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Assembly of a functional replication complex without ATP hydrolysis: A direct interaction of bacteriophage T4 gp45 with T4 DNA polymerase

(accessory proteins/holoenzyme/polyethylene glycol/processivity/sliding clamp)

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ABSTRACT **The seven-protein bacteriophage T4 DNA replication complex can be manipulated *in vitro* to study mechanistic aspects of the elongation phase of DNA replication. Under physiological conditions, the processivity of DNA synthesis catalyzed by the T4 polymerase (gp43) is greatly increased by the interaction of this enzyme with its accessory proteins (gp44/62 and gp45) and the T4 single-stranded DNA binding protein (gp32). The assembly of this T4 holoenzyme requires hydrolysis of ATP by the gp44/62 complex. We demonstrate here that processive T4 holoenzyme-like DNA synthesis can be obtained without hydrolysis of ATP by simply adding gp45 to the T4 DNA polymerase at extremely high concentrations, effectively bypassing the ATPase subunits (gp44/62) of the accessory protein complex. The amount of gp45 required for the gp43–gp45 heteroassociation event is reduced by addition of the macromolecular crowding agent polyethylene glycol (PEG) as well as gp32. A chromatographic strategy involving PEG has been used to demonstrate the gp43–gp45 interaction. These results suggest that gp45 is ultimately responsible for increasing the processivity of DNA synthesis via a direct and functionally significant interaction with the T4 DNA polymerase. A corollary to this notion is that the specific role of the gp44/62 complex is to catalytically link gp45 to gp43.**

The past few years have seen a coalescence of mechanistic themes in DNA replication. This is especially true of the emerging role of the polymerase accessory proteins in forming a replication holoenzyme that is capable of elongating DNA in a highly processive manner. In T4, *Escherichia coli*, and many eukaryotic systems, the assembly of the functional holoenzyme appears to require hydrolysis of ATP by accessory protein subunits complexed with DNA (see ref. 1).

The T4 holoenzyme contains the T4 DNA polymerase together with the gp44/62 ATPase complex, the gp45 protein, and the gp32 single-stranded DNA binding protein. The assembly of these components into a processive holoenzyme is driven by ATP binding and hydrolysis, although the precise molecular details of this process remain unknown (for recent reviews, see refs. 2 and 3). The functional role of the accessory proteins in establishing the holoenzyme appears to be to form a "sliding clamp" (4, 5) that holds the polymerase to the DNA template.

Recent progress in understanding the *E. coli* DNA polymerase III holoenzyme system (for review see refs. 6 and 7) suggests how the T4 proteins might form such a complex. The *E. coli* γ complex (which consists of five subunits) hydrolyzes ATP and, in so doing, transfers β subunits (as a dimer) onto the primer template DNA substrate. The β dimer then interacts directly with the core DNA polymerase (8, 9) to form a processive DNA replication holoenzyme. The recent elucidation of the three-dimensional structure of the β dimer has elegantly revealed the molecular basis of this process; the β dimer forms a ring that topologically encircles the DNA, whereby it presumably clamps the *E. coli* polymerase to the DNA (10). The γ complex of *E. coli* acts catalytically in this assembly process (11, 12) and is not functionally required once the β dimer has encircled the DNA (9).

Since gp44/62 and gp45 appear, respectively, to be the functional analogs in T4 of the γ complex and β subunit of the *E. coli* system (13, 14), the role of the gp44/62 ATPase complex may also be to load the gp45 trimer onto DNA, which then also forms a sliding clamp to tether the T4 polymerase to the DNA template. This hypothesis is supported by previous findings that suggested indirectly a functional (5, 15, 16) or physical (17–19) interaction between gp43 and gp45. In this study, we confirm and extend these observations.

MATERIALS AND METHODS

Templates. Oligonucleotides were prepared as described (20) and their concentrations were determined by using calculated molar extinction coefficients (21). Purified single-stranded circular phage M13 DNA was from United States Biochemical. Primer strands were radioactively labeled at their 5′ ends and annealed to their complementary templates as described (22).

