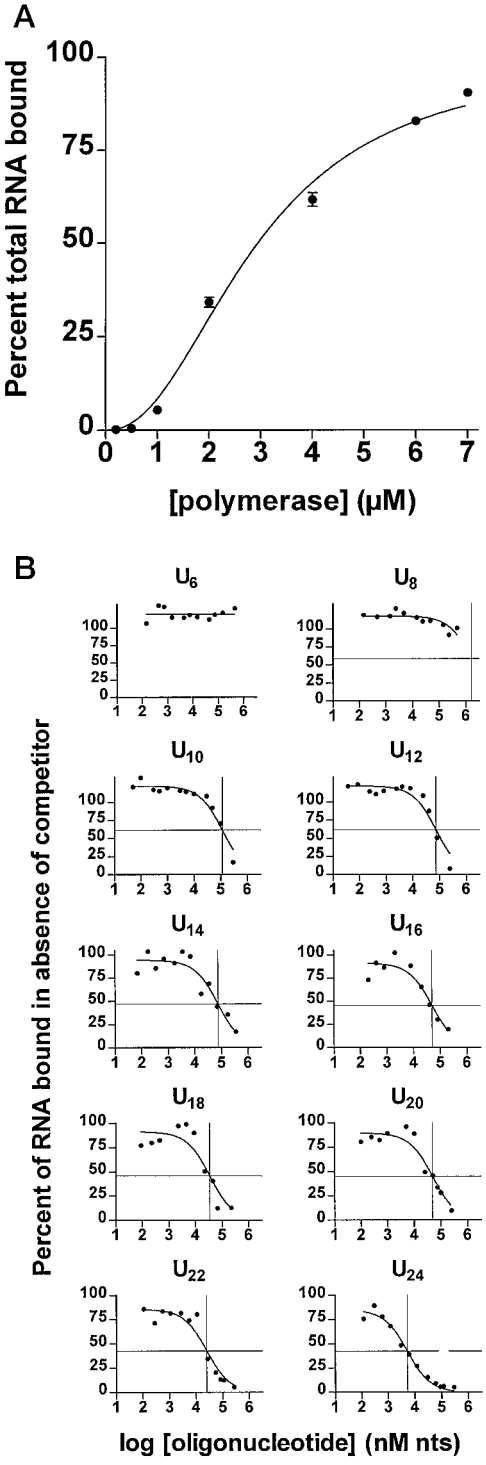

FIG. 3. **Cooperative binding of polymerase to 116-nt heteropolymeric RNA and competition by oligo(U) of different lengths.** *A*, percentage of total 116-nt RNA bound as a function of increasing polymerase concentration. Equation 1 (——) gives values of $n = 2.2$, $K = 10.8\ \mu M^{2.2}$, and $R^2 = 0.99$. *B*, competition of polymerase binding to the 116-nt RNA by oligo(U) of different lengths. For each oligo(U), *cross-hairs* depict IC_{50} (in nanomolar nucleotides).

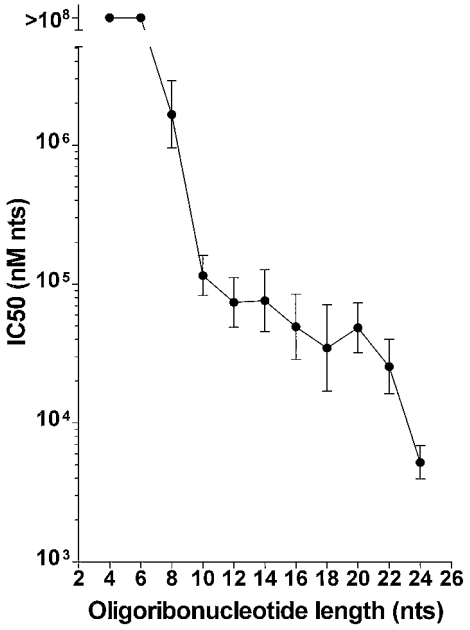

FIG. 4. **IC_{50} values decrease as a function of oligonucleotide length.** IC_{50} values from the experiments shown in Fig. 3*B* are plotted as a function of oligo(U) length. *Error bars* represent the 90% confidence interval determined from each individual data set.

addition of 2 nt to an RNA oligonucleotide of 10 nt decreased the IC_{50} by only 2-fold. It is not likely that the change in affinity for polymerase at 10 nt represents the length of RNA to which two polymerase molecules can be cooperatively bound, both because the site size of cooperative polymerase binding is likely to be at least 8 nt to span between polymerase active sites and because no appreciable binding affinity was observed for RNA oligonucleotides smaller than 10 nt in length. We consider it likely that the RNA-binding site is ~10 nt and that a binding site that is fully occupied has much greater affinity for its RNA substrate than a binding site that is only partially filled by its RNA substrate.

