Interactions of Bacteriophage T4-coded Primase (gp61) with the T4 Replication Helicase (gp41) and DNA in Primosome Formation*  

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One primase (gp61) and six helicase (gp41) subunits interact to form the bacteriophage T4-coded primosome at the DNA replication fork. In order to map some of the detailed interactions of the primase within the primosome, we have constructed and characterized variants of the gp61 primase that carry kinase tags at either the N or the C terminus of the polypeptide chain. These tagged gp61 constructs have been probed using several analytical methods. Proteolytic digestion and protein kinase protection experiments show that specific interactions with single-stranded DNA and the T4 helicase hexamer significantly protect both the N- and the C-terminal regions of the T4 primase polypeptide chain against modification by these procedures and that this protection becomes more pronounced when the primase is assembled within the complete ternary primosome complex. Additional discrete sites of both protection and apparent hypersensitivity along the gp61 polypeptide chain have also been mapped by proteolytic footprinting reactions for the binary helicase-primase complex and in the three component primosome. These studies provide a detailed map of a number of gp61 contact positions within the primosome and reveal interactions that may be important in the structure and function of this central component of the T4 DNA replication complex.

Most known DNA replication complexes, including the bacteriophage T4-coded system, are organized into subassemblies of proteins that perform equivalent functions within the moving replication fork. Since the T4 complex is probably the simplest replication system that includes separate processivity clamp and clamp loading proteins and primosome subassemblies, the T4 system has often served as a useful paradigm to elucidate and model some of the mechanistically important interactions that also occur within the replication complexes of higher organisms.

The central replication machinery of bacteriophage T4 consists of seven phage-encoded proteins that work together with...
stranded DNA. This ATPase is coupled to a directional (5'→3') double-stranded DNA unwinding (helicase) activity, which is further stimulated by binding of the gp61 primase (12–14). The primase itself serves as a limited polymerase in that, in the presence of single-stranded DNA, it can catalyze a limited template-dependent polymerization of ribonucleoside triphosphates, yielding mainly dimers at high primase concentrations (15). The addition of the gp41 helicase stimulates this RNA synthesis activity of the gp61 primase and switches the reaction primarily to the template-directed formation of specific pentameric primers (15), which in turn serve to prime the synthesis of Okazaki fragments in lagging strand synthesis (15). The addition of the gp41 helicase stimulates this RNA template-dependent polymerization of ribonucleoside triphosphates. The presence of single-stranded DNA, it can catalyze a limited primase itself serves as a limited polymerase in that, in the double-stranded DNA unwinding (helicase) activity, which is determined by UV absorbance at 280 nm, using a molar extinction coefficient (ε280) of 7.6 × 104 M−1 cm−1 for gp41 and 6.9 × 104 M−1 cm−1 for gp61. These extinction coefficients were calculated from amino acid residue composition data as described elsewhere (23, 24).

A functional association of primase and helicase also operates within the DNA replication systems of bacteriophage T7 (19) and Escherichia coli (20, 21). In T7, the helicase and primase domains are fused within the same polypeptide chain, while in E. coli and T4 these functions are in separate subunits. Although many studies have demonstrated that manifestation of the full physiological activity of both the helicase and the primase components of these systems depend on the combined presence of both proteins, the details of the direct physical interactions between them have not been mapped in any system.

In this paper, we report the construction of gp61 primase variants that carry a kinase tag at either the N terminus or the C terminus of the gp61 polypeptide chain. The interactions of these tagged gp61 variants within the primosome have been defined using proteolytic digestion and kinase protection footprinting techniques. By employing these methods in combination to probe the interactions that occur between the primase and helicase, in both the presence and the absence of single-stranded DNA, we have been able to identify a number of macromolecular interaction sites and conformational changes within the primosome. These studies comprise the beginnings of a detailed interaction map of subunit contacts and interaction sites within the T4 DNA replication complex.

MATERIALS AND METHODS

Reagents and Enzymes—[γ-32P]ATP was obtained from NEN Life Science Products. Vent DNA polymerase for the polymerase chain reaction (PCR) and DNA restriction enzymes were from New England Biolabs. Unlabeled nucleotides were from Amersham Pharmacia Biotech. Oligonucleotides was obtained from Genosys (The Woodlands, TX). Endoproteases, bovine heart muscle protein kinase (HMPK), and other reagents were obtained from Sigma.

Constructions of Primase Plasmids—Three gp61 derivatives were constructed for these studies and are shown schematically in Fig. 1. The first construct (designated H-gp61) carries a hexa-His tag at the N terminus, while the second (H-gp61-K) carries a hexa-His tag at the N terminus and a bovine HMTag (sequence RRRRR) at the C terminus. The third construct (HTK-gp61) contains a hexa-His tag, followed by a thrombin cleavage site (sequence LVPGRGRRASV) to permit removal of the His tag if necessary and a HMTag at the N terminus.

The procedures used to construct these plasmid vectors are outlined in Appendix A and B. The coding sequence for the full-length gp61 primase was PCR oligonucleotide-amplified using pDH111 (22) as the template strand. The purified PCR products of pDH111 and pDH111-K were inserted into pQE30 vector (Qiagen) at BamH I and Smal sites, and the resulting constructs were verified by DNA sequencing. The pQE30 vector (Qiagen) encodes an N-terminal hexa-His tag, and transcript synthesis was initiated at a phage T5 promoter. The pQE30-HTK-gp61 construct was made using pQE30-His-gp61 as a template for a one-step PCR to insert both the thrombin site and the HMTag downstream of the His tag at the N terminus of the primase DNA. The gel-purified PCR products were self-ligated.