Proteins. All T4 proteins were purified from overproducing strains (gifts of T. C. Lin from the Konigsberg laboratory). T4 DNA polymerase (gp43) (22), gp32 and gp44/62 (23), and gp45 (24) were purified as described. All protein preparations were >98% pure and contained no contaminating nuclease or ATPase activities. The concentrations of gp32 and gp43 are reported in units of monomers, gp45 as trimers, and gp44/62 as 4:1 complexes (23) and were measured spectrophotometrically with calculated molar extinction coefficients (18).

Synthesis Assays. All T4 replication proteins were diluted as described (22) and were preincubated for 5 min at the reaction temperature with the indicated amounts of DNA substrate. Mg^{2+} was omitted from the preincubations and was added to initiate the reaction. The final reaction solutions (10 μl) contained 25 mM Hepes (pH 7.5), 160 mM KOAc, 5 mM dithiothreitol, 0.2 mM EDTA, 125 μM of all four dNTPs, 1 mM ATP, 0.2 mg of bovine serum albumin per ml, and 6 mM $Mg(OAc)_2$. Reaction mixtures with polyethylene glycol (PEG) contained 7.5% (wt/vol) PEG 12,000 (average molecular weight) from Fluka. Complete reaction mixtures were incubated for 15 sec, quenched by the addition of 40 μl of a formamide dye mixture, and subjected to denaturing gel electrophoresis as described (22). Radioactive gels were quantitated on an Ambis 4000 radioanalytic scanner (Ambis Systems, San Diego).

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Single-Stranded DNA Cellulose Chromatography. The DNA cellulose matrix was prepared as described (25). Columns were packed to a final bed vol of 1 ml. All chromatographic procedures were performed at ≈21°C (room temperature). Columns were gravity run, with flow rates of ≈15 ml/hr. T4 proteins at a total vol of 500 μl were preincubated in the buffer used to equilibrate the matrix [25 mM Hepes, pH 7.5/50 mM NaCl/0.5 mM dithiothreitol/1 mM EDTA/25% (vol/vol) glycerol or 7.5% PEG]. The following amounts of proteins were loaded, either individually or in combination: 0.5 nmol of gp43, 1.5 nmol of gp44/62, and 5.0 nmol of gp45. After the proteins were loaded, the column was washed with 5 bed vol (5 ml) of equilibration buffer and subsequently step eluted with three 4-ml washes of equilibration buffer containing 0.2, 0.4, and 0.6 M NaCl. One-milliliter fractions were collected.

RESULTS

Addition of gp45 Alone Permits T4 DNA Polymerase to Bypass Template Sequence-Specific Stop Sites. The sequence of the primer template used as a model for lagging-strand synthesis is shown in Fig. 1*A*. The design of these oligonucleotide constructs avoided inclusion of sequences that might form stable stem–loop structures.

The synthesis experiments shown in Fig. 2 were performed by using a large molar excess of primer template over polymerase and allowing <10% of the starting primers to be extended. These single-hit conditions permit determination of the processivity of synthesis (see ref. 18). Assay of the polymerase alone showed a distinctive pattern of synthesis (Fig. 2, lane 0) with ≈45% of the extended primers reaching full length (49 and 50 residues) and an almost equivalent fraction of the extended primers prematurely terminated at residues 45 and 46. However, it was observed that addition of gp45 alone resulted in a significant increase in the fraction of primers extended to full-length product (Fig. 2). This increase in processive synthesis due to addition of gp45 is independent of the presence (or depletion) of either ATP or the gp44/62 accessory protein complex (data not shown).

