Structural Analyses of gp45 Sliding Clamp Interactions during Assembly of the Bacteriophage T4 DNA Polymerase Holoenzyme

III. THE gp43 DNA POLYMERASE BINDS TO THE SAME FACE OF THE SLIDING CLAMP AS THE CLAMP LOADER*

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In the preceding paper (Latham, G. J., Bacheller, D. J., Pietroni, P., and von Hippel, P. H. (1997) J. Biol. Chem. 272, 31677–31684), we demonstrated that the T4 gp44/62-ATP clamp loader binds to the C-terminal face of the gp45 sliding clamp. Here we extend these results by exploring the structural relationship between the gp43 polymerase and the gp45 sliding clamp. Using fluorescence intensity and polarization techniques, as well as photo-cross-linking methods, we present evidence that gp43, like gp44/62, binds to the C-terminal face of gp45. In addition, we show that gp43 binds to the gp45 clamp in two distinct interaction modes, depending on the presence or absence of template-primer DNA. When template-primer DNA is present, gp43 binds tightly to gp45 to form the highly processive DNA polymerase holoenzyme. Gp43 also binds to gp45 in the absence of template-primer DNA, but this interaction is more than 100 times weaker than gp43-gp45 binding on DNA. Specific interactions between gp43 and the C-terminal face of gp45 are maintained in both modes of binding. These results underscore the pivotal role of template-primer DNA in modulating the strength of protein-protein interactions during DNA synthesis and provide additional insight into the structural requirements of the replication process.

Stable interactions between the sliding clamp and the DNA polymerase within the replication holoenzyme are essential for processive and efficient replication of genomic DNA in both prokaryotes and eukaryotes. Indeed, phage T4 DNA polymerase (gp43)^1 dissociates from the template-primer DNA after incorporating only a few nucleotides into the nascent chain (1). Although gp43 is characterized by an intrinsic rate of synthesis (>400 s⁻¹)(2) that is comparable to the rate of DNA replication in vivo (600 s⁻¹) (3), the frequency of polymerase dissociation events in the absence of other replication factors would increase the time required for T4 genome synthesis from the observed 2–5 min to hours or even days (4). As a consequence, it has been suggested that the role of the polymerase accessory proteins is to increase the affinity of the polymerase for the template DNA during replication rather than to increase the inherent rate of chain extension (5). This proposal is particularly appealing, since the functional role of gp45 sliding clamp can now be understood in terms of its structure. Moarefi and Kuriyan^2 have recently determined that the gp45 trimmer is a torus characterized by an internal diameter that is more than sufficient to encircle duplex DNA. Thus, if gp45 binds to DNA as a surrounding ring, as biochemical studies suggest (7–10), then binding between gp45 and gp43 would tether the polymerase to the DNA via a protein-protein interaction with the sliding clamp. In this way, inefficient polymerase dissociation and reassociation events can be avoided. In addition, regulation of the stability of the gp43-gp45 complex also provides a means of integrating the discontinuous synthesis that occurs on the lagging strand into the dynamics of the overall replication process.

In the preceding paper (11) we described the use of three different cysteine mutants of gp45 to probe the polarity and general topography of interaction of the gp45 sliding clamp with the gp44/62-ATP clamp loader complex. In this work, we employ these gp45 mutants as tools to map regions on the sliding clamp surface that are at or near the binding site(s) of gp45 for gp43 in the T4 replication holoenzyme. We find that the gp43 polymerase binds to the gp45 clamp on the same C-terminal face with which the gp44/62 clamp loader interacts. Our results also demonstrate that gp45 can bind to gp43 in two interaction modes, depending on whether or not template-primer DNA is present. When template-primer DNA is available, gp43 and gp45 form a tight complex with a dissociation constant no greater than a few nM. In the absence of template-primer DNA, gp43 binds to gp45 much more weakly, with a dissociation constant of approximately 500 nM. These results are consistent with our current understanding of the regulation of protein-protein interactions within the replication complex during leading and lagging strand DNA synthesis.