Overexpression and Purification of the Primase Variants—The gp61 plasmids were transformed into the E. coli strain 100009/pRE14 (host carrying the pRE14 repressor plasmid and were expressed and purified using the Qiagen QIAexpression system (Qiagen). The 100009/pRE14 strain carrying the gp61 derivatives was grown overnight in Luria broth at 30 °C. The following day, the culture was then diluted 1:100 to a final concentration of 0.2 mM and shifted to 37 °C for 2–3 h. The cells were harvested by centrifugation and stored at −80 °C until used. The His-tagged gp61 proteins were purified using a nondenaturing native metal ion chromatography procedure on Ni2+-nitrilotriacetic acid-agarose (Qiagen), followed by passage through a DE52 column.

Preparation of T4 Helicase and Protein Concentration Determinations—The T4 helicase (gp41) protein was overexpressed in E. coli strain OR1264/pDH15 (22) and purified as described previously (6). This procedure yielded a clear preparation that migrates as a single Coomasie Blue G-250-stained band in SDS-polyacrylamide gel electrophoresis. The concentrations of purified gp41 and gp61 samples were determined by UV absorbance at 280 nm, using a molar extinction coefficient (ε280) of 7.6 × 104 M−1 cm−1 for gp41 and 6.9 × 104 M−1 cm−1 for gp61. These extinction coefficients were calculated from amino acid composition data as described elsewhere (23, 24).

Quantitative Kinase Protection Assays—The rate of ATP hydrolysis was measured by tracking both the disappearance of ATP and the formation of pyrophosphate (PPi) as a function of time, using the AMBIS (San Diego) radioactivity scanning and analysis system.

Gel Mobility Shift Assays to Measure the Interaction of the gp61 Variants with gp41—The ability of the various gp61 derivatives to participate in primosome formation reactions was tested using a gel shift assay as described previously (7). In brief, 10 nm concentrations of single-stranded M13mp18 DNA and 0.5 μM concentrations of gp61 (wild-type or variants, either phosphorylated or not phosphorylated at kinases) were mixed with 1 mM ATP and 3 μM concentrations of gp41 (monomer units) in a 20-μl reaction volume. The mixtures were incubated at room temperature for 1–2 min before loading onto a 0.5% nondenaturating gel, and the gel was subjected to SYBR Green I staining (Molecular Probes, Inc. Eugene, OR). The rate of ATP hydrolysis was measured by tracking both the disappearance of ATP and the formation of pyrophosphate (PPi) as a function of time, using the AMBIS (San Diego) radioactivity scanning and analysis system.
DNA concentrations, primosome formation goes to completion on a single-stranded DNA template (6).

Kinase tag phosphorylation reactions were initiated by adding 10 units of reconstituted catalytic subunit of HMPK (Sigma P-26445) with 0.1 mCi of [γ-32P]ATP. Ten-μl aliquots were removed at various times and mixed with 10 μl of GDP-γ-S and 

The time course of kinase-catalyzed incorporation of phosphate groups onto the serine residue of the HMPK tag of gp61 derivatives under various conditions was assayed by separating the protein components on 10% SDS-polyacrylamide gels and quantitating the [32P]-labeled bands with a model 860 Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Quantitative Tryptic Footprinting Assays—Tryptic digestion footprinting of native gp61 derivatives was performed using 25 ng of trypsin in TAMIK buffer at 37 °C in 100-μl reaction volumes containing 0.5 μM C-terminal [32P]ATP-labeled H-gp61-K ([32P]gp61K). For studies in the presence of single-stranded DNA and/or gp41, 5 μM concentrations of dT50 and/or 0.5 μM concentrations of gp41 hexamer (formed in the presence of 0.5 mM GTP-S) were added. The total concentration of protein in each sample was adjusted to 210 μg/ml by adding BSA. Twenty-μl aliquots were taken after 0.2, 0.5, 1, or 5 min of incubation, and proteolysis was terminated by adding 5 μl of H2O and 5 μl of 5× protein loading buffer (200 mM Tris-HCl, 20% β-mercaptoethanol, 8% SDS, and 50% glycerol) and immediately cooling the samples to 70 °C by plunging into dry ice. The resulting tryptic digestion products were incubated at 80 °C for 5 min before loading onto a Tricine-SDS-polyacrylamide gel. The resulting gel patterns were quantitated by PhosphorImager analysis.

Internal molecular weight markers were prepared by partial proteolytic digestion of [32P]gp61CK and were run in parallel with the footprinting reactions. These partial digestes were prepared by mixing 0.5 μg of [32P]gp61K in 100 μl of 1× TAMIK buffer containing 0.1% SDS and either 20 ng of endoprotease Lys-C or 10 ng of endoprotease Asp-N. (These proteases cleave at lysine and aspartic acid residues, respectively.) Thirty-μl aliquots were removed after 1, 5, and 10 min of incubation at 37 °C. Partial tryptic digestion was also used to obtain molecular weight marker ladders. For this purpose, cleavage was performed using 0.5 μM concentrations of [32P]gp61CK in a 70-μl volume of TAMK solution containing 0.1% SDS and 20 ng of trypsin. Thirty-five-μl aliquots were removed after 30 s or 1 min of incubation. All samples were quenched by adding 6 μl of protein loading buffer to each tube and incubating at 80 °C for 5 min before loaded on a Tricine-SDS-polyacrylamide gel. A plot of the logarithm of the molecular weights of these gp61 peptides as a function of distance migrated in a gel electrophoresis experiment was almost linear (see Fig. 8B) and was used as a standard curve to establish the sizes of the gp61 fragments obtained in the tryptic footprinting studies.