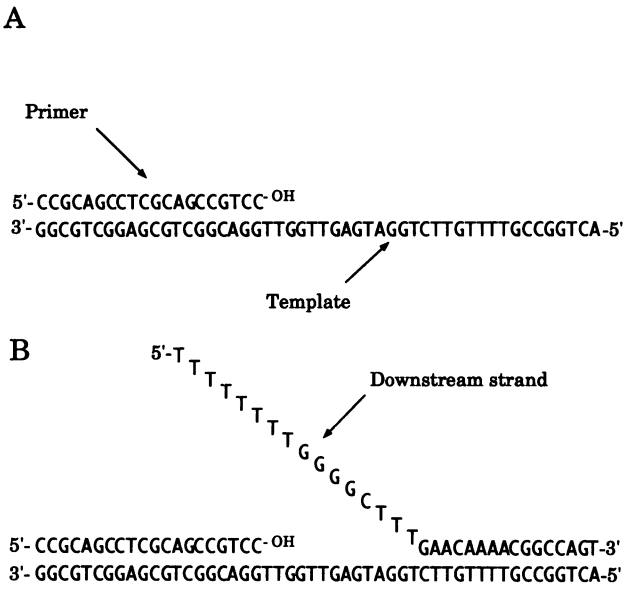

FIG. 1. DNA sequence of two model oligonucleotide constructs. (*A*) A primer template construct, used as a model for lagging-strand DNA synthesis, is formed upon annealing a 20-nucleotide primer to a 50-nucleotide template strand. (*B*) A forked primer template construct, used as a model for leading-strand DNA synthesis, is formed upon annealing a 32-residue oligonucleotide to the primer template structure shown in *A*. T4 DNA polymerase catalyzes synthesis from the 3'-hydroxyl residue (-OH) as shown.

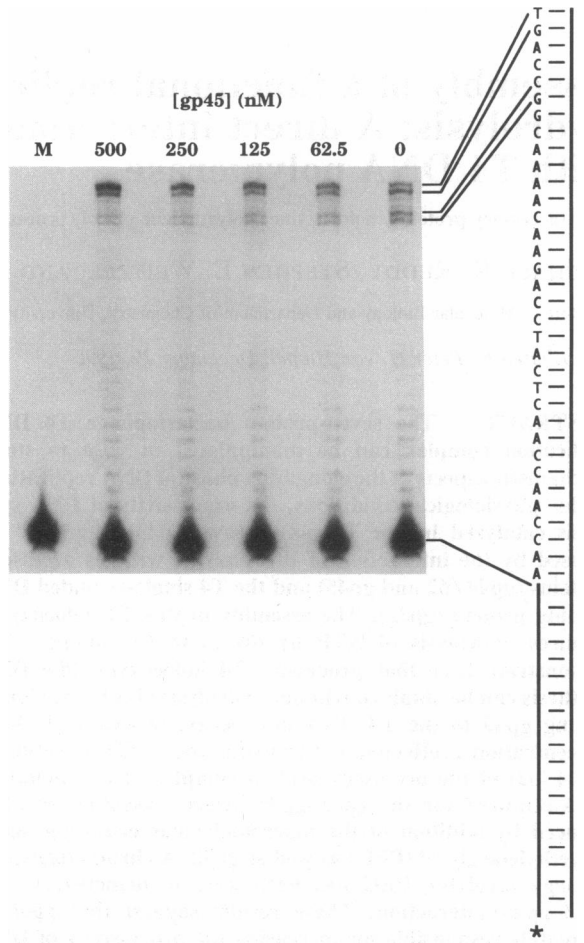

FIG. 2. Addition of gp45 increases the processivity of T4 DNA polymerase (gp43). Synthesis assays were performed at 37°C on the ^{32}P-labeled (*) primer template construct of Fig. 1*A*, as described. The products were subjected to electrophoresis on a 12% acrylamide (20:1)/8 M urea DNA sequencing gel. All the reaction mixtures contained 1 nM gp43, 100 nM primer template (molecules), and the indicated molarities of gp45 (in units of trimers). The primer template construct used is shown on the right. Sequences shown represent nucleotides complementary to the template strand that are synthesized by extending the 3' terminus of the 20-nucleotide primer strand. Lane M, unextended primer strand only.

The premature termination in synthesis at residues 45 and 46 (Fig. 2) does not depend on the proximity of this site to the 5' terminus of the template strand, since it is known that T4 DNA polymerase can synthesize processively to the very end of other oligonucleotide templates (for example, see ref. 22). Also, this sequence induces dissociation (or stalling) of T4 polymerase when present on a circular M13 DNA template (ref. 26; M.K.R., unpublished data). Furthermore, Bedinger *et al.* (27), also using large circular viral templates, showed that T4 polymerase pauses at 4-residue palindromic sequences similar to the stop site demonstrated above.