The IC_{50} values decrease somewhat linearly with the length of the oligo(U) from oligo$(U)_{12}$ to oligo$(U)_{22}$. Modest increases in affinity with increasing oligonucleotide lengths, even if only one polymerase can be bound to each oligonucleotide, are expected due to "lattice effects": in a longer polymer, there are more ways to occupy a site of defined length than in a shorter one (24). At 24 nt, a larger apparent increase in oligo(U) affinity was seen that is likely to correspond to the addition of a second polymerase molecule (Fig. 4).

Direct Binding of Polymerase to Oligo$(U)_{12}$ and Oligo$(U)_{24}$—If the minimal binding site for a single polymerase is 10–12 nt in length, then polymerase binding to oligo(U) molecules that contain only a single binding site may reveal the intrinsic affinity of polymerase for RNA, separated from the protein-protein interactions that are likely to dominate the energetics of polymerase binding to longer RNAs. Direct binding of polymerase to oligo$(U)_{24}$, predicted to contain two binding sites for polymerase, would then reflect the intrinsic affinity of two polymerase molecules for RNA as well as the protein-protein interactions between the polymerases. Fig. 5 compares the direct binding of wild-type polymerase to oligo$(U)_{12}$ and oligo$(U)_{24}$. The affinity of polymerase for oligo$(U)_{12}$ was much less than half of that seen for oligo$(U)_{24}$. Furthermore, the apparent cooperativity of binding to oligo$(U)_{12}$ was less than binding to oligo$(U)_{24}$ (Fig. 5). While these observations are consistent with the hypothesis that one polymerase molecule binds to oligo$(U)_{12}$ and two polymerase molecules bind cooperatively to oligo$(U)_{24}$, it is interesting that the binding of polymerase to oligo$(U)_{12}$ still displayed some cooperativity. It may be that interactions between RNA-bound polymerase and free polymerase can increase the strength of RNA binding.

Competition of Gene 32 Protein Binding to RNA and DNA Polynucleotides by DNA Oligonucleotides of Different Lengths—To test the method of titration with oligonucleotides

Downloaded from www.jbc.org at Stanford University on February 4, 2007

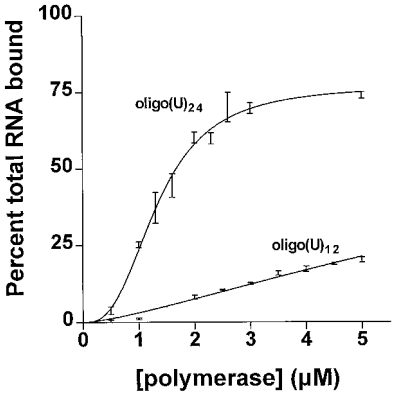

FIG. 5. **Direct binding of wild-type polymerase to oligo(U)$_{12}$ and oligo(U)$_{24}$.** Shown is the percentage of total oligo(U)$_{12}$ and oligo(U)$_{24}$ bound as a function of polymerase concentration. The data were fit to Equation 1 with a maximum RNA bound of 77% and give values of n = 1.4, K = 24 μM^n, and R^2 = 0.98 for oligo(U)$_{12}$ and n = 2.8, K = 2.3 μM^n, and R^2 = 0.98 for oligo(U)$_{24}$. ●, oligo(U)$_{24}$; ○, oligo(U)$_{12}$.