The time courses of digestion of H-gp61-K with Asp-N endoproteinase were obtained by mixing 0.5 μg of H-gp61-K with Asp-N at a molar ratio of 100:1, in either the absence or presence of 0.1% SDS, in a 100-μl reaction volume. Twenty-μl aliquots were removed after 0, 5, 15, and 30 min of incubation at 37 °C and quenched by the addition of 4 μl of protein loading buffer. The proteolytically cleaved fragments were separated on 10% SDS-polyacrylamide gels and displayed by autoradiography.

Tricine-SDS-Polyacrylamide Gel Electrophoresis—Cleaveage products were analyzed by discontinuous Tricine-SDS-polyacrylamide gel electrophoresis as described (26). This gel system can resolve cleavage products differing length by 5 residues or less in the molecular weight range used here. Electrophoresis was performed on three-layer cross-linked 20 × 22 cm slab gels that were 0.75 mm in thickness. The separating gel (16.5% T, 6% C with 6 M urea) was overlaid by a 2–3-cm cross-linked 20% gel. The gels were prepared as described (26). Samples were initially run through the stacking gel at 90 V for 1–2 h and then electrophoresed at 180 V for 15–20 h at room temperature. Gels were dried and quantitated using the ImageQuant software provided by Molecular Dynamics.

Analysis of the Proteolytic Digestion Footprinting Data—These data were analyzed essentially as described by Heyduk and co-workers (27). PhosphorImager intensities of all lanes were integrated across the full widths of the lanes and plotted versus electrophoretic mobility using the ImageQuant software. Gel loading was normalized to constant efficiency across the entire lane, including the portions corresponding to uncleaved full-length protein. The resulting plots of intensity as a function of lane position were aligned to correct for lane-to-lane gel distortion using the ALIGN program (27). Data from multiple lanes were averaged. Difference plots of cleavage patterns for averaged data for each complex (ssDNA-gp61, gp41-gp61, and ssDNA-gp61-gp41) were compared with difference plots obtained using averaged data obtained with [32P]gp61K alone by calculating the value of (I2 – Itot)/Itot, for each complex as described above, I2 represents the corrected intensity data for each complex and Itot represents the total intensity calculated for each pattern. Final difference plots were constructed by plotting the averaged values of (I2 – Itot)/Itot for each complex versus residue number. Residue numbers were obtained for gp61 from the electrophoretic mobility data, using polynomial curve fitting with KaleidaGraph.

RESULTS

Design and Characterization of gp61 Constructs—Fig. 1 shows the gp61 variants (and their designations) that we constructed for this study. Successful protein footprinting measurements require the use of protein probes that have been labeled to high specific radioactivity at defined sites. These modified protein probes must also be fully functional. After checking for activity (see below), we monitored the rates of phosphorylation of specific kinase tag sequences by cAMP-dependent BHMP kinase, as well as by proteolytic cleavage reactions, to examine intermolecular interactions of the primase within the primosome.

For this purpose, a five-amino acid residue kinase (HMPK) recognition motif (tag) was introduced at the N or the C terminus of the gp61 polypeptide chain. A, H-gp61. This derivative carries a His tag at the N terminus of the gp61 polypeptide chain. B, H-gp61-K. This derivative carries a His tag at the N terminus and a kinase tag at the C terminus. C, HTK-gp61. This derivative carries a His tag, a thrombin cleavage site, and a kinase tag in sequence at the N terminus of the otherwise wild-type gp61 sequence.

FIG. 1. Schematic diagrams of the gp61 derivatives used in these studies. A, H-gp61. This derivative carries a His tag at the N terminus of the gp61 polypeptide chain. B, H-gp61-K. This derivative carries a His tag at the N terminus and a kinase tag at the C terminus. C, HTK-gp61. This derivative carries a His tag, a thrombin cleavage site, and a kinase tag in sequence at the N terminus of the otherwise wild-type gp61 sequence.

As shown in Fig. 3, the N- and the C-terminal kinase-tagged gp61 derivatives were phosphorylated by kinase at approximately equal rates, which also corresponds closely to the rate of phosphorylation of the C-terminal kinase-tagged β-subunit of DNA polymerase III holoenzyme of E. coli under the same conditions (28). As a control, a gp61 derivative carrying a His tag at the N terminus, but no HMPK tag, was subjected to the same kinase treatment. No significant phosphorylation was observed (see Fig. 3). These results with the T4 primase, taken
together with those on the β-subunit of E. coli, suggest that the kinase recognition sites at both termini of gp61 are very accessible to phosphorylation by kinase and may be fully exposed on the protein surface. We note that the kinase reaction rates for both free gp61 derivatives are linear in time of incubation well beyond 10 min, validating our use of the extent of phosphorylation at 8 min (see below) as a measure of relative kinase-dependent phosphorylation rates for the various binary and ternary complexes studied with the kinase protection assay.

The labeled gp61 derivatives used in this study represent significant modifications of wild type gp61, not only because several amino acid residues have been added to the termini of the polypeptide chains but also because phosphorylation of the kinase tag inserts a charged phosphate group. Thus, the activities of the labeled gp61 derivatives must be compared with those of wild-type gp61 to ensure that these modifications do not alter essential primase functions. To this end, we measured both the magnitude of the gp61-dependent stimulation of gp41 ATPase activity and the ability of gp61 derivatives to form primosomes as partial assays for the functional activities of our gp61 constructs (see "Materials and Methods").

The T4 helicase (gp41) carries a single-stranded DNA-dependent ATPase/GTPase activity that is significantly stimulated by the addition of stoichiometric concentrations (one primase subunit per six helicase subunits) of gp61 (7, 13). Fig. 4A shows a plot of the rate of \( \gamma^{32} \text{P} \text{ATP} \) hydrolysis catalyzed by fixed concentrations of gp41 and ssDNA cofactor in the presence and absence of gp61 (solid triangles), gp61-K (solid circles), and HTK-gp61 (open triangles) were measured by monitoring the labeling of these proteins with \( \gamma^{32} \text{P} \text{ATP} \) (in units of cpm) as a function of time.