EXPERIMENTAL PROCEDURES

Materials and Proteins—The sources and methods of purification of most of the materials and proteins used in this study are described in the preceding paper (11) with the exception of the purification of the T4 DNA polymerase. The gp43 polymerase was isolated by the method of Rush and Konigsberg (12), except that the order in which the columns were used was changed; the single-stranded DNA-cellulose column was used first in the preparative procedure, followed by the phosphocellulose and DE52 columns. In addition, the DE52 column replaced the Affi-Gel 10 column used by Rush and Konigsberg (12). All other procedures used have been presented previously (11) or are described in the figure legends or tables.

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1 The abbreviations used are: gp43, T4 gene 43 protein; gp45, T4 gene 45 protein; gp44/62, a 4:1 complex of the T4 gene 44 protein and gene 62 protein; IA, 5-[[2-iodoacetyl]amino(ethyl)amino]napthalene-1-sulfonic acid; T3, N-(4-azido-2,3,5,6-tetrafluorobenzy)-3-maleimidopropionamide; SA, streptavidin.
2 I. Moarefi and J. Kuriyan, manuscript in preparation.
T4 Polymerase Binds to the C-terminal Face of Sliding Clamp

RESULTS

Experimental Rationale—Elsewhere (11) we have described the biochemical and structural characteristics of three separate cysteine mutants of gp45 that we have designated gp45-S19C, gp45-S45C, and gp45-K81C. The indicated amino acid residues were chosen for site-specific mutation on the basis of their location within the three-dimensional structure of the gp45 protein ring. Thus, Ser-19 is situated on the C-terminal face of gp45 near the hole within the ring; Ser-45 is positioned on the opposite N-terminal face and slightly closer to the edge of the ring; and Lys-81 is located in the middle of the rim of the ring near the gp45-gp45 subunit interface (see Fig. 1 of Ref. 11). We have utilized the single cysteine sulphydryl group within each mutant subunit to attach several different types of reporter groups to determine how the environment at these positions changes in the presence of the gp44-gp46 clamp loader and various protein and nucleotide cofactors. In summary, our results showed that the gp44-gp46 subassembly binds to the C-terminal (and not the N-terminal) face of the gp45 sliding clamp ring. In this work we extend these findings by probing the site(s) of interaction of the gp45 sliding clamp with the gp43 polymerase.

The Fluorescence of the IA-gp45-S19C Sliding Clamp on End-blocked Template-Primer DNA Constructs Must Be End-blocked to Observe a Fluorescence Change in IA-gp45-S19C upon Adding gp43—Studies in several laboratories have revealed that the DNA processivity clamps of different replication systems can slide off a linear template-primer DNA if the ends are not blocked (8, 14). Indeed, the DNA polymerase holoenzymes from phage T4 and Escherichia coli both absolutely require either an end-blocked linear or circular template-primer construct to allow stable interactions on DNA between the sliding clamp and the polymerase (10, 14). To demonstrate that the gp43-dependent fluorescence increase in DNA-bound IA-gp45-S19C reflects assembly of the T4 DNA polymerase holoenzyme, we repeated the experiment shown in Fig. 1 using an unblocked linear template-primer DNA (13). The subsequent addition of the gp43 polymerase caused a 5% increase in the fluorescence of the IA-gp45-S19C trimmer ring (Fig. 1, filled squares). Thus, this fluorescence increase offset more than half of the quenching induced by the addition of template-primer DNA.

TABLE I

| Sample Change in fluorescence intensity |
|----------------------------------------|
| 100 nM IA-gp45-S19C + 1 mM ATP         | +22% |
| + 120 nM gp44/62                       |     |
| + 73 nM SA-50/30                       |     |
| + 100 nM gp43                          | +5%  |
| 100 nM IA-gp45-S19C + 1 mM ATP         |     |
| + 120 nM gp44/62                       |     |
| + 73 nM SA-50/30                       |     |
| + 100 nM gp43                          |     |
| 100 nM IA-gp45-S45C + 1 mM ATP         |     |
| + 120 nM gp44/62                       |     |
| + 73 nM SA-50/30                       |     |
| + 100 nM gp43                          |     |
| 200 nM IA-gp45-S19C + 311 nM gp43      | +2%  |
| + 311 nM gp43                          |     |
| 200 nM IA-gp45-S45C + 311 nM gp43      |     |

* NS, not significant (<2% change in fluorescence).