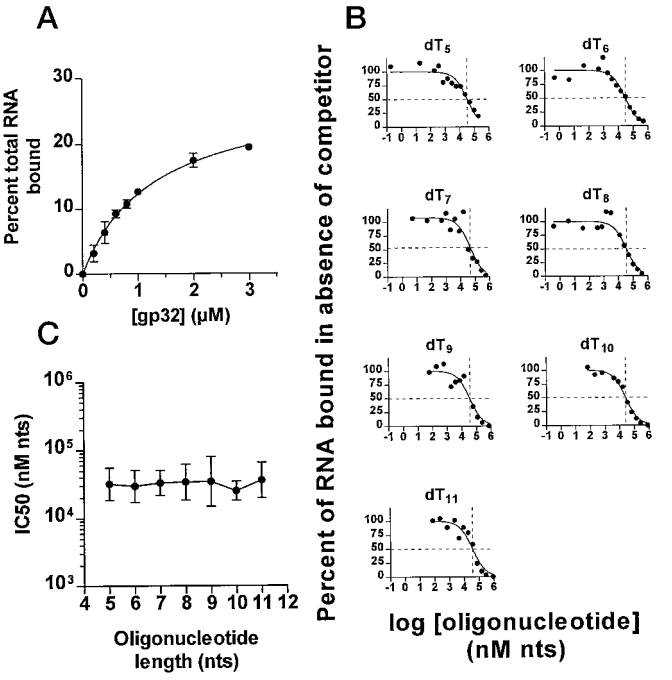

FIG. 6. **Noncooperative binding of gene 32 protein to 116-nt heteropolymeric RNA and competition by oligo(dT) of different lengths.** *A*, percentage of total 116-nt RNA bound as a function of increasing gene 32 protein concentration. The theoretical curve describes simple binding where B_{max} = 29% RNA bound, K = 1.3 μM, and R^2 = 0.97. *B*, competition of gene 32 protein binding to the 116-nt RNA by oligo(dT) of different lengths. For each competition curve, *cross-hairs* depict IC$_{50}$ (in nanomolar nucleotides). *C*, IC$_{50}$ values from *B* plotted as a function of oligo(dT) length. *Error bars* represent the 90% confidence interval determined from each individual data set.

of increasing length on a well known cooperative single-stranded binding protein, we tested the effect of oligonucleotide competition on the binding of bacteriophage T4 gene 32 protein to both the 116-nt RNA and a 46-nt heteropolymeric single-stranded DNA. Site sizes of binding for gene 32 protein monomers from 5 to 10 nt have been reported (21, 24–26), with a consensus at 6 nt in recent literature (21). The extent of cooperativity can be varied by changing the binding conditions: the use of RNA-binding substrates, for example, is known to disfavor cooperative binding under many conditions (26, 27).

A simple binding curve of gene 32 protein to the 116-nt RNA at relatively low salt concentrations is shown in Fig. 6*A*. The noncooperative nature of this binding (n = 1.0) is readily seen. The effect of competing the RNA-binding signal observed at 0.6 μM gene 32 protein with varying lengths of oligo(dT) (Fig. 6*B*) and the derived IC$_{50}$ values as a function of deoxyoligonucleotide length (Fig. 6*C*) were determined. The IC$_{50}$ values remained relatively constant over the range of deoxynucleotide lengths tested.

To test whether DNA oligonucleotides could be bound cooperatively under these conditions, the binding of gene 32 protein to a 46-nt single-stranded DNA heteropolymer was performed (Fig. 7*A*). A simple binding curve proved to be highly cooperative with respect to gene 32 protein concentration (n = 2.7). When the DNA-binding signal observed at 0.22 μM gene 32 protein was competed using oligo(dT) molecules of increasing length (Fig. 7*B*), very little effect of deoxyoligonucleotide length on the IC$_{50}$ values was again observed (Fig. 7*C*). The IC$_{50}$ values plotted against deoxyoligonucleotide length resulted in the graph shown in Fig. 7*C*. A small drop in IC$_{50}$ was reproducibly observed between oligo(dT)$_8$ and oligo(dT)$_9$.

DISCUSSION

Significance of Cooperative Binding of Poliovirus Polymerase to Single-stranded RNA to the Poliovirus Replicative Cycle—The expression strategy of all picornaviruses, including poliovirus, dictates that approximately equimolar amounts of viral enzymes (such as proteases and the RNA-dependent RNA polymerase 3D) and capsid proteins are synthesized. Sixty molecules of each capsid proteins are required to encapsidate each RNA genome. Thus, given the proportionately large quantities of polymerase available, it is possible that the 3D polypeptide also functions as a single-stranded binding protein.

