The bacteriophage T4 replication complex is composed of eight proteins that function together to replicate DNA. This replisome can be broken down into four basic units: a primosome composed of gp41, gp61, and gp59; a leading strand holoenzyme composed of gp43, gp44/62, and gp45; a lagging strand holoenzyme; and a single strand binding protein polymer. These units interact further to form the complete replisome. The leading and lagging strand polymerases are physically linked in the presence of DNA or an active replisome. The region of interaction was mapped to an extension of the finger domain, such that Cys-507 of one subunit is in close proximity to Cys-507 of a second subunit. The leading strand polymerase and the primosome also associate, such that gp59 mediates the contact between the two complexes. Binding of gp43 to the primosome complex causes displacement of gp32 from the gp59-gp61-gp41 primosome complex. The resultant species is a complex of proteins that may allow coordinated leading and lagging strand synthesis, helicase DNA unwinding activity, and polymerase nucleotide incorporation.

The bacteriophage T4 DNA replication complex is composed of eight proteins that form a highly efficient machine responsible for accurate DNA synthesis. Central to the replication process is the T4 DNA polymerase (gene product 43 (gp43)), which catalyzes the incorporation of nucleotides in the 5’ to 3’ direction and contains a 3’ to 5’ exonuclease proofreading activity (1). Because the polymerase only synthesizes DNA in the 5’ to 3’ direction, the lagging strand must be synthesized discontinuously in stretches of oligonucleotides known as Okazaki fragments. In the absence of other proteins, the processivity of the polymerase is limited, and a trimeric sliding clamp (gp45) is required to enhance processivity (2, 3). The toroidal sliding clamp circumscribes DNA and is assembled onto DNA by a clamp loader (gp44/62) (4).

A helicase is required to unwind dsDNA ahead of the polymerase. The bacteriophage T4 replicative helicase (gp41) forms a hexameric ring that encircles the lagging strand and unwinds the DNA duplex with a 5’ to 3’ polarity by hydrolyzing either ATP or GTP (5). Single-stranded regions of DNA exposed by the action of the helicase are covered with single-stranded binding proteins (gp32). gp32 binding prevents formation of DNA secondary structure and reannealing of the duplex, thereby enhancing gp43 DNA polymerase activity (6). A helicase assembly protein (gp59) is required for the initial loading of the helicase onto single-stranded DNA (ssDNA)1 coated with single-stranded binding proteins (7).

The activity of the helicase is enhanced by binding of a primase (gp61) (8, 9). The primase synthesizes pentanucleotide primers on the lagging strand that are extended by the polymerase into stretches of Okazaki fragments. The primers are later removed, and ligases join the Okazaki fragments to form a continuous complementary strand.

Previously, the T4 replisome has been studied as four basic units: a primosome formed from the helicase, primase, and helicase loading protein that is responsible for unwinding of duplex DNA and synthesis of RNA primers on the lagging strand; a holoenzyme containing the polymerase coupled to the sliding clamp on the leading strand; a lagging strand holoenzyme; and a single-stranded DNA-binding protein. However, it is unlikely that these units function separately; more likely, the activities of all four are coupled. For instance, Salinas and Benkovic (10) demonstrated coupling between leading and lagging strand DNA synthesis and suggested that the two polymerases are physically linked. It also seems apparent that the movement of the holoenzyme needs to be coupled with that of the primosome as it unwinds double-stranded DNA during leading strand synthesis. Studies in similar systems have demonstrated this type of interaction. The bacteriophage T7 gene 4 helicase/primase protein has been shown to directly interact with the T7 DNA polymerase (11). Furthermore, the σ subunit of the DNA polymerase III holoenzyme in Escherichia coli contacts the DnaB helicase, thereby increasing its unwinding activity 10-fold (12).