**FIG. 3. Kinetics of kinase-catalyzed phosphorylation of H-gp61-K, HTK-gp61, and H-gp61 alone.** Kinase phosphorylation reactions contained 0.5 \( \mu \text{M} \) purified H-gp61-K, HTK-gp61, or H-gp61, 5 \( \mu \text{l} \) of 3000 \( \mu \text{Ci} \) of \( \gamma^{32} \text{P} \text{ATP} \), and about 25 units of reconstituted kinase (HMPK) in a final reaction volume of 120 \( \mu \text{l} \). Two-\( \mu \text{l} \) aliquots were taken after 0, 2, 5, 15, 30, 45, 60, and 150 min of incubation at 25 °C, spotted on polyethyleneimine-cellulose paper, and subjected to thin layer chromatography. The rates of phosphorylation of gp61-wt. (solid triangles), H-gp61-K (solid circles), and HTK-gp61 (open triangles) were measured by monitoring the labeling of these proteins with \( \gamma^{32} \text{P} \text{ATP} \) (in units of cpm) as a function of time.

**FIG. 2. Construction of the gp61 derivative plasmids.** A, the pQE30-gp61-K plasmid. The 5′-primer (N terminus) has a BamHI site, and the 3′-primer (C terminus) has a protein kinase recognition sequence and a SmalI site. The gp61 wild-type sequence was used as a template. The PCR products obtained were digested with both BamHI and SmaI restriction enzymes and were ligated into a pQE 30 vector at the same sites. B, the pQE 30-HTKgp61 plasmid. The pQE 30-gp61-wt plasmid was used for this one-step PCR. The plasmid of pQE 30-gp61-wt was constructed as in A, except that the 3′-primer has a SmalI site only. Primer 1 contains a kinase tag and gp61-wt sequences that anneal to the gp61 part of the template and run in a clockwise direction. Primer 2 has a thrombin site and a His tag sequence, which anneals to the template at the His tag region and runs counterclockwise. Overall, this protocol allows insertion of a thrombin site immediately after the His tag, followed by a kinase tag, in one PCR step. The PCR products were phosphorylated and self-ligated to form a pQE 30-HTK-gp61 plasmid.
FIG. 4. A, the stimulation of gp41 ATPase by gp61 and gp61 derivatives. Reaction mixtures were prepared as described under "Materials and Methods." The rate of \( [\gamma-\text{32P}]\text{ATP} \) hydrolysis (plotted as a percentage of total P) produced by a 50 nM concentration of gp41 hexamers, activated by a 1 \( \mu \)M concentration of dT\(_{50}\) molecules, is indicated by solid circles. The ATPase rate in the presence of wild-type gp61 (50 nM) is indicated by solid squares; the rate in the presence of a 50 nM concentration of H-gp61-K is indicated by open squares, and the rate in the presence of 50 nM HTK-gp61 is indicated by open triangles. B, gel mobility shift analysis of primosome assembly. Native agarose gel shift electrophoresis was used to detect the formation of protein-DNA complexes of phosphorylated and unphosphorylated gp61 and gp61 derivatives with single-stranded M13mp18 alone or with M13 DNA and gp41 hexamer. The DNA-containing band positions were visualized by staining with SYBR-Green I. The DNA-containing band positions were visualized by staining with SYBR-Green I. The outside lanes contain free single-stranded M13 DNA only. Lanes marked by lowercase letters contained M13 ssDNA, together with wild-type gp61 (lane a), unphosphorylated H-gp61-K and HTK-gp61 (lanes b and c), and phosphorylated H-gp61-K and HTK-gp61 (lanes d and e). Lanes labeled with capital letters contained M13mp18 DNA and stoichiometric concentrations of gp41 hexamers, together with wild-type gp61 (lane A) or gp61 derivatives (lanes B–E).

A gel retardation assay was used to test the ability of the various gp61 derivatives to participate in primosome assembly with gp41 hexamers and M13mp18 single-stranded DNA (7). The assembly of primosomes using both tagged gp61 constructs, either phosphorylated (lanes d, e, D, and E) or unphosphorylated (lanes b, c, B, and C), was compared with assembly using wild-type gp61 (lanes a and A) with the same components. The results are shown in Fig. 4B. M13mp18 ssDNA contains multiple DNA hairpins, each of which has the essential geometry of a small DNA replication fork at room temperature. Therefore, M13 ssDNA can be used as a DNA template to reconstitute primosome complexes. The results show that all the gp61 derivatives used in this study can both bind DNA (lanes a–e) and participate effectively and stoichiometrically in the assembly of complete primosomes (lanes A–E).

Taken together, these ATPase stimulation and the gel retardation assays suggest that the modified termini of the gp61 constructs do not interfere significantly with the protein-protein and protein-DNA interactions of gp61 protein that are involved in primosome assembly and function.

Partial Asp-N Endoproteinase Digestion Shows That gp61 Is a Structured Protein—All of our kinase-tagged gp61 derivatives show high levels of radioactive labeling and biological activity, indicating that these derivatives can serve as useful probes of the interactions of gp61 with the other components of the primosome. Sequence analysis has shown that the T4 gp61 primase is significantly homologous to the primases of E. coli, and of bacteriophages T7, T3, and P4, in that all of these primases contain six similar regions of conserved sequence (29), although no three-dimensional structural information is available for any of these proteins. Partial endoproteinase digestion experiments can provide information regarding the degree of folding of various portions of the polypeptide chain of the primase. To determine the distribution of structured domains within the native gp61 primase monomer, the C-terminal kinase-labeled gp61 (H-gp61-K) was subjected to partial proteolytic digestion under both native and denaturing solution conditions.