Thus far, the poliovirus polymerase has not been reported to bind specifically to any RNA sequence. However, any specificity could easily have been masked by the high degree of cooperativity. Now it can be appreciated that fairly small RNAs must be screened for those that contain specific polymerase-binding sites that might nucleate RNA binding. The single-stranded RNA-binding activity could play numerous roles in the infectious cycle, as suggested by the functions served by other single-stranded RNA-binding proteins. For example, T4 gene 32 protein has been shown to decrease the error rate of T4 DNA polymerase (21, 28), and RecA is absolutely required for replication and recombination of the *E. coli* genome (29). Gene 32 protein down-regulates the translation of its own mRNA by binding to an RNA pseudoknot that includes the initiating AUG codon (30). A role for a single-stranded nucleic acid-binding protein in the packaging of single-stranded nucleic acid into virions has been established for gene V protein of M13 phage, which binds to newly synthesized single-stranded phage DNA molecules, preventing their further replication and transferring them, by an as yet unknown mechanism, to the empty phage capsid (reviewed in Ref. 31).

The homo-oligomerization of polymerase molecules has no precedent in the DNA-dependent polymerases. Among the reverse transcriptases, human immunodeficiency virus type 1 reverse transcriptase forms a heterodimer between one full-length and one proteolytically processed subunit (32, 33); Moloney murine leukemia virus reverse transcriptase has been reported to show some cooperative binding to DNA templates and is likely to act as a dimer (34). Whether the cooperative binding of poliovirus RNA-dependent RNA polymerase to RNA is shared with the polymerases of other positive-stranded RNA viruses, such as the closely related rhinoviruses and the more distantly related hepatitis C virus, remains to be tested.

Stoichiometry of Polymerase Binding to RNA—Does the low efficiency of RNA binding by purified poliovirus polymerase reflect a low affinity for RNA templates, or does it reflect the activity of only a subset of the purified polymerase molecules?

Downloaded from www.jbc.org at Stanford University on February 4, 2007

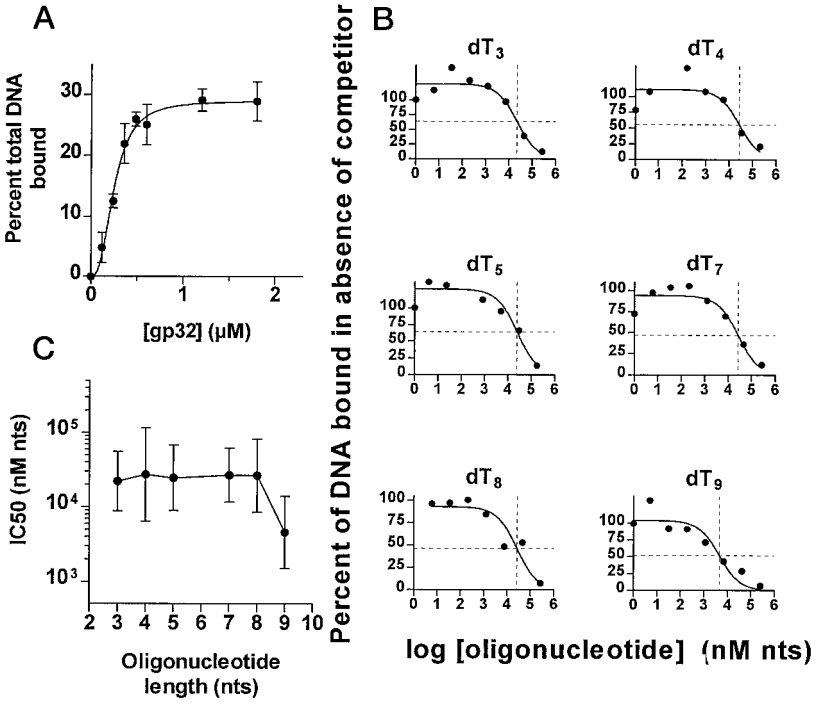

FIG. 7. **Cooperative binding of gene 32 protein to 46-nt heteropolymeric DNA and competition by oligo(dT) of varying lengths.** *A*, percentage of total 46-nt DNA bound as a function of increasing gene 32 protein concentration. The cooperative binding curve (see "Experimental Procedures") had a maximum of 29% DNA bound and gives values of n = 2.7, K = 0.025 $\mu M^{2.7}$, and R^2 = 0.93. *B*, competition of gene 32 protein binding to the 46-nt DNA by oligo(dT) of different lengths. For each oligo(dT), *cross-hairs* depict IC_{50} (in nanomolar nucleotides). *C*, IC_{50} values from the experiments in *B* plotted as a function of oligo(U) length. *Error bars* represent the 90% confidence interval determined from each individual data set.