Numerous studies have suggested that there is a “functional” interaction between the holoenzyme and the primosome in the T4 replisome. Cha and Alberts (13) demonstrated that only the helicase was needed for strand displacement synthesis by

1 The abbreviations used are: ssDNA, single-stranded DNA; FRET, fluorescence resonance energy transfer; BMH, 1,6-bis-maleimidohexane; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption ionization; gp44c, gp44 with an extra cysteine added at the C terminus; SA-HRP, streptavidin linked to horseradish peroxidase; CPM, 7-diethylamino-3-(4-methylphenyl)-4-methylcoumarin; OG, Oregon Green 488 maleimide; TMR, tetramethylrhodamine 5'-maleimide; OGN, Oregon Green 488 carboxylic acid, succinimidyl ester, 5-isomer; gp59(CPM), gp59 labeled at Cys-49 with CPM; gp61(TMR), gp61 labeled at Cys-144 with TMR; gp41(TMR), gp41 labeled at Cys-316 with TMR; gp41(OG), gp41 labeled at Cys-316 with OG; gp43(OGN), gp43 labeled at the N-terminal with OGN; gp32(OG), gp32 labeled at Cys-166 with OG; HPLC, high pressure liquid chromatography; AMCA, sulfoSuccinimidyl-7-amino-4-methylcoumarin-3-acetic acid; TMEA, Tris(2-maleimidethanolamine).
the holoenzyme; the primase and gp32 had no effect on this rate of synthesis. These results indicated that there was molecular communication between the helicase and polymerase. Shrock and Alberts (14) later suggested that the helicase and polymerase interact, through changes in the processivity of the helicase in a reconstituted replisome. Moreover, Dong et al. (15) showed that rapid and processive strand displacement synthet- sis could be observed with only the helicase and polymerase in the presence of a macromolecular crowding agent (polyethylene glycol). Presumably, the crowding agent drives the helicase onto the fork, where in turn it interacts with the polymerase to give the observed results (15, 16). An interaction between the polymerase and helicase in the absence of DNA, however, was not found by analytical ultracentrifugation (16). This led to the hypothesis that the coupling between the two proteins might be mediated by formation of a ternary complex with DNA (16).

All of these studies provide indirect evidence of a physical interaction between the holoenzyme and the primosome in bacteriophage T4. Potential interactions between the holoen- zyme and the primase and gp59 were not analyzed. Moreover, recent studies by the Drake and Von Hippel laboratories (16, 17) have indicated that the leading and lagging strand holoen- zymes do not actually form a physical complex. To further investigate the formation of complexes between the two holoen- zymes and between the holoenzyme and primosome, a number of cross-linking and fluorescence resonance energy transfer (FRET) experiments were conducted. These experiments demon- strate that 1) the polymerase forms a dimer, but only in the presence of DNA or an active replication fork, 2) a physical interaction exists between the leading strand holoenzyme and the primosome, mediated primarily through gp59 and gp43, and 3) the polymerase displaces the gp32 single-stranded bind- ing protein from gp59 upon binding. These results are dis- cussed herein.

**EXPERIMENTAL PROCEDURES**

**Cross-linking between gp43 Subunits—**gp43 was purified as described previously (18). The protein was dialyzed into 20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol before being subjected to cross-linking. The homobifunctional, thiol-thiol cross-linker 1,6 bis-maleimidohexane (BMH) was dissolved in N,N-dimethylformamide to a concentration of 10 μM. One μl of this solution was added to 19 μl of a solution containing 1 μM gp43 in the absence or presence of 1 μM of a partially double- stranded DNA substrate (34/62-mer, the sequence is as fol- lows: 5′-ACT CCT TCC CCA GGT AAT TTT TGA CCG ACG TTT G and 5′-ACA CAG ACG TAC TAT CAT GAC GCC ATC AGA CAA CGT CGT AAA TTA CGT CCG GAA GGT G). The reaction was allowed to proceed for 3 min and was quenched with 50 mM dithiothreitol (DTT) followed by the addition of 0.5 μl of SDS-PAGE buffer (150 mM Tris, pH 6.8, 4% SDS, 0.1% bromphenol blue, 30% glycerol). The samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane (micron), and subjected to a Western blot using an anti-gp43 antibody. The membrane was blocked for 2 h at 25 °C with 3% bovine serum albumin in 20 mM Tris, pH 7.6, 140 mM NaCl, and 0.1% Tween 20 (TBST) and then incubated with a 1:5000 dilution of the anti-gp43 antibody (3% BSA, 1% milk, 5% AB) and probed with 1:10000 dilution of horseradish peroxidase (SA-HRP), as described previously (20).