The gp61 polypeptide contains 20 aspartic acid residues that are widely distributed throughout the sequence. The time course of digestion of the primase with Asp-N endoproteinase at 37 °C, at a molar gp61:Asp-N protease ratio of 100:1, resulted in reproducible cleavage of the gp61 chain at a number of defined Asp sites (Fig. 5). The rates of cleavage of this subset of proteolytic target sites under solution conditions favoring the native state of the protein should reflect the accessibility of these sites within folded protein structure. Under denaturing conditions (0.1% SDS), four major bands (corresponding to cleavage at residues 202, 227, 238, and 267) that were not present in the native cleavage pattern were observed, and one minor band (corresponding to cleavage at residue 295) disappeared from the digestion pattern. These results suggest that the region of the polypeptide chain located between Asp-207 and -267 is stably folded into a conformation that is not accessible to the Asp-N endoproteinase in the native state.

In contrast, peptide bonds in sequences located near both termini of gp61 were available for digestion in the native protein, suggesting (on the basis of this cleavage criterion) that they are exposed on the surface of the native structure. This conclusion is supported by our observation that kinase tags located at the N and C termini of the chain could be readily phosphorylated (see Fig. 3, and see below) and also be readily phosphorylated derivatives (data not shown).
cleaved by trypsin in the native structure (see below).

Quantitative Kinase Protection Assays—The DNA replication primases of bacteriophage T4, T7, and E. coli all contain a potential zinc-binding motif located near the N terminus of the primase chain (29–31). This motif is thought to be partially responsible for the recognition by primase of potential priming sites on the DNA template (31). For the E. coli primase subunit (DnaG), the eight amino acid residues located at the C terminus are required for interaction with DnaB helicase (33). To determine whether the N and C termini of the T4 bacteriophage primase are also involved in interactions within the primosome, we placed a five-residue kinase recognition sequence (HMPK tag) at either the N or the C terminus of gp61 (Fig. 1).

By comparing the relative rates of kinase-catalyzed phosphorylation of these tags in the absence and presence of various primosome cofactors, we obtained a rough estimate of the positions and strengths of interactions (or at least the positions of contacts) of both termini of gp61 within the primosome.

As shown in Fig. 3 (above), the HMPK tag at either terminus of gp61, in the absence of other primosome cofactors, is phosphorylated with essentially equal efficiency by the kinase. The catalytic subunit of the bovine heart muscle kinase has a molecular mass of ~40 kDa, which is comparable with that of gp61. Previous studies (7) have shown that the gp61 primase binds strongly to appropriate single-stranded DNA templates (e.g. dTn, with n > 15), that the gp41 helicase forms a hexamer when activated by the binding of purine ribonucleoside triphosphates, and that the gp61 primase binds to the gp41 helicase with a 1:6 subunit stoichiometry. We used the technique of protein kinase protection (34) to investigate the involvement of both termini of gp61 in interactions within the primosome that might reduce the access of the kinase to the HMPK tags.

Fig. 6A shows the time courses of phosphorylation of kinase tags located at the C or N termini of gp61 variant monomers in the absence or presence of single-stranded dT50, gp41 helicase, or both, under conditions that favor the formation of binary (gp61-ssDNA or gp61-gp41) or ternary (gp61-ssDNA-gp41) complexes. To quantitate the phosphorylation rate at each time point in Fig. 6A, we plotted the levels of phosphorylation achieved for two gp61 HMPK-tagged derivatives as a function of reaction time in the absence and presence of various primosome components in Fig. 6B. The results are compared and summarized in Fig. 7, where we plot (as percentages of the rates of phosphorylation of the gp61 derivatives alone) the phosphorylation rates taken from the linear portions of the graphs of Fig. 6B.

As Fig. 7 shows, the addition of hexameric gp41 to the gp61 derivatives reduces the rates of phosphorylation of both termini to 28 ± 6% (columns 3 of Fig. 7) of the rates measured with gp61 alone (columns 1). The addition of dT50 results in much more protection at the N terminus (9 ± 1%) and less at
The phosphorylation of kinase tags located at either end of gp61 is inhibited by various cofactors that interact with gp61. (The labeling rates for the gp61 monomers alone were set at 100% accessibility.) The solid bars represent the phosphorylation of H-gp61-K (C-terminal labeling), and the striped bars represent the phosphorylation of the HTK-gp61 derivative (N-terminal labeling). Columns 1 show the rates of phosphorylation of the gp61 derivatives alone (100% by definition), columns 2 show the inhibition of phosphorylation induced by the addition of saturating concentrations of dT50 to both derivatives, columns 3 show the inhibition induced by the addition of stoichiometric concentrations of gp41 hexamer, and columns 4 show the virtually complete blocking of phosphorylation of both kinase tags induced by the presence of both dT50 and the gp41 hexamer.