The results of Fig. 2 demonstrate that gp45 can increase the processivity of synthesis via a direct, albeit weak, interaction with T4 DNA polymerase. A similar series of reactions were performed in the presence of 7.5% PEG 12,000 (average M_r). The choice of these conditions was motivated by the previous observations of Jarvis *et al.* (18). Our present results (Fig. 3) demonstrated that the gp43–gp45 heteroassociation is strengthened ≈10- to 20-fold by addition of PEG. The experiments described in the rest of this paper were all performed in the presence of 7.5% PEG.

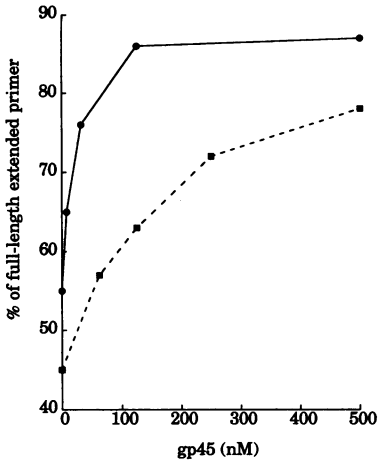

FIG. 3. Full-length synthesis products as a function of gp45 concentration in the presence and absence of PEG. Results from the experiment of Fig. 2 are plotted to show the fraction of extended primers that are 49 and 50 residues long in reaction mixtures that did not (■) contain PEG. Also plotted are results from a similar series of reactions performed in the presence (●) of 7.5% PEG.

Addition of gp32 Stimulates Processive gp45-Dependent Strand-Displacement Synthesis on a Leading-Strand DNA Construct. Hybridization of a 32-residue oligonucleotide (in which 16 of the residues are complementary to the downstream 5′ end of the template strand) to the primer template construct of Fig. 1*A* results in the forked structure shown in Fig. 1*B*. This construct mimics what a holoenzyme complex "sees" during leading-strand DNA synthesis. Using this forked template, it was observed that strand-displacement synthesis does not occur to any significant extent in polymerase-only reaction mixtures or in reaction mixtures containing polymerase plus the gp44/62 complex. However, full-length strand-displacement synthesis resulted when large amounts (>1 μM) of gp45 were included in reaction mixtures containing gp43 (data not shown). Finally, it was determined that the degree of gp43–gp45 processive full-length synthesis on the forked template was rather low. Thus, this protocol provided a good test of the effects of gp32 in further reconstituting a fully functional processive holoenzyme.

Fig. 4 displays the effect of increasing gp32 addition on the processivity of DNA synthesis by the T4 polymerase in the presence and absence of gp45. We note that gp32 alone cannot displace the downstream strand of the construct under the experimental conditions and protein concentrations of Fig. 4. As stated above, synthesis by T4 DNA polymerase alone proceeded only to the junction of the annealed strand (Fig. 4, lane 2) in the absence of both gp32 and gp45. Addition of gp32 resulted in decreased primer usage (lanes 3 and 4), reflecting a decrease in the dissociation rate of gp43 brought about by direct interaction of the polymerase with gp32 (for example, see refs. 17 and 28). However, there is no significant increase in full-length synthesis.

A strikingly different pattern of synthesis results when gp45 is included in the reaction mixture together with gp32 (Fig. 4, lanes 5–7). Upon addition of one or two molecules of gp32 per primer template construct, there is a significant increase in processive synthesis of full-length, strand-displaced product. Fully processive synthesis occurs when ≈8 gp32 molecules are present per DNA construct. Again, these observations were independent of the presence of ATP and were not obtained if the gp44/62 accessory protein complex (plus ATP) was added instead of gp45 (data not shown).