**Cross-linking between the Primosome and gp43—**gp43 was labeled with the trifunctional cross-linker (Fig. 2C). gp43(OGN) was then dialyzed in labeling buffer at pH 6.8 and then labeled with a 3-fold excess of OGN labeling buffer containing 10 mM N-ethylmaleimide (NEM) in the presence of ATP (3 mM final). The reaction was quenched by the addition of 10 μl DTT. The DNA was removed from gp43 by applying the mixture to a mono-Q fast protein liquid chromatography column. Cross- linked, dimeric gp43 was then separated from monomeric gp43 by a 2000 HPLC column (Superdex-200) and the dimeric gp43 was then cross-linked and applied to a C-18 column as previously described (20). The cross-linked peptides were isolated by collecting the fraction that absorbed at 345 nm (the absorbance of the AMCA probe). This fraction was dried in a speed vac concentrator and analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry as described previously (19).

**Labeling of Proteins with a Trifunctional Cross-linker—**gp41, gp59, and gp61 were purified as previously published (19, 21, 22). Before labeling, each protein was dialyzed into 20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol. gp41, gp59, and gp61 each contain one free, solvent- exposed cysteine residue: Cys-316, Cys-42, and Cys-144, respectively. The functional cross-linking shown in Fig. 3A was dissolved in N,N- dimethylformamide to a concentration of 500 μM, and 5 μl of this solution was added to 50 μl of a 5 μM solution of either gp41, gp59, or gp61. The proteins were labeled for 12 h at 4 °C in the dark to ensure complete labeling of the cysteine residues by the cross-linker. Excess label was removed by applying the mixture to a 1-mL Sephadex G-25 spin column. Another form of gp41, gp41 containing a cysteine added to the C terminus as previously described (19) (gp41c), was also labeled in this manner.

gp43 was labeled with the trifunctional, amine-reactive probe Sulfo- SBD (Pierce). The protein (500 μl of a 5 μM solution) was dialyzed into 20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol and mixed with 50 μl of a 10 mM solution of Sulfo-SBD (dissolved in dimethyl- amide). The reaction was allowed to proceed for 4 h and quenched by the addition of 20 mM Tris, pH 7.5 (final concentration). The protein was then dialyzed into 20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol. Cross-linking between the Primosome and gp43—gp41, gp61, or gp59 labeled with the trifunctional cross-linker (at a final concentration of 3 μM) was mixed with gp43 (final concentration of 0.5 μM) in the presence of the other two primosome proteins (3 μM each) and a forked DNA substrate (0.5 μM) in a final volume of 20 μl. gp43 labeled with Sulfo- SBD was cross-linked to primosome proteins following the same procedure.

Two forked DNA substrates were used in these experiments. The first was composed of a 34-mer primer annealed to a 62-mer leading strand (with the sequence given above) and a partially complementary lagging strand of 75 nucleotides (with the sequence 5′-TGGTTGGGGAGGGAGTGGGATGATA- GTACGTCTGTGT). The reaction was initiated by exposure to an ultraviolet lamp for 7 min followed by the addition of 5 μl of SDS-PAGE buffer (20). In the lanes where reduction of the disulfide was desired, DTT at a final concentration of 100 mM was added. The proteins were immediately separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with a rabbit antibody against horseradish peroxidase (SA-HRP), as described previously (20).