The following controls were performed to detect and avoid possible methodological artifacts. A constant ratio of kinase to total protein was maintained by conducting all the kinase protection reactions in the presence of a constant (429 μg/ml) total concentration of protein, which was achieved by adding appropriate amounts of BSA into the final 70-μl reaction volume of each sample. In addition, a 10-residue peptide that contains the bovine HMPK recognition sequence was synthesized and subjected to kinetic-catalyzed phosphorylation under the same reaction conditions used here, and similar reactions were performed with the C-terminal kinase-tagged β-subunit of DNA polymerase III holoenzyme of *E. coli*. No notable changes in the rate of phosphorylation of either the peptide or the β-subunit were observed upon the addition of dT50, gp41, or both together (data not shown), indicating that these nonhomologous components do not interact and thus inhibit kinase activity under these conditions. In addition, we also showed that the helicase-prime interactions detected by this technique with the T4 components are specific for the primosome proteins, since replacement of the gp41 helicase by either the T4 gp45 ring-shaped processivity clamp (data not shown) or the hexameric *E. coli* transcription termination helicase Rho (a helicase that is very similar in size and shape to gp41) also had no effect on the rate of phosphorylation of either terminus of gp61 (Fig. 6, A and B).

**Fig. 7. Summary of the kinase protection of the N- and C-terminal kinase tags of the gp61 primase.** The phosphorylation of kinase tags located at either end of gp61 is inhibited by various cofactors that interact with gp61. (The labeling rates for the gp61 monomers alone were set at 100% accessibility.) The solid bars represent the phosphorylation of H-gp61-K (C-terminal labeling), and the striped bars represent the phosphorylation of the HTK-gp61 derivative (N-terminal labeling). Columns 1 show the rates of phosphorylation of the gp61 derivatives alone (100% by definition), columns 2 show the inhibition of phosphorylation induced by the addition of saturating concentrations of dT50 to both derivatives, columns 3 show the inhibition induced by the addition of stoichiometric concentrations of gp41 hexamer, and columns 4 show the virtually complete blocking of phosphorylation of both kinase tags induced by the presence of both dT50 and the gp41 hexamer.

**Quantitative Tryptic Footprinting of the H-gp61-K Construct—**The above results (e.g. kinase tag phosphorylation, partial Asp-N digestion, and kinase protection) all suggest that both the N and the C termini of gp61 are effectively exposed in the gp61 primase monomer in solution and that both interact specifically with single-stranded DNA and the gp41 helicase hexamer in forming the functional T4 primosome. Quantitative proteolytic footprinting was used to further explore the details of the interactions of gp61 within the primosome.

The HTK-gp61 derivative was less suitable for quantitative proteolytic protection studies than the H-gp61-K construct. This follows because both the His tag for protein purification and the HMPK tag for kinase labeling are positioned at the same N terminus on the HTK-gp61 derivative, meaning that adventitiously truncated gp61 products (which can still be radiolabeled by kinase) may be copurified with the full-length gp61 derivative and interfere with subsequent data analysis (e.g. see Fig. 9, lane 2). As a consequence, we performed these proteolytic protection studies primarily with the C-terminally labeled H-gp61-K derivative. This primase construct was end-labeled with [γ-32P]ATP at the C-terminal kinase tag to form [32P]gp61CF, and the native protein was subjected to controlled tryptic digestion in TAMK buffer.

Fifty-nine (17%) of the amino acid residues of gp61 are arginine and lysine, and these residues are widely distributed along the polypeptide chain. As a result, trypsin can serve as a suitable footprinting “probe” of the structure and interactions of this protein. Partial protease digests (Fig. 8A) were used to create ladders of internal markers to allow identification of the various tryptic cleavage sites in the native gp61 protein. For this purpose, samples of end-labeled [32P]gp61CF were partially digested with the proteases Asp-N, Lys-C, or trypsin in TAMK buffer containing 0.1% SDS as a denaturing agent to obtain peptide markers of known molecular length. Fig. 8B shows an almost linear dependence of the measured gel mobilities on the logarithm of the known (from the amino acid sequence) lengths of the peptides produced by the protease cleavage reactions.

To establish optimal single hit footprinting conditions for the
The total protein in each sample was brought to 210 m accessible site on the full-length [32P]gp61CK chain. This fact also broadens the range of conditions over studies. However, since the kinase tag is used to label the chain, this approach allows single hit trypsin footprints to be obtained from the full-length gp61 derivative (data not shown). We found that only a subset of the potential trypsin cleavage sites along the gp61 chain resulting from the addition of ssDNA, gp41 hexamer, or both. The bars indicate protected regions along the gp61 chain resulting from the addition of ssDNA, gp41 hexamer, or both.

Tryptic protein footprinting of native gp61 with different cofactors. Tricine-SDS-PAGE electrophoresis gels of tryptic cleavages of [32P]gp61CK are shown in the presence or absence of different cofactors. Lane 1 contains fragments of [32P]gp61CK cleaved by Asp-N, to form an internal ladder of molecular weight markers. Lane 2 contains [32P]gp61CK cleaved by Asp-N, showing that mixed multiple lengths of THK-gp61 protein perturb the quantitative monitoring of the protease cleavage reactions (see text). Lanes 4–6 contain [32P]gp61CK alone without (lane 4) or with trypsin digestion (reactions stopped after 30 or 15 s of digestion, respectively) as controls. Lanes 7–9 contain [32P]gp61CK with dT50 in the absence (lane 7) or presence (lanes 8 and 9) of trypsin after 30 or 15 s of digestion. Lanes 10–12 contain [32P]gp61CK with gp41 hexamer in the absence (lane 10) or presence (lanes 11 and 12) of trypsin after 15 or 30 s of digestion. Lanes 13–15 contain [32P]gp61CK with gp41 hexamer and dT50 in the absence (lane 13) or presence (lanes 14 and 15) of trypsin after 15 and 30 s of digestion. The arrows marked 1–4 point to the cleavage bands that are strongly enhanced (arrows 1, 2, and 4) or bands that are newly formed (arrow 3) upon interaction of gp61 with gp41 hexamer. The bars indicate protected regions along the gp61 chain resulting from the addition of ssDNA, gp41 hexamer, or both.