Template-Primer DNA Constructs Must Be End-blocked to Observe a Fluorescence Change in IA-gp45-S19C upon Adding gp43—Fig. 1 presents data obtained under three conditions for binding to the DNA, holoenzyme was still unable to assemble into a stable complex. Since, as described above, steady-state fluorescence assays have shown that IA coupled to template-primer DNA (13). The subsequent addition of the gp43 polymerase caused a 5% increase in the fluorescence of the IA-gp45-S19C trimmer ring (Fig. 1, filled squares). Thus, this fluorescence increase offset more than half of the quenching induced by the addition of template-primer DNA.

The Fluorescence of the IA-gp45-S45C Sliding Clamp on End-blocked Template-Primer DNA Is Unchanged by the Addition of the gp43 DNA Polymerase—Since, as described above, steady-state fluorescence assays have shown that IA coupled to template-primer DNA (13). The subsequent addition of the gp43 polymerase caused a 5% increase in the fluorescence of the IA-gp45-S19C trimmer ring (Fig. 1, filled squares). Thus, this fluorescence increase offset more than half of the quenching induced by the addition of template-primer DNA.

The Fluorescence of the IA-gp45-S45C Sliding Clamp on End-blocked Template-Primer DNA Is Unchanged by the Addition of the gp43 DNA Polymerase—Since, as described above, steady-state fluorescence assays have shown that IA coupled to...
the S19C residue of gp45 reports a change in the local environment when gp43 is added, the most straightforward interpretation is that gp43 contacts the gp45 ring at or near the S19C residue on the C-terminal face. Inasmuch as the S45C mutant residue is located on the N-terminal side of the ring (see Fig. 1 of Ref. 11), we can use this residue in the same way to determine whether the N-terminal face of the ring is involved in gp43 binding as well. To this end, we labeled gp45-S45C with IA (three Cys-45 residues/gp45 trimer) and characterized the fluorescence properties of this protein during clamp loading and holoenzyme assembly. Although the IA-gp45-S45C protein was efficiently labeled (2.2 IA fluorophores/gp45 trimer), this and holoenzyme assembly. Although the IA-gp45-S45C protein is unable to bind gp43, perhaps due to the introduction of the amino acid change at S45C or to covalent attachment of the IA fluorophore. To rule out this possibility, two control experiments were performed to show that both IA-gp45-S45C and the IA-gp45-S19C trimer can bind polymerase. First, the fluorescence anisotropy of DNA-bound IA-gp45-S19C and IA-gp45-S45C clamps was measured in the presence of increasing concentrations of gp43. As Table II shows, the anisotropy of both IA-gp45-S19C and IA-gp45-S45C increased when substoichiometric concentrations (50 nM) of gp43 were added, indicating that the gp43 protein decreases the rate of rotational diffusion of the IA-gp45 trimer rings. This result is consistent with the formation of a gp43-gp45 complex on template-primer DNA. When saturating gp43 (101 nM) was added, the anisotropy reached a plateau. This finding indicates that all of the gp43 was bound at this point. Thus, it is clear that gp43 changes the rotatory diffusion rate of the IA-gp45-S19C and IA-gp45-S45C clamps in a dose-dependent manner and that saturation is reached at a stoichiometric concentration of gp43.

Although we cannot accurately calculate a dissociation constant for the gp45 interaction with gp43 in the presence of template-primer DNA on the basis of these limited data, the results do show that the binding interaction between the two proteins is very tight, with a $K_d$ in the low nanomolar range or less. In contrast, parallel attempts to measure a binding constant between gp43 and IA-gp45-S19C on linear, unblocked template-primer DNA did not result in a significant change in the IA-gp45-S19C anisotropy at the concentrations used in the experiment ($\leq 100$ nM). This negative result was expected, since the polymerase holoenzyme cannot be stably assembled on a linear template-primer construct with unblocked termini (10).