**Labeling of Proteins with Fluorophores—**Accessible cysteine residues at gp32/Cys-166, gp59/Cys-42, gp41/Cys-316, and gp61/Cys-144 were labeled with 7-diethylamino-3-(4′-maleimidophenyl)-4-methyl- coumarin (CPM), Oregon Green 488 maleimide (OG), or tetramethylrhodamine 5′-maleimide (TRITC) as required. Unlabeled were dialyzed in labeling buffer (25 mM HEPES, pH 7.3, 150 mM NaCl, and 10% glycerol) and labeled with a 5-fold excess of the appropriate fluorescent dye for 1–4 h at 4 °C. The labeled protein was then dialyzed in labeling buffer containing 10 μM β-mercaptoethanol and frozen in aliquots at −70 °C. Alternatively, the N terminus of gp43 was specifi- cally labeled in a lower gel buffer using Oregon Green 488 carboxylic acid, succinimidyl ester, 5-isomer (OGN). Unlabeled gp43 was dialyzed in labeling buffer at pH 6.8 and then labeled with a 3-fold excess of OGN for 4 h at 4 °C. gp43(OGN) was then dialyzed in labeling buffer at pH 7.5 and frozen in aliquots at −70 °C. All labeling processes were per-
formed in less than 30 h to minimize any reduction in wild-type activity. The activity of the labeled proteins was confirmed using conventional activity assays. gp59(CPM) and gp41(OG) were analyzed using the ATPase assay (23), and no significant difference was found in the rate of ATP hydrolysis from that of the wild-type gp59 and gp41 combination. The priming activity of gp59(TMR) was tested using the priming assay (22) or minicircle assay (10). Labeling of gp56 resulted in a 15% loss in priming activity and a 25% loss in synthesis of lagging strand products. gp43(OGN) was tested using with the ATPase assay (24) and the strand displacement assay (25). The addition of gp43(OGN) in the ATPase assay shut down ATP hydrolysis to 75% of that of unlabeled gp43, suggesting an interaction with gp43 causing the sequestering of the holoenzyme onto DNA. The strand displacement assay was conducted in a benchtop format to check the ability of gp43(OGN) to displace a 36-mer from the 34/62/36-mer DNA substrate (26) and extend the 34-mer to a 62-mer. Extension of a 5’-32P-labeled 34-mer oligonucleotide results in full-length 62-mer 32P-labeled products. The ratio of strand-displaced full-length products to unextended 34-mer was used to analyze the polymerization property of gp43(OGN). gp43(OGN) displacement of CPM was observed at 390 nm. Unlabeled gp43.

**RESULTS**

**Cross-linking Studies to Analyze gp43-gp43 Interactions**—Using a two-hybrid approach, Salinas and Benkovic (10) identified a region of gp43 that was critical for dimerization. This

**FIG. 1. Cross-linking of gp43 on a primer template DNA substrate.** A, gp43 was cross-linked with the thiol-thiol cross-linker BMH in the presence or absence of partially duplex DNA as described under “Experimental Procedures.” The cross-linked species were separated by SDS-PAGE and subjected to a Western blot using an anti-gp43 antibody. Lane 1 corresponds to gp43 in the absence of DNA and gp43 was mixed with BMH in the absence (lane 2) or presence (lane 3) of DNA. B, the cross-linking experiment was repeated on a rolling minicircle as replication was occurring. gp43 was mixed with all of the replicase proteins, dNTPs, rNTPs, and BMH in the absence (lane 1) or presence (lane 2) of minicircle DNA.
some proteins, gp41, gp59, and gp61, were specifically labeled with the trifunctional cross-linker shown in Fig. 3A at Cys-316, Cys-42, and Cys-144. The trifunctional cross-linker contains a biotin affinity handle, a photoactivable arylazide, and a cleavable disulfide bond. The structure of the cross-linker is such that after the labeled protein is cross-linked to an interacting protein, cleavage of the disulfide bond results in transfer of the biotin group to the interacting protein. In these experiments, gp43 was mixed with all of the primosome proteins (gp41, gp43, and gp61) in the presence of a forked DNA substrate (either the 34/62/73-mer or 34/62/36-mer), where one of the primosome proteins was labeled with the trifunctional cross-linker.