For each of the experiments presented here, micromolar concentrations of polymerase were required to saturate complex formation with labeled RNAs present at nanomolar concentrations. For example, in Fig. 3*A*, ~3 μM polymerase was required to retain half of the 116-nt labeled RNA, present at 1 nM strands, in protein-RNA complexes on nitrocellulose filters. The likelihood that multiple polymerases bind to each RNA is obviously not sufficient to explain the necessity for a 3000-fold molar excess of polymerase for half-maximal RNA binding. Titration of complex formation between polymerase, present at 2 μM, with increasing amounts of the 116-nt RNA has shown that complex formation saturates at ~0.2 μM RNA, at a polymerase/RNA ratio of 10:1 (data not shown). Therefore, there is not likely to be a large population of polymerase that is inactive in RNA binding. Despite the low affinity of poliovirus polymerase for its RNA substrates, it is possible that, within infected cells, other viral or host proteins in the replication complex serve to increase the affinity and specificity of poliovirus polymerase for RNA (8, 10, 35–37). For poliovirus polymerase, understanding the nature of the contacts made between the polymerase and RNA in isolation will give us a starting point to understand the function of accessory proteins in the RNA replication complex.

The Binding Site for a Single Polymerase Molecule Is 10–12 nt in Length—Due to the low affinity of poliovirus polymerase for RNA, and especially for small oligonucleotides (Fig. 5), we used competition experiments to test the affinities of RNA oligonucleotides of different lengths (Figs. 3 and 4). RNA oligonucleotides 10 nt and longer show a large increase in affinity for poliovirus polymerase, as judged by their ability to compete with longer heteropolymeric RNAs for polymerase binding (Fig. 4). This could result from one of two possible effects of oligo(U) length. We favor the interpretation that, at 10 nt, the RNA has reached a length that can completely or almost completely occupy the RNA-binding site in one polymerase molecule. There are several arguments that favor this interpretation. First, 10 nt could readily span the 46-Å distance between polymerase active sites, given two polymerase molecules juxtaposed at the substantial interface (Interface I) shown in the three-dimensional structure (11). Depending on the path of the RNA between polymerase molecules, the actual distance traversed is likely to be greater than the direct distance of 46 Å. The internucleotide distance of the bound RNA traversing the polymerase molecule would then be, on average, at least 4.6 Å/nt, comparable to similar distances observed in complexes of RNA- and DNA-binding proteins with single-stranded nucleic acids (38, 39). Second, another substantial drop in IC_{50} was not observed until another 12–14 nt were added to the length of the oligo(U) competitor (Fig. 4). It is likely that competition with oligonucleotides can result in an underestimation of the actual site size because bound proteins can occlude more nucleotides than they interact with physically (40).

Direct binding of polymerase to $oligo(U)_{12}$ and $oligo(U)_{24}$ (Fig. 5) supported the hypothesis that a single polymerase molecule could bind to oligo(U) molecules 10–12 nt in length, and 24 nt was sufficient to span two polymerase molecules. Polymerase binding to $oligo(U)_{24}$ was highly cooperative and showed an apparent affinity much greater than twice that observed for binding to $oligo(U)_{12}$ (Fig. 5).

Another interpretation of the decrease in IC_{50} observed with $oligo(U)_{12}$ is that, when the oligo(U) reaches 10–12 nt in length, two polymerase molecules can bind. If this were the case, another drop in IC_{50} at 16–18 nt might be expected due to binding of a third polymerase; this was not observed (Fig. 4). To span 46 Å from active site to active site, even at the very extended single-stranded configuration of 6 Å/nt observed in the complex of single-stranded RNA with the U1 small nuclear ribonucleoprotein (Code 1URN, Protein Data Bank, Brookhaven National Laboratory (20)), would require at least 8 nt.