As a second test to show that the derivatized gp45 mutant proteins were functionally unaffected by the modification (either IA or T3; see below), the capacity of the modified mutant gp45 clamps to form functional polymerase holoenzyme complexes was monitored in two separate assays: (i) an M13 primer extension assay and (ii) a strand displacement assay (Fig. 4). In the M13 primer extension assay, the reaction conditions were carefully chosen such that the synthesis of multikilobase products is possible with the T4 polymerase holoenzyme but not with the gp43 polymerase alone. The efficiency of product formation was then determined at different concentrations of wild-type gp45 and compared with the processive synthesis activity conferred by similar concentrations of the various derivatized gp45 mutant clamps. The results show that each of the modified gp45 mutants is capable of stimulating significant synthesis activity that can only arise from the action of polymerase holoenzyme complexes. Furthermore, the relative activity of the gp45 mutants calculated from this assay was comparable with the activity stimulated by the unmodified, unmutated wild-type gp45 clamp.
the 50 nM wild-type gp45 reaction was arbitrarily set to 1.00. The relative activity of each strand assay was determined by comparing the sum of the intensities of all product molecules longer than 1 kilobase pair, where the total intensity of said products from the 50 nM wild-type gp45 reaction was arbitrarily set to 1.00. Bottom, strand displacement assays were conducted at 30 °C for 30 s each using a template-primed construct (a 30-mer primer annealed to 3′-biotinylated 50-mer) that also had a “flap” 32-mer partially annealed to it (see Ref. 28 for a description of this construct, which is identical to the one discussed here except that a 30-mer primer, instead of a 20-mer primer, was used). Reactions contained 3 nM gp43, 250 nM gp44/62, 1 mM ATP, 0.5 μM gp32, 250 nM biotin-50/30/32-mer DNA, 160 mM potassium acetate, 115 μM dNTPs (each), and various concentrations of gp45. The relative activity of each polymerase holoenzyme complex was assessed by determining the fraction of polymerase molecules that can displace the flap 32-mer, once synthesis on the template-primed construct is initiated. All values were calculated relative to the efficiency of strand displacement in the presence of 250 nM wild-type gp45 (arbitrarily set to 1.00). F30, the 30-mer primer; T50, the 3′-biotinylated 50-mer template; F32, the flap 32-mer.

A strand displacement assay was also performed to test the ability of the mutant gp45 proteins to participate in holoenzyme assembly and function. In this assay, the fraction of holoenzyme molecules can be measured from the percentage of polymerase molecules that can displace a “flap” 32-mer oligonucleotide partially annealed downstream of the primer. Strand displacement is not observed in the absence of gp45, and the efficiency of displacement of the “flap” DNA strand is dependent on the concentration of gp45 available in solution. As Fig. 4 shows, the various IA- and T3-modified gp45 clamps assemble into fully functional polymerase holoenzyme molecules that strand displace at levels nearly identical to the efficiency of strand displacement in the presence of comparable concentrations of the wild-type gp45 clamp. Thus, in two separate assays the various modified, mutant gp45 clamps were found to form functional polymerase holoenzyme complexes that were nearly indistinguishable from holoenzymes assembled with wild-type gp45.

Photo-cross-linking Studies Reveal a Specific gp43-gp45 Interaction at the C-terminal Face of the Sliding Clamp—As shown in the companion papers (11, 15), site-specific photo-cross-linking is an excellent method for pinpointing potential sites of interaction between macromolecules. To provide further evidence that the S19C residues of gp45 are at or near the gp43 binding site, the sulfhydryl groups of gp45-S19C and gp45-S45C were covalently attached to the T3 cross-linker (see Ref. 15 for a discussion of the properties of this cross-linking agent). Both proteins were efficiently labeled by T3 (11), and polymerase holoenzyme activity assays were used to demonstrate that T3 modification does not perturb the formation of a functional gp43-gp45 complex on template-primer DNA (Fig. 4). Cross-linking results for the interaction of T3-gp45 with other components of the T4 DNA replication machinery are presented in Fig. 5.