The samples were exposed to ultraviolet light to initiate cross-linking, separated by SDS-PAGE in the presence or absence of DTT, and then subjected to a Western blot using SA-HRP as a probe.

When labeled gp59 was mixed with gp43 and exposed to UV light, a higher molecular weight band was seen on the Western blot, consistent with a cross-link between gp43 and gp59 (Fig. 4A, lane 2). The addition of DTT results in cleavage of the disulfide bond in the cross-linker and the resultant transfer of the biotin label to gp43 (Fig. 4A, lane 4), thus verifying the interaction between gp59 and gp43. There was no difference in cross-linking between assembly of the complex on the 34/62/73 fork versus the 34/62/36 fork (data not shown). In contrast, cross-links between gp41 and gp43 or between gp61 and gp43 were not observed consistently, and when they were observed, only trace amounts of cross-linking could be seen (data not shown). gp41c (gp41 with a cysteine added to the C terminus) was also labeled and subjected to cross-linking with gp43 but did not show any interaction.

Since the labels attached to gp41 and gp61 may not have been in close proximity to gp43, gp43 was labeled with the trifunctional cross-linker Sulfo-SBED and mixed with the primosome proteins. Sulfo-SBED is similar to the trifunctional cross-linker used above, with the exception that it is amine-reactive instead of thiol-reactive. Due to the large number of lysine residues on gp43, numerous labels can be placed throughout the protein, maximizing the probability of capturing an interacting protein. In the presence of the primosome proteins and fork DNA, no cross-linking was observed between gp43 and gp41 or gp61. However, cross-linking between gp43 and gp59 was again observed, since the biotin label was transferred from gp43 to gp59 upon reduction of the cross-linker (Fig. 4B, lane 4).

The interaction between gp59 and gp43 was investigated further on an active rolling minicircle. The replisome was assembled on DNA in the presence of the minicircle using gp59 labeled with the thiol-reactive trifunctional cross-linker. After the initiation of replication, cross-linking was induced by exposure to UV light. The products of the reaction were subjected to SDS-PAGE and Western blot as described above. Cross-link bands are observed, and when DTT is added, the biotin label is transferred to proteins corresponding to the molecular weights of gp61 and gp43, indicating that interactions between gp59, the primase, and the polymerase occur during active replication (Fig. 4C, lane 4).

These collective results suggest that the primary protein-protein interaction between gp43 and the primosome occurs via gp59, whereas gp41 and gp61 do not interact with gp43, or the interaction is too transient to capture with this technique. Additionally, the anticipated contact between the primase and gp59 protein is verified.

**Interaction between gp59 and gp43 by FRET**—Interactions between the primosome and holoenzyme proteins were also explored by FRET. gp43(OGN) was mixed with either gp59(CPM), gp61(TMR), or gp41(TMR) in the presence of the other two unlabeled primosome proteins and the 32/64/73-mer fork substrate. No FRET was observed between gp43(OG) and gp41(TMR) or gp61(TMR), consistent with the cross-linking results above that failed to demonstrate an interaction between gp43 and gp41 or gp61. However, FRET was observed between...
gp59(CPM) and gp43(OGN), as shown in Fig. 5A. Fluorescence emission was lost from gp59(CPM) (donor quenching) and gained by gp43(OGN) (acceptor sensitization). A FRET signal shows an $R_0$ distance dependence on fluorophore separation (see “Experimental Procedures”) with an $R_0$ value of 48 Å (50% of the maximum, obtainable signal) for the CPM-OG pair. As such, detection of FRET-induced spectral changes requires intimate protein-protein contacts. A distance of 44 ± 5 Å was calculated between the FRET pair on gp43 and gp59, underscoring the requirement of intimate protein-protein contacts for FRET detection.