Conformational Change in Polymerase upon RNA Binding—The 20-fold decrease in IC_{50} from $oligo(U)_8$ to $oligo(U)_{10}$ is likely to reflect either a large conformational change upon binding those RNA molecules that can completely occupy the RNA-binding site of the polymerase or the existence of widely spaced binding determinants in the RNA-binding site. A large conformational change upon substrate binding has been documented for human immunodeficiency virus type 1 reverse transcriptase, for example. Compared with the crystal structure of the free enzyme (32), the thumb domain of human immunode-

Downloaded from www.jbc.org at Stanford University on February 4, 2007

ficiency virus type 1 reverse transcriptase is pivoted away from the fingers domain by 30 Å in the structure of a co-crystal with a template-primer DNA (16). Other possible conformational changes upon RNA binding might involve increased affinity for other polymerase molecules. Widely spaced binding determinants are thought to be responsible for the extreme sensitivity of cyclic AMP-binding protein to DNA length (19).

Interestingly, little inflection in the IC_{50} values was observed when T4 gene 32 protein binding to either RNA or DNA was competed with oligo(dT) of varying lengths that spanned the 6-nt site size. An artifactual explanation of this observation might be that, when gene 32 protein is in "oligonucleotide-binding mode," it binds only to the ends of small oligonucleotides and is insensitive to their length or base composition (26). If this were the case in Figs. 6 and 7, the IC_{50} (in nanomolar nucleotides) should increase with increasing oligonucleotide length, which it does not. Rather, it seems likely that the nucleic acid-binding site of gene 32 protein can bind regions of single-stranded nucleic acid shorter than its complete binding site with as high an affinity/nucleotide as to complete sites. The three-dimensional structure of gene 32 protein in the absence of the protein-protein interaction domains shows 4 nt of DNA resolved in the binding site of the "core" protein. The other 2 nt in the 6-nt DNA site are thought to be contacted less closely by gene 32 protein and are disordered in the crystal (21). The ability to fill a binding site partially might be useful in the function of gene 32 protein in stabilizing the unfolded state of partially single-stranded DNA molecules during replication, recombination, and repair. The structure of gene 32 protein in the absence of complexed DNA has not been determined, so any conformational changes that occur upon nucleic acid binding have not been characterized structurally. However, gene 32 protein contains an N-terminal peptide that is thought to occupy the nucleic acid-binding site in the free protein, but to move out of the binding cleft when it is occupied by nucleic acid (41). Then, this N-terminal peptide, which is required for the cooperativity of single-stranded nucleic acid binding, is free to interact with other gene 32 protein molecules. In this context, the inflection in IC_{50} at 9 nt (Fig. 7*C*) is interesting: it is perhaps not until another gene 32 protein monomer can be contacted that any increase in energy as a function of oligonucleotide length can be realized. In any case, the gene 32 protein studies reveal that the method of oligonucleotide titration is a useful method to determine site size of protein binding only for certain proteins, probably those that undergo large conformational changes upon full occupation of their nucleic acid-binding sites or that contain widely spaced binding determinants.

Acknowledgments—We thank Yousif Shamoo for supplying the gene 32 protein used in this study; Kay Sanders for assistance with protein purification; Peter Sarnow, Steve Schultz, and John Lyle for critical reading of the manuscript; and John Lyle for assistance with the figures. We are especially grateful to Steve Schultz and Jeff Hansen for extensive discussions of the three-dimensional structure of poliovirus polymerase.