Adding BSA as needed. Control experiments showed that these amounts of BSA do not perturb the rates of trypsin digestion under the conditions of the experiment, nor does BSA interfere with the interactions of the gp61 derivatives with either gp41 or ssDNA (data not shown). The cleaved products of such tryptic footprinting reactions were separated on a high resolution Tricine-SDS-polyacrylamide gel (Fig. 9) and quantitated by PhosphorImager analysis (Fig. 10) as described below.

In Fig. 9, lanes 1 and 2 show ladders of internal molecular weight markers produced by Asp-N cleavage of [32P]gp61CK (lane 1) and [32P]gp61NK (lane 2). As pointed out, mixtures of truncated and full-length protein in Asp-N digests of [32P]gp61NK did not give clear (single hit kinetics) results. Lanes 4, 5, and 6, containing [32P]gp61CK alone, were run either in the absence (lane 4) or in the presence (lanes 5 and 6) of trypsin, which was allowed to cleave the [32P]gp61CK chain for 15 and 30 s at 37°C. Thus, these lanes serve as controls for the quantitative analysis. Lanes 7, 8, and 9 represent [32P]gp61CK in the presence of dT50 without (lane 7) or with (lanes 8 and 9) the identical trypsin treatment. Lanes 10, 11, and 12 contain [32P]gp61CK in the presence of gp41 hexamer only, either in the absence (lane 10) or in the presence (lanes 11 and 12) of trypsin. Lanes 13, 14, and 15 contain [32P]gp61CK with both gp41 hexamer and dT50, either in the absence (lane 13) or in the presence (lanes 14 and 15) of trypsin.

All of the footprinting data were quantitated and analyzed by the methods of Heyduk et al. (27) to compare the cleavage patterns of the different binary and ternary complexes to those obtained with [32P]gp61CK alone. Fig. 10I shows the aligned and corrected data for each complex (including gp61-ssDNA, gp61-gp41, and gp61-ssDNA-gp41) compared with [32P]gp61CK alone as a function of the apparent electrophoretic mobility of each band, while Fig. 10II shows the same data calculated and replotted as a function of amino acid residue position. Cleavage intensities measured on lanes containing [32P]gp61CK alone were taken (by definition) as 0%. At any gp61 position within each complex, if the difference quotient (Ii - Igp61)/Itotal was a negative number, the result was recorded as a negative percentage (compared with the control cleavage intensity of gp61 alone). If this quotient was positive, the result was recorded as a positive percentage. Thus, negative percentages imply that the region of the gp61 protein near the potentially cleavable bond interacts directly with, or is shielded by, one of the components of the primosome. In contrast, regions characterized by positive percentages must reflect a local conformational change that occurs upon cofactor binding, resulting in increased exposure of that region of the gp61 polypeptide to the tryptic probe.

Fig. 10II (A) shows an experimental differential cleavage pattern obtained by subtracting the normalized intensities of one [32P]gp61CK alone cleavage pattern from those of an identical sample run in the same way. Clearly (as expected) the calculated amplitude of the difference intensity signal is close to 0% across the entire sequence, confirming the validity of our differential analytical procedure.

Fig. 10II (B) represents a difference pattern corresponding to the cleavage pattern of the binary ssDNA-[32P]gp61CK complex subtracted from that of [32P]gp61CK alone. Protection occurs for gp61 sites located near both the N and C termini of this gp61 construct in the presence of single-stranded dT50. These sites correspond to residues 1–50 at the N terminus, with difference cleavage intensities of −25 ± 4% to −89 ± 9% compared with measured difference cleavage intensities of 0% for [32P]gp61CK alone. At the C terminus, the HMPK tag of the smallest peptide of [32P]gp61CK was protected to an extent of −116 ± 6% relative to the control. There is also an enhanced site at amino acid residue 334 that is differentially exposed at a level of +75 ± 10%.
Fig. 10. Differential analysis of the tryptic digestion footprinting data. I, tryptic footprinting data analysis using the ALIGN program. Aligned and corrected tryptic cleavage patterns of gp61 alone are compared with those of complexes of gp61 in the presence of dT₅₀ gp41 hexamer, or both. Dashed lines show corrected intensities obtained from tryptic digestion patterns of the various complexes tested (gp61-ssDNA (B), gp61-gp41 (C), gp61-ssDNA-gp41 (D)) aligned with patterns obtained with gp61 alone (A) (solid lines), as described in the text. Aligned intensities are plotted versus migration mobility. II, difference intensity plots of tryptic footprinting data. Difference plots show corrected intensities versus residue number (see text). A, gp61 versus gp61 control; B, gp61-dT₅₀ versus gp61 alone; C, gp61-gp41 versus gp61 alone; D, gp61-dT₅₀-gp41 versus gp61 alone. Positions at which the corrected differential intensities for the complexes differ from those of [γ-³²P]gp61 alone by more than 30% are marked with horizontal ticks (decreases) or arrows (increases).
terminus of gp61 upon the addition of gp41 hexamer, either with or without ssDNA, strongly implies that a conformational change occurs upon binding of gp41 hexamer to gp61. A control experiment in which gp41 was replaced with \textit{E. coli} transcription termination factor Rho was performed also as a part of this series of tryptic protection experiments. No significant interactions between Rho and gp61 were observed in either the presence or the absence of ssDNA (data not shown), suggesting that the interactions observed between gp61 and the components of the primosome by these methods are indeed specific.