Kinetics of gp43 Binding to gp59—A presteady-state analysis of the binding to gp43 to the primosome was conducted. gp43(OGN) was mixed with gp59(CPM) associated with gp41, gp61, and DNA in a stopped flow fluorimeter, and the change in FRET was monitored over a 2-s time course (Fig. 6A). The trace obtained fit best to a double exponential, where the initial binding step of gp43(OGN) to gp59(CPM) was fluorescently silent. The binding step was simulated using an approximate $K_0$, with KinTekSim to obtain a forward rate constant of $300 \pm 100 \text{ M}^{-1} \text{s}^{-1}$ and a reverse rate constant of $30 \pm 10 \text{ s}^{-1}$. The proposed kinetic scheme for binding of gp43 to the primosome is given in Fig. 6B. The first observable fluorescent change is a first order process (changing the concentration of gp43(OGN) relative to gp59(CPM) causes no change in rate) that corresponds to a conformational change or orientation change between the two proteins such that the two probes move away from each other. This is followed by another first order step that is rate-limiting for the complex assembly.

Displacement of gp32 upon gp43 Binding—We demonstrated that the gp32 single-stranded binding protein remains part of the primosome (bound proximal to gp59) even after gp59 has assembled gp61 and gp41 onto DNA. This raises the question as to whether gp43 binding would displace gp32. A FRET experiment was conducted such that gp59(CPM) was mixed with gp32(OG) in the presence of gp41, gp61, and DNA. FRET (quenching of donor emission and enhancement of acceptor emission) is observed between gp59(CPM) and gp32(OG) under these conditions (Fig. 5B). However, when gp43 is added, the FRET signal is lost, indicating that gp32 has been displaced from gp59. It is not yet clear whether gp43 competes with gp32 for a binding site on gp59 or whether it induces a conformational change in the helicase-loading protein, causing a decreased affinity for gp32.

DISCUSSION

Formation of protein complexes is a common feature in biological processes. In DNA replication in particular, formation of protein complexes drives DNA synthesis. In the past, the bacteriophage T4 DNA replication complex has been studied pri-

F. T. Ishmael, M. A. Trakselis, and S. J. Benkovic, manuscript in preparation.
Interactions between the Primosome and Holoenzyme—The interaction between primosome proteins and the holoenzyme complex was investigated using cross-linking and fluorescent techniques. This allowed the study of protein interactions either on DNA or, as in the case of cross-linking, during active DNA replication. FRET experiments showed an interaction between gp59 and gp43 in the presence of gp41, gp61, and DNA. The distance between the fluorescent probes (Cys-42 of gp59 and the N terminus of gp43) was calculated to be 44 Å, reflective of the close physical contact. The kinetics for establishing this contact follow a three-step process involving binding.

Dimerization of gp43—Salinas and Benkovic (10) proposed that coupling may be at least in part mediated by an interaction between the two polymerases. Using a two-hybrid system, they showed that a region containing amino acids 401–600 was capable of dimerization (10). Moreover, Alberts and co-workers (30) showed that a gp43 affinity column retained gp43 when exposed to a clear lysate of T4-infected E. coli that contained radiolabeled T4 early proteins. However, more recently, studies aimed at detecting interactions between gp43 subunits have failed to demonstrate a dimerization. Kadyrov and Drake (17) investigated the quaternary nature of gp43 by size exclusion chromatography and did not observe any higher order species. Moreover, Delagoutte and von Hippel (16) studied interactions between gp41 and gp43 as well as gp43 and gp43 by analytical ultracentrifugation and also failed to see any complex formation between either of the species. However, both of these studies were conducted in the absence of DNA. Since DNA could induce conformational changes in gp43 that stabilized dimerization, we used a cross-linking approach to study oligomerization of the protein in the presence of a DNA substrate. gp43 was found to dimerize in the presence of a partially double-stranded DNA (34/62-mer), indicating that interaction of the protein with DNA could induce changes that allowed dimerization. No dimerization was observed in the absence of DNA. A second experiment was conducted to study association of gp43 subunits during active DNA replication. gp43 dimerization was seen on a replicating rolling minicircle, indicating that the interaction occurs during the course of replication and is physiologically relevant.