Synthesis from a Singly Primed Circular Single-Stranded M13 DNA Template. As a stringent assay for holoenzyme formation, processive DNA synthesis was monitored on a circular M13 single-stranded DNA template with a 17-residue oligonucleotide as primer. As Fig. 5 (lane 2) shows, even in

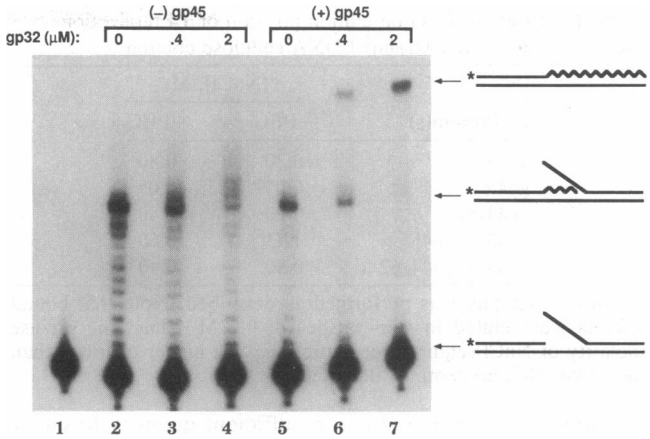

FIG. 4. Processive synthesis on the forked primer template construct as a function of gp45 and gp32. Synthesis assays were performed at 30°C, as described, in the absence or presence of 250 nM gp45. Each reaction mixture contained 2 nM gp43, 250 nM forked primer template molecules, 7.5% PEG, and the indicated amount of gp32 (monomers). On the right are shown representations of the extended primer at various stages of the synthesis reaction. The starting ^{32}P-labeled substrate is shown at the bottom (*). Product for which synthesis has proceeded up to the junction of the downstream strand is shown in the middle. Full-length (50 nucleotides) product is depicted at the top (the displaced, downstream strand is not shown). Lane 1, unextended primer only.

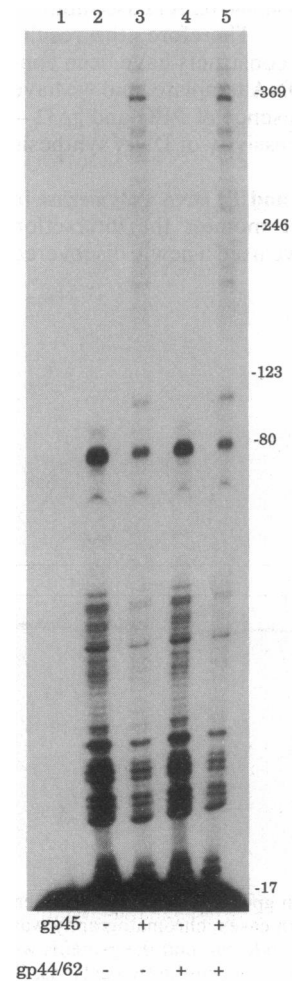

FIG. 5. Processive synthesis on singly primed M13 DNA by various T4 replication proteins. A ^{32}P-labeled 17-nucleotide primer (5′-GTAAAACGACGGCCAGT-3′) was annealed to M13mp8 at positions 6286–6270. Synthesis assays were performed at 30°C with this primer template construct as described. Each reaction mixture contained 0.1 nM gp43, 10 nM primer template (molecules), 5 μM gp32, 7.5% PEG, and, when present (see chart at bottom), 125 nM gp45 (trimers) and 25 nM gp44/62 (4:1 complexes). Products were subjected to electrophoresis on a 5% acrylamide (20:1)/8 M urea DNA sequencing gel; their approximate lengths (nucleotides) are indicated on the right. Lane 1, unextended primer only.

Table 1. Effect of PEG on elution position of T4 replication proteins from a single-stranded DNA cellulose column

Protein(s)	[NaCl], M −PEG	[NaCl], M +PEG
gp43	0.20	0.40
gp45	0.05	0.05
gp44/62	0.20	0.40
gp43 + gp45	NC	0.50
gp43 + gp44/62	0.20	0.40

Chromatography was performed as described. Note that bound species were eluted in step washes of 0.2 M. Thus, the precise molarity of NaCl required for elution may be higher or lower than indicated. NC, no complex detected.

the presence of gp32 (added in sufficient quantity to cover ≈50% of this template based on a site size of 7 nucleotide residues per gp32 molecule), the T4 DNA polymerase alone was unable to synthesize processively through a strong (i.e., 12-bp stem) hairpin structure located ≈80 nucleotides from the 5′ end of the primer. The same result was obtained upon addition of gp44/62 (and ATP) to the reaction mixture (lane 4). Earlier work demonstrated that processive synthesis past this hairpin requires assembly of the functional holoenzyme (18).