In the presence of T3-gp45-S19C and gp44/62-ATP, cross-linked bands were observed between gp45-gp62 and gp45-gp44 (Fig. 5, lane 2) (11, 15). However, when gp43 and end-blocked SA-50/30-mer template-primer DNA were added under polynucleotide idling conditions, the cross-linking efficiency of gp45 to gp44/62 decreased, and several new bands appeared that moved with electrophoretic mobilities less than that of gp43 (Fig. 5, lane 3). Since the formation of these bands required gp43 and the band mobilities were too small to represent any reasonable combination of cross-linked gp45-gp44/62 multimers, these new bands must represent cross-linked products of gp43 and gp45. We note that the formation of gp43-dependent cross-links with gp45 was equally efficient when unblocked linear 50/30-mer template-primer DNA constructs were used in place of the end-blocked DNA (Fig. 5, lane 4), indicating that gp45 and gp43 can interact at least transiently on this nonstabilizing template-primer construct. Cross-linking between T3-gp45-S45C and gp43 (or T3-gp45-S45C and gp44/62-ATP) was not observed when either end-blocked or unblocked template-primer DNA were added (Fig. 5, lanes 5 and 6). Thus, the T3 cross-linker reports a gp43-gp45 interaction on DNA when attached to S19C but not when attached to S45C.

When T3-gp45-S19C is photoactivated in the presence of idling conditions, the gp43-dependent cross-linking efficiency of gp45 to gp44/62 decreased, and several new bands appeared that moved with electrophoretic mobilities less than that of gp43 (Fig. 5, lane 3). Since the formation of these bands required gp43 and the band mobilities were too small to represent any reasonable combination of cross-linked gp45-gp44/62 multimers, these new bands must represent cross-linked products of gp43 and gp45.
Analyses of the T3-gp45-S19C mutant (with 82% of its Cys-19 residues modified with IA) and the T3-gp45-S19C mutant (>90% of the Cys-19 residues modified with T3) provided clear evidence for protein-protein interactions between gp45 and gp43. However, in no experiment were we able to detect interactions between gp43 and reporter groups attached to the S45C residue of gp45. This result is significant, since both IA-gp45-S45C (72% of Cys-45 residues modified) and T3-gp45-S45C (>90% of Cys-45 residues modified) were extensively labeled with reporter groups in these fluorescence and photo-cross-linking studies. Thus, the negative results obtained for both experiments indicate that none of the three S45C residues in the gp45 trimer lie within the interaction distance of the gp43 protein in the T4 polymerase holoenzyme. Sexton et al. (18) have reported that a fluorophore attached to the TT7C residue of gp45, which lies on the same side of the trimer complex as S45C, but closer to the hole in the ring, also fails to undergo a change in fluorescence upon assembly of the T4 polymerase holoenzyme. This determination means that at least four and possibly six sites (three S45C sites plus at least one and perhaps three TT7C sites) on the N-terminal face of gp45 to date are known to be inaccessible to the gp43 polymerase.

The lack of gp43-gp45 photo-cross-linking with the T3-gp45-S45C trimer offers additional evidence for the absence of gp43-gp45 interactions on the N-terminal side of the ring. Since the T3 cross-linker is 13 Å in length, the interaction distance between S45C residue and any part of the gp43 polymerase should be greater than this distance. Although it is possible that the distance between S45C and the nearest residue on gp43 is within cross-linking range and that an unfavorable orientation of the cross-linker prevents cross-linking, we feel this is unlikely given the many positive results using T3 cross-linker in other studies (15, 19, 20). In fact, in the second paper of this series (11) we report that T3-gp45-S45C does not cross-link to gp44/62-ATP. However, we also presented evidence in that work using an alternate method that demonstrated that the lack of T3-gp45-S45C cross-linking to gp44/62 was consistent with the conclusion that the T3 cross-linker was simply too far away from gp44/62 to react. Thus, the most reasonable explanation for these data is that the T3 cross-linker of T3-gp45-S45C is also too far away from gp43 to permit cross-linking.

**Polymerase-Sliding Clamp Interactions on the Edge of the gp45 Ring**—We have also shown that T3-gp45-K81C cross-linking to gp43 is much less efficient than T3-gp45-S19C cross-linking to gp43. Since the K81C residue lies on the edge of the gp45 ring, this result suggests that at least one of the K81C residues of the trimer ring must lie within ~13 Å of gp43. Indeed, distance measurements taken from the gp45 crystal structure indicate that the side chain of K81C is approximately 13 Å from the plane of the C-terminal face of the protein ring.
Thus, gp43 could bind exclusively to the C-terminal (S19C) face of gp45 and still show some inefficient cross-linking to T3-gp45-K81C. However, we cannot rule out some binding interactions between gp43 and gp45 at a site(s) on the rim of the sliding clamp.