The site of cross-linking between gp43 subunits was mapped using an approach described previously (19). The region of interaction, Cys-507 of one subunit to Cys-507 of a second subunit, lies in an antiparallel coiled-coiled extension of the finger domain. This structure has previously been suggested to play a role in the interaction of two polymerases and was shown to be capable of dimerization in a two-hybrid system (10, 31, 32). A model of the gp43-gp43 interaction is shown in Fig. 7A. The gp43 three-dimensional structure was created from a homology model of the bacteriophage RB69 polymerase by submission to SWISS-MODEL (33–35). The two proteins are identical or chemically similar at 74% of all amino acid sites. In this model, the protrusion of the extension of the finger domain is in close proximity to the same structure on a second subunit, such that Cys-507 of one subunit and Cys-507 of a second subunit are juxtaposed. It is presumed that this structure forms only in the presence of DNA, although the DNA was omitted in this model to simplify the diagram. The linked polymerases are shown such that they are antiparallel. However, the relative orientation of the two subunits is unknown and is currently being investigated further. Nevertheless, this physical coupling of the two holoenzyme complexes could ensure the rapid and efficient transfer of the lagging strand polymerase to the next primer after completion of an Okazaki fragment.

Interaction between the Primosome and Holoenzyme—The interaction between primosome proteins and the holoenzyme was treated as distinct complexes. Moreover, the holoenzyme on leading strand DNA and that on the lagging strand have also been considered separate entities. However, since these parts function together as a whole to synthesize DNA, they must communicate with each other, through noncovalent physical interaction.

Previously, Salinas and Benkovic (10) demonstrated coordinated leading and lagging strand synthesis and suggested that coupling of the polymerases may occur by formation of a gp43 dimer. However, more recent studies failed to find any stable interaction between the gp43 protein subunits (16, 17). Second, coordinated replication requires molecular interactions between the holoenzyme component and primosome. Efficient T4 DNA replication proceeds through the unwinding of double-stranded DNA by the helicase component of the primosome. On the leading strand, the helicosome may travel juxtaposed to the primosome, incorporating nucleotides into the growing daughter strand. The obvious question then arises as to whether the holoenzyme and primosome are coupled through noncovalent interactions. It is difficult to imagine that these two units function separately, especially in light of studies conducted on similar systems, which demonstrated this type of contact. For example, the helicase/primase protein in bacteriophage T7 forms a complex with the T7 polymerase, as does the replicative helicase and DNA polymerase III in E. coli (11, 12).

We investigated complex formation between components of the bacteriophage T4 replisome and showed that 1) gp43 dimersizes in the presence of DNA and is dimeric in an actively replicating rolling circle system, 2) a physical interaction between the gp59 in the primosome and polymerase in the holoenzyme exists, and 3) the polymerase displaces gp32 from gp59 as part of the primosome upon binding.

**Fig. 6.** Presteady-state FRET detects the kinetic interaction between gp43 and gp59. A, the kinetics of gp43 binding to gp59 were monitored by time-dependent gp59(CPM) quenching in the presence of gp43(OGN). B, the data were fit using KinTekSim to the model shown to obtain rate constants for the process.
primesome assembly (25, 36, 37). No FRET was observed between gp43 and gp41 or gp61.