However, DNA synthesis past this hairpin does occur upon addition of gp45 (Fig. 5, lane 3). Furthermore, the product distributions obtained are identical to those found with a holoenzyme assembled in the presence of a full complement of accessory proteins and ATP (lanes 3 and 5). Thus, the pattern of synthesis obtained by adding gp45 directly to gp43 is the same as the processive synthesis found with holoenzyme assembled by the gp44/62 ATPase subunits of the accessory protein complex. Therefore, the results obtained with the oligonucleotide constructs have been confirmed by using this much larger DNA template, and we have shown that gp45 alone—in the presence of PEG and gp32—can significantly increase the processivity of DNA synthesis catalyzed by gp43.

Direct Interaction Between gp45 and T4 DNA Polymerase in the Presence of PEG. To further monitor the interaction between these two proteins we have used a newly discovered form of column chromatography. In this procedure, proteins were preincubated, either alone or in combination with other replication proteins, in buffer in the presence or absence of PEG. The protein samples were then loaded onto a small single-stranded DNA cellulose column that had been equilibrated in preincubation buffer. Bound proteins were step eluted from the column by increasing the NaCl concentration. The results are summarized in Table 1.

The SDS/polyacrylamide gel at the top of Fig. 6 shows that gp45 does not bind to the single-stranded DNA cellulose column in the presence of 7.5% PEG. The same result is obtained in the absence of PEG (Table 1). Consistent with the weak association between these two proteins, no interaction was detected between gp45 and gp43 in the absence of PEG within the (lengthy) time scale of this experiment (Table 1). However, a different result was obtained when gp45 was preincubated with gp43 in the presence of PEG. The elution profile obtained using this protocol is shown in the lower gel of Fig. 6. Although the majority of gp45 once again does not bind to the column, a significant amount of gp45 does bind and is coeluted with the gp43 at ≈0.5 M NaCl. Note that, unlike the gp43–gp45 complex, a putative gp43–gp44/62 complex, if formed, elutes at the same salt concentrations as do its individual protein components chromatographed separately (Table 1).

DISCUSSION

Before this study, several pieces of evidence had appeared that pointed to an interaction between gp45, gp43, and primer template DNA. Thus, affinity column measurements performed in the Alberts laboratory (17) implied a direct interaction between gp45 and gp43. However, crude lysates rather than purified proteins were used in these studies, and therefore it could not be ruled out that the observed retention of a given protein on the column might have been mediated by another protein present in the lysate. In another type of experiment, gp45 appeared to enhance the fidelity of DNA synthesis by gp43. These workers (29) interpreted this result in terms of a direct interaction between gp45 and gp43.

A previous study from our laboratory (18) suggested that the primary effect of the addition of PEG to DNA synthesis