**Polarity of Interaction of the gp45 Clamp with Other Proteins**—Taken together, the above data strongly support a model in which gp43 binds to the C-terminal, and not the N-terminal, face of the gp45 ring on DNA. If the gp45 ring is topologically committed to the DNA, as numerous biochemical experiments implicating a sliding mechanism have suggested (7, 8), then the two sides of the structurally asymmetrical gp45 ring<sup>2</sup> will face in opposite directions along the DNA template. Consequently, when the polymerase associates with DNA that already carries a sliding clamp, one face of the gp45 torus will be far better positioned to bind the polymerase than the opposing face. Indeed, experiments from the Geiduschek laboratory (7, 21) have shown convincingly that the gp44/62 clamp loader mounts the gp45 clamp onto the template-primer DNA construct with a defined polarity. The sliding clamp polarity induced by this loading event then determines the face of gp45 (here acting as a mobile enhancer) that can slide along the DNA and interact with the RNA polymerase transcription initiation complex to stimulate late transcription (7). Recently, a direct interaction between gp45 and the promoter recognition protein gp55 has been demonstrated in the absence of RNA polymerase (22). In toto, these results show that the same (C-terminal) face of gp45 interacts with at least three protein complexes, i.e. gp44/62 (11), gp43, and gp55 (22). An interaction between gp45 and the transcriptional coactivator gp33 protein has also been implied but not conclusively demonstrated (23).

**Polarity of Sliding Clamp Interactions with Proteins from Other DNA Replication Systems**—The structural polarity of the gp43 polymerase-gp45 sliding clamp interaction is also supported by biochemical studies using replication proteins from other organisms. For example, O’Donnell and colleagues (24) have reported that the C terminus of the β-clamp (which protrudes from one face of the β ring) plays a key role in binding both δ (the subunit of the γ-complex clamp loader that binds β) and α (an E. coli DNA polymerase III subunit). Both the L2 and R64 residues in *Schizosaccharomyces pombe* proliferating cell nuclear antigen lie on the same face of the ring, and, significantly, L2V and R64A mutations cause marked reductions in the processivity of the polymerase δ holoenzyme (25). Fukuda *et al.* (26) have also identified several residues (including the N terminus) that share the same face of human proliferating cell nuclear antigen and that are essential for processivity by the polymerase δ holoenzyme. Thus, polarized binding between the sliding clamp and the replicative polymerase within the DNA polymerase holoenzyme appears to be a conserved feature of the DNA replication machinery from a variety of organisms.

**Template-Primer DNA Modulates the Affinity of the Polymerase for the Sliding Clamp**—The demonstration that gp45 can bind to gp43 both on and off template-primer DNA raises a number of interesting points. First, to the level of resolution possible in these experiments, the polarity of gp43 binding to the gp45 ring is maintained even when the two proteins interact free in solution; *i.e.* the qualitative change in fluorescence observed with IA-gp45-S19C and IA-gp45-S45C is the same in the presence and absence of DNA. For example, in the presence of DNA IA-gp45-S19C and IA-gp45-S45C are the same in the presence and absence of DNA. However, if the presence of DNA IA-gp45-S19C reports a 5% increase in steady-state fluorescence when the polymerase holoenzyme is assembled (Fig. 1 and Table 1). In contrast, the IA-gp45-S45C clamp exhibits a slight decrease in fluorescence under the same conditions (Fig. 3 and Table 1). In the absence of DNA, the addition of gp43 also increases the fluorescence intensity of IA-gp45-S19C, whereas the IA-gp45-S45C fluorescence is unchanged. Thus, binding between gp43 and IA-gp45-S19C induces a fluorescence increase when the gp43-gp45 complex is either on or off the template-primer DNA. Similarly, a slight quench or no fluorescence change is observed when gp45-IA-gp45-S45C binds to gp43 in both the presence and absence of DNA. We interpret these data to mean that gp43-dependent increases in the fluorescence intensity observed with IA-gp45-S19C indicate gp45 binding to gp43, regardless of the presence of template-primer DNA. This conclusion is supported by the anisotropy results summarized in Table II and Fig. 6. The lack of an increase in the fluorescence of the IA-gp45-S45C clamp probably reflects the fact that gp43 does not contact the N-terminal side of the gp45 ring.