REFERENCES

1. Racaniello, V. R., and Baltimore, D. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4887–4891
2. Morrow, C. D., Warren, B., and Lentz, M. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6050–6054
3. Richards, O. C., Ivanoff, L. A., Bienkowska-Szewczyk, K., Butt, B., Petteway, S. R., Jr., Rothstein, M. A., and Ehrenfeld, E. (1987) *Virology* **161,** 348–356
4. Rothstein, M. A., Richards, O. C., Amin, C., and Ehrenfeld, E. (1988) *Virology* **164,** 301–308
5. Plotch, S. J., Palant, O., and Gluzman, Y. (1989) *J. Virol.* **63,** 216–225
6. Neufeld, K. L., Richards, O. C., and Ehrenfeld, E. (1991) *J. Biol. Chem.* **266,** 24212–24219
7. Paul, A. V., Cao, X., Harris, K. S., Lama, J., and Wimmer, E. (1994) *J. Biol. Chem.* **269,** 29173–29181
8. Harris, K. S., Xiang, W., Alexander, L., Lane, W. S., Paul, A. V., and Wimmer, E. (1994) *J. Biol. Chem.* **269,** 27004–27014
9. Pata, J. D., Schultz, S. C., and Kirkegaard, K. (1995) *RNA* **1,** 466–477
10. Hope, D. A., Diamond, S. E., and Kirkegaard, K. (1997) *J. Virol.* **71,** 9490–9498
11. Hansen, J. L., Long, A. M., and Schultz, S. C. (1997) *Structure* **5,** 1109–1122
12. Shamoo, Y., Adari, H., Konigsberg, W. H., Williams, K. R., and Chase, J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8844–8848
13. Sarnow, P. (1989) *J. Virol.* **63,** 467–470
14. Icho, T., and Wickner, R. B. (1989) *J. Biol. Chem.* **264,** 6716–6723
15. Hill, T. L. (1957) *J. Polymer Sci. Part D Macromol. Rev.* **23,** 549–562
16. Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, A. D., Raag, R., Nanni, R. G., Hughes, S. H., and Arnold, E. (1995) *Biochemistry* **34,** 5351–5363
17. Kumar, K. A., Mahalakshmi, S., and Muniyappa, K. (1993) *J. Biol. Chem.* **268,** 26162–26170
18. Stole, E., and Bryant, F. R. (1995) *J. Biol. Chem.* **270,** 20322–20328
19. Liu-Johnson, H. N., Gartenberg, M. R., and Crothers, D. M. (1986) *Cell* **47,** 995–1005
20. Oubridge, C., Ito, N., Evans, P. R., Teo, C.-H., and Nahai, K. (1994) *Nature* **372,** 432–438
21. Shamoo, Y., Friedman, A. M., Parsons, M. R., Konigsberg, W. H., and Steitz, T. A. (1995) *Nature* **376,** 362–366
22. Arnott, S., Hukins, D. W., Dover, S. D., Fuller, W., and Hodgson, A. R. (1973) *J. Mol. Biol.* **81,** 107–122
23. Kowalczykowski, S. C., Paul, L. S., Lonberg, N., Newport, J. W., McSwiggen, J. A., and von Hippel, P. H. (1986) *Biochemistry* **25,** 1226–1240
24. Jensen, D. E., Kelly, R. C., and von Hippel, P. H. (1976) *J. Biol. Chem.* **251,** 7215–7228
25. Kelly, R. C., Jensen, D. E., and von Hippel, P. H. (1976) *J. Biol. Chem.* **251,** 7240–7250
26. Kowalczykowski, S. C., Lonberg, N., Newport, J. W., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145,** 75–104
27. Newport, J. W., Lonberg, N., Kowalczykowski, S. C., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145,** 105–121
28. Sandhu, D. K., and Keohavong, P. (1994) *Gene* (*Amst.*) **144,** 53–58
29. Takahashi, M., and Norden, B. (1994) *Adv. Biophys.* **30,** 1–35
30. McPheeters, D. S., Stormo, G. D., and Gold, L. (1988) *J. Mol. Biol.* **201,** 517–535
31. Stassen, A. P. M., Folmer, R. H. A., Hilbers, C. W., and Konings, R. N. H. (1995) *Mol. Biol. Rep.* **20,** 109–127
32. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) *Science* **256,** 1783–1790
33. Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, L. A., Chirino, A. J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3911–3915
34. Telesnitsky, A., and Goff, S. P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1276–1280
35. Roehl, H. H., Parsley, T. B., Ho, T. V., and Semler, B. L. (1997) *J. Virol.* **71,** 578–585
36. Plotch, S. J., and Palant, O. (1995) *J. Virol.* **69,** 7169–7179
37. Blyn, L. B., Chen, R., Semler, B. L., and Ehrenfeld, E. (1995) *J. Virol.* **69,** 4381–4389
38. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) *Nature* **355,** 318–325
39. Takahashi, M., Kubista, M., and Norden, B. (1989) *J. Mol. Biol.* **205,** 137–147
40. Keyes, R. S., and Bobst, A. M. (1993) *Biophys. Chem.* **45,** 281–303
41. Casas-Finet, J. R., and Karpel, R. L. (1993) *Biochemistry* **32,** 9735–9744

Downloaded from www.jbc.org at Stanford University on February 4, 2007