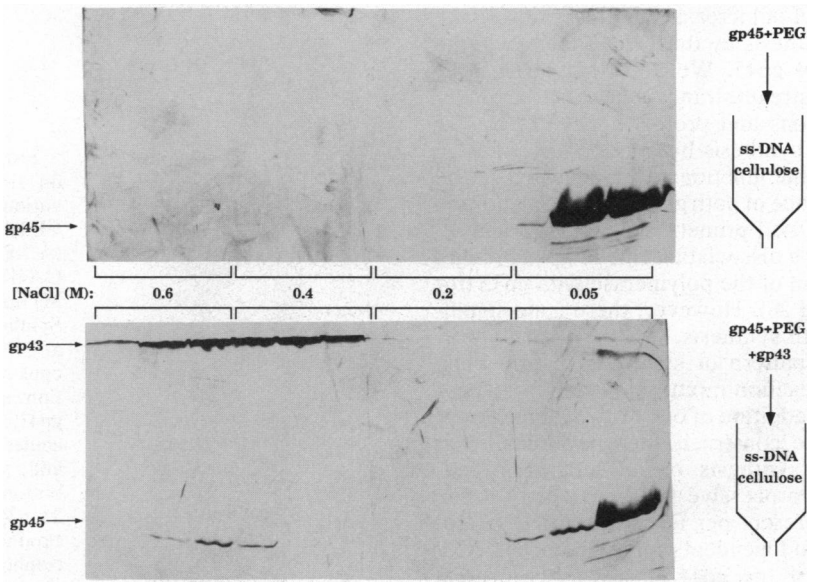

FIG. 6. Direct association between gp45 and gp43 in the presence of PEG. Two separate chromatographic runs were performed using the protocols depicted on the right. In both cases, chromatography was performed in the presence of 7.5% PEG. Aliquots of eluted fractions were separated on an SDS/15% polyacrylamide gel, and the proteins were visualized by staining with silver. Concentrations (molar) of NaCl used in the various elution steps are shown between the two gels. ss, Single stranded.

reaction mixtures, in the presence of the T4 accessory proteins, was to increase the affinity of the holoenzyme complex for gp45. Recently, Munn and Alberts (19) observed an enlargement of the DNA footprint of gp43 upon addition of large amounts of gp45 to the reaction mixture. Finally, N. G. Nossal (personal communication) observed formation of a putative complex between gp43 and gp45 by gel-shift methods.

We have demonstrated that a large molar excess of gp45 to gp43 is required to observe a functional change in processivity of DNA synthesis by the T4 polymerase. This result is comparable to the earlier observation, made with the *E. coli* DNA polymerase III holoenzyme system (8, 30), that addition of very large amounts (up to a 5000-fold molar excess) of β protein to the core polymerase leads to a bypass of the requirement for the other accessory proteins of the *E. coli* holoenzyme and for ATP.

The fact that the functional interaction between gp45 and gp43 is weak may explain why two previous studies concluded that gp45 alone does not potentiate the exonuclease activity of gp43 (15, 16). In these studies, the ratio of gp45 to gp43, as well as the absolute amounts of gp45 added, was considerably less than used here. Whether the exonuclease activity of gp43 is stimulated by large molar excesses of gp45 remains to be investigated.

The requirement for gp32 to obtain processive strand-displacement synthesis (Fig. 4) is consistent with previous observations on the important role of this protein in T4 holoenzyme function. For example, addition of gp32 decreased substantially the amount of either the accessory proteins or the T4 DNA polymerase required to obtain a footprint on DNA (19). Also relevant is the observation (31) that assembly of gp44/62 and gp45 on a primer template construct in the presence of adenosine 5′-[γ-thio]triphosphate required gp32. Finally, Gogol *et al.* (32) used cryoelectron microscopy to show that the ATP-dependent assembly of a distinctive accessory protein structure (originally termed a "hash-mark" but now more appropriately called a T4 sliding clamp) was greatly stimulated by gp32.

The experiments presented in Fig. 6 represent concurrent use of column chromatography and macromolecular crowding to probe weak protein–protein and protein–DNA interactions. The observation that chromatography in the presence of PEG results in a significant increase in the concentration of salt required to elute T4 DNA polymerase from single-stranded DNA is entirely consistent with a previous study of the effect of PEG on DNA synthesis by a variety of polymerases, including that of T4 (33). Chromatography in the presence of PEG might be exploited not only for analytical purposes, but also as a preparative strategy for the isolation of weakly interacting multicomponent protein assemblies from *in vivo* systems (an approach of this type has been suggested; see ref. 34).

Finally, we have recently learned that the Geiduschek laboratory (35) has independently applied a similar molecular crowding approach to the T4 accessory protein-dependent enhancement of T4 late transcription and has reached the complementary conclusion that gp45 is directly responsible for transcriptional activation (G. Sanders, G. Kassavetis, and E. P. Geiduschek, personal communication).

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