The cross-linking results presented in Fig. 5 provide additional support for the conclusion that the “sidedness” of the gp45 binding to gp43 is the same whether or not DNA is involved in the binding complex. In the presence of DNA, several cross-linked gp43-gp45 bands are seen on the gel, including one intense band and two less intense bands. These three bands are also observed when DNA is omitted from the cross-linking reaction. Thus, the interaction between gp43 and gp45 in the absence (and presence) of DNA is topographically specific, in that cross-linking to random sites in the gp45 protein is not observed (T3-gp45-S45C does not cross-link to gp43). Moreover, the T3-gp45-S19C can be cross-linked to gp43 even when template-primer DNA is absent. It is tempting to speculate that the three cross-linked bands involving gp45 and gp43 represent a monomer, dimer, and trimmer of gp45 cross-linked to gp43, although this possibility has not yet been thoroughly investigated.

It is also clear, however, that the gp45 clamp and the gp43 polymerase do not interact in exactly the same way when the binding complex is formed in the presence and absence of template-primer DNA. The fluorescence anisotropy results presented in Table II suggest that gp43-gp45 binding on template-primer DNA to form the polymerase holoenzyme is very strong, with a dissociation constant in the nanomolar range or less. Consistent with this conclusion, a recent report from the Benkovic laboratory has estimated the dissociation constant for gp43-gp45 binding in the DNA-bound polymerase holoenzyme to be 30 pm (27). In contrast, our results show that when the gp43 and gp45 proteins are free in solution, they bind at least 100-fold more weakly (*K<sub>d</sub> = 480 ± 100 nm*). This finding fits well with our inability to detect gp43-gp45 binding in the absence of template/primer DNA by conventional gel shift techniques, although a weak association of gp43 and gp45 in solution has been demonstrated by macromolecular crowding (28).

### Notes

4 The cross-linking reactions shown in lanes 9 and 10 of Fig. 5 were photoactivated under more favorable conditions than were the reactions shown in lanes 2–8. The three gp43-gp45 bands visible in lane 10 are also visible in lanes 3, 4, 7, and 8, although the bands are less intense. Thus, it would be inappropriate to compare the cross-linking efficiency of products shown in lane 10 with those shown in lanes 3, 4, 7, and 8.

5 Berdis and Benkovic (30), in their measurement of the “shutdown” of the gp44/62 ATPase, have found that the binding of gp43 to gp45 in the polymerase holoenzyme increases linearly with the addition of increasing numbers of equivalents of gp43. Since this “shutdown” is directly dependent on the concentration of gp45 free in solution versus gp45 associated with the polymerase in the holoenzyme, we can conservatively estimate that the dissociation constant between gp43 and gp45 on DNA is at least 10 times lower than the concentration of gp45 and template-primer DNA used in their experiment (250 nm). Thus, the *K<sub>d</sub>* for gp43-gp45 binding in the polymerase holoenzyme is less than 25 nm. This calculation is in good agreement with our assertion that gp43 binds tightly to gp45 (*K<sub>d</sub>* ≈ few nm) on template-primer DNA.

6 S. Weitzel and M. Reddy, unpublished data.
polymerase III and the β-clamp is 250 nM in the absence of DNA (14) but less than 5 nM in the presence of DNA (24). Thus, as suggested also for the *E. coli* complex (24, 29), these changes in binding affinity must reflect conformational changes in either the sliding clamp or the polymerase (or both) that result in increased binding affinity between the two proteins when template-primer DNA is present.