The interaction between gp59 and gp43 also was detected on a static fork using a specific trifunctional cross-linker. The experiment was conducted using two types of forks, one containing a 55-nucleotide lagging strand (32/64/36-mer) and the other with an 18-nucleotide (32/64/36-mer) lagging strand. The substrate containing the shorter lagging strand region is not long enough to support binding of the polymerase when gp41 and gp61 are present. Since cross-linking is seen between gp59 and gp43 on both substrates, it is the leading strand holoenzyme most likely that interacts with gp59. Interaction between the lagging strand holoenzyme and gp59 may still be possible but will require further study.

When the cross-linking experiment was repeated in an active replication system, cross-linking between gp59 and gp43 was again observed, demonstrating that this interaction occurs during active DNA synthesis. Cross-links were observed between gp59 and gp61 as well under these conditions. No interactions between gp61 and gp59 were observed on a static fork, indicating that conformational changes may occur during the course of replication that place the label on gp59 closer to the cross-linking site on gp61. One cannot, however, rule out cross-linking within interactive replisomes. Cross-linking between gp59 and gp32 was not observed nor between gp59 and gp41. In the latter case, the label on gp59 (at Cys-42) is not in close proximity to gp41 (19). However, it was previously demonstrated that gp59 and gp32 formed a complex where Cys-42 of gp59 is in close proximity to gp32 (21). The cross-linking experiment on the rolling circle was engineered such that initiation of cross-linking did not occur until after a few minutes of DNA synthesis, thus allowing time for assembly of the replisome. Most likely, the gp32-gp59 interaction occurs as the primesome is assembled but disappears once replication has started.

Previous kinetic evidence suggested an interaction between the primesome proteins, gp41 and gp43. Dong et al. (15) showed that a minimal complex of gp41, gp43, and DNA can perform strand displacement synthesis at close to physiologic rates in the presence of polystyrene glycol (15). However, no interaction between gp41 and gp43 (or gp61 and gp43) was detected by either FRET experiments or cross-linking. Moreover, analytical ultracentrifugation experiments conducted in the von Hippel laboratory also failed to detect any interaction between gp43 and gp41 (16). It is possible that the interaction between gp41 and gp43 is unstable but is affected by the addition of polystyrene glycol. More likely, the contact between the primesome and holoenzyme is mainly mediated through gp59. If any area of contact between gp43 and gp41 exists, it may be small, resulting in only transient interactions between the two proteins in the absence of gp59.

FRET studies between gp32(OG) and gp59(CPM) in the presence of gp43, gp41, gp61, and DNA support the departure of gp32 from gp59 after primesome assembly and their lack of cross-linking during active replication. We have demonstrated that gp32, gp59, gp61, and gp41 formed a complex on DNA. However, the addition of gp43 results in loss of the FRET signal between gp59(CPM) and gp32(OG), indicating a loss of close contact between these two proteins. Presumably, displacement of gp32 is needed for polymerase binding. gp59 has been shown to contact both gp61 and gp41, perhaps having only a limited area exposed for other protein binding. It is not clear, however, whether gp32 remains part of the primesome. Previous studies showed that gp32 and gp61 can interact, implicating this complex in transfer of the primer synthesized by the primase to the lagging strand gp43 (22).

The bacteriophage T4 replisome is comprised of eight proteins that form distinct units within the replication complex. These units, the primosome, leading and lagging strand holoenzyme, and single-stranded binding proteins, combine to form the replisome that is responsible for efficient DNA replication. A summary model of the contacts demonstrated to date between the elements of the primosome and holoenzyme is shown in Fig 7B. In this diagram, the primesome is coupled to the holoenzyme on the leading strand via interactions between gp59 and gp43. The lagging strand holoenzyme is linked to the leading strand holoenzyme via contacts in the extension of the finger domain. These physical linkages allow molecular communication between the primesome and holoenzyme as well as leading and lagging strand holoenzymes and may serve to coordinate both leading and lagging strand synthesis as well as DNA unwinding and polymerase synthesis.

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