The Clamp Loader and the Polymerase both Bind to the C-terminal Face of the gp45 Ring—Berdis et al. (27) have recently shown that the C-terminal six amino acid residues of the gp43 polymerase are critical for assembly of the polymerase holoenzyme. In their report they also demonstrated that an oligopeptide composed of these same six C-terminal residues from gp43 inhibits the gp44/62 clamp loader when the gp45 clamp and template-primer DNA are present, implying that the oligopeptide binds to gp45 at a site that the gp44/62 clamp loader also uses for binding to gp45. These results are in excellent agreement with the conclusion supported by the data presented here and in the companion paper (11); i.e. the gp43 polymerase and the gp44/62 clamp loader share a binding site(s) on the gp45 ring (see below). In addition, our results also indicate that the location of this coincident binding site(s) is restricted to the C-terminal (S19C) face of the gp45 clamp.

How can we explain the fact that the gp43 polymerase and the gp44/62 clamp loader both bind the C-terminal face of the gp45 clamp? This result is extremely appealing when one considers the coordination of protein-protein interactions required to meet the demands of the replication process. As shown in Fig. 7, in the absence of DNA the gp45 clamp binds to gp44/62-ATP with much higher affinity (Kd, app < 10 nM) (11, 13) than to gp43 (Kd ~ 500 nM). The clamp loader thus outcompetes the polymerase for the sliding clamp under these conditions, and most of the gp45 rings are sequestered in a binding complex with gp44/62-ATP. However when template-primer DNA is present, the DNA facilitates the dissociation of the gp45-gp44/62-ADP complex (13, 15), and the gp45 sliding clamps are loaded onto the template-primer junction. Under these conditions, the clamp can bind tightly to the DNA polymerase.

The net result is that the template-primer DNA modulates
the affinity of the replication proteins for one another at different stages in the assembly of the replication holoenzyme. If the clamp loader could still bind the sliding clamp when bound to DNA, then this binding would block assembly of the holoenzyme, since the clamp loader and the polymerase both contact the C-terminal face of the gp45 clamp. This scenario would greatly impair the overall rate of chain elongation by decreasing the efficiency of strand synthesis. Yet this possibility is circumvented, since the clamp loader does not have access to the clamp when the clamp is bound to the polymerase on the DNA. Likewise, the polymerase does not have access to (or the requisite binding affinity for) the sliding clamp before the DNA. The polymerase does not have access to (or the requisite binding affinity for) the sliding clamp before the DNA, since the clamp loader does not have access to the clamp when the clamp is bound to the polymerase on the DNA. Similarly, the polymerase does not have access to (or the requisite binding affinity for) the sliding clamp before the DNA, since the clamp loader does not have access to the clamp when the clamp is bound to the polymerase on the DNA. Likewise, the polymerase does not have access to (or the requisite binding affinity for) the sliding clamp before the DNA, since the clamp loader does not have access to the clamp when the clamp is bound to the polymerase on the DNA. Similarly, the polymerase does not have access to (or the requisite binding affinity for) the sliding clamp before the DNA, since the clamp loader does not have access to the clamp when the clamp is bound to the polymerase on the DNA.

These conclusions are fully supported by the cross-linking data presented in Fig. 5. T3-gp45-S19C cross-linking to gp62 and gp44 is only slightly decreased by the addition of gp43. However, when template-primer DNA is added, cross-linking between gp45 and gp44/62 decreases dramatically, and conversely, cross-linking to gp43 increases. This result is consistent with the ability of the clamp to bind more tightly to the gp44/62-ATP clamp loader in the absence of DNA and more tightly to the gp43 polymerase in the presence of DNA. Although we note that the photo-cross-linking interactions described here do not, by definition, represent equilibrium protein-protein interaction states, the qualitative nature of the observed changes in cross-linking efficiency strongly support the above physiological inferences.

Conclusions—In summary, our results show that both the T4 polymerase and the T4 clamp loader bind to the gp45 sliding clamp through its C-terminal face and that the strength of this binding is modulated by template-primer DNA. These results are in excellent agreement with those described for the E. coli replication proteins (24). Inasmuch as the T4, E. coli, and human SV40 replication systems share significant structural and functional homology, it will be interesting to see if the mechanisms for the control of protein-protein interactions in human DNA replication resemble those described for T4 and E. coli.

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