Figs. 9 and 10 show that only a subset of the arginine and lysine residues of gp61 are cleaved by trypsin in this proteolytic digestion assay. Clearly, the observed cleavages occur at the most exposed of the potentially cleavable peptide bonds, since the arginine residues of the HMPK kinase tag on gp61 are also significantly exposed on the surface, and thus the radiolabel we used to monitor the reaction is also subject to removal by trypsin fairly early in the reaction process. As a consequence, it is possible that only partial information about the interaction of gp61 with its cofactor(s) within a primosome is revealed by tryptic protein footprinting studies under native conditions using this labeling technique.

In order to circumvent this problem, as well as to achieve higher resolution footprinting, we also used the iron-EDTA cleavage method (35–37) on these complexes (data not shown). In this technique, a diffusible hydroxyl free radical is generated in solution from the iron-EDTA-peroxide reagent, and this radical rapidly and non-sequence-specifically cleaves the exposed peptide bonds of the folded protein. Under proper conditions, the rates of cleavage of specific peptide bonds will reflect the degree of exposure of the associated protein surfaces. We have used this method to demonstrate protection of gp61 sequences in the presence of ssDNA in the same regions protected in our tryptic digestion studies. Thus, in these experiments we observed strongly enhanced bands near the N terminus of gp61 in the presence of gp41 hexamer alone or in the presence of both ssDNA and gp41 in these reactions, just as in the tryptic protection studies. Those enhanced bands can even be observed, although to a lesser extent, in the presence ssDNA and gp41 in the absence of GTP\textsubscript{S}\&. Under these conditions gp41 interacts as a dimer with the gp61 monomer, as demonstrated by analytical ultracentrifuge studies (data not shown). No significant protection of gp61 was obtained with this iron-EDTA method for the binary gp61-gp41 complexes. This latter result probably reflects the fact that the interaction of gp61 with the gp41 hexamer, especially in the absence of ssDNA, is not sufficiently tight to differentially shield the peptide bonds of the gp61 chain from attack by the very small (and highly diffusible) hydroxyl free radical species.

**DISCUSSION**

The T4 primase (gp61) plays several important roles in DNA replication. As a limited DNA-dependent RNA polymerase, it catalyzes the specific template-directed synthesis of the ribopoligouronucleotides that prime lagging strand DNA synthesis. It also interacts with the ring-shaped hexameric replication helicase of T4 (gp41) to facilitate the ATP-dependent unwinding of the double-stranded DNA template ahead of the replication fork. Finally, it has a significantly higher affinity for single-stranded DNA than does the helicase and thus probably plays an important role in helping to physically couple the helicase to the DNA of the replication fork. Understanding the interactions between the primase, helicase, and DNA components of the primosome will allow us to begin to define the protein-protein and protein-DNA interactions that are essential for coordination of the multiple reactions that occur within the DNA replication complex as it functions at the moving replication fork.

As detailed above, functional assays have made it clear that the primase and the helicase of T4 must interact physically within both the primosome and the complete replication complex, since the presence of the helicase is required for the synthesis by the primase of lagging strand primers of physiologically relevant length and sequence (15), and the presence of the primase is required to strengthen and coordinate the interactions of the helicase with the DNA fork that facilitate processive DNA unwinding by the primosome (12–14). However, the binary interaction between the primase and the helicase has been found to be too weak and transient to detect by direct physical methods (32, 33).

To overcome these difficulties and also to learn more about the molecular details of the relevant interactions, we have used “cumulative” (nonequilibrium) protection methods to map protein-protein and protein-DNA contacts within the primosome. The findings presented in this paper provide compelling evidence of a physical interaction between gp61 and the gp41 hexamer, in both the presence and the absence of single-stranded DNA. The results also reveal the specific domains within the gp61 polypeptide chain that are involved in the interactions of this entity with the helicase and DNA components of the primosome.

To initiate these studies, a His\textsubscript{6} tag and a kinase tag were engineered into each terminus of gp61 polypeptide chain, both to facilitate purification and to permit radioactive labeling at defined positions. The addition of these tags did not significantly alter the physiological characteristics of the primase, as judged both by the extent of primosome formation and by the magnitude of the primase-induced stimulation of the DNA-dependent ATPase activity of the gp41 helicase.

In principle, a kinase motif can usefully be placed in the polypeptide chain at any exposed site, as long as the kinase protein has sufficient access to permit reasonable rates of phosphorylation. However, the results of such experiments have not always turned out as expected. Thus, O’Donnell and co-workers (28) placed a kinase tag at either end of the \(\beta\)-subunit of the \textit{E. coli} DNA replication polymerase holoenzyme III, as well as at two internal chain positions. All these positions were expected to be significantly exposed to the solution (and therefore to the kinase) on the basis of the crystal structure of the \(\beta\)-clamp processivity factor, yet experiments showed that only the kinase tag placed at the C terminus of the \(\beta\)-subunit could be effectively phosphorylated (28). In contrast, we found that both the N- and C-terminal kinase tags that were added to gp61 could be phosphorylated with approximately equal efficiency by the bovine heart muscle kinase (Fig. 3).

This result suggested that both termini of gp61 are significantly exposed on the protein surface in free native gp61, and this suggestion was further supported by the results of the Asp-N cleavage and protein kinase protection assays. A partial Asp-N protease digestion, performed with both native and denatured gp61, has revealed that the native protein is indeed extensively structured (Fig. 5). By following the rate of phosphorylation of the kinase recognition sequences at either end of gp61 in the presence of excess protein kinase, protection measurements suggest that gp61 interacts in binary complexes with both single-stranded DNA and the gp41 hexamer and that these interactions in both binary complexes involve both termini of the gp61 protein. Furthermore, the interaction affinities between the components are significantly increased within the ternary (primosome) complex. The interaction with ssDNA is much stronger at the N terminus of gp61, where kinase protection showed that the rate of phosphorylation of the ki-
nase tag is reduced to 9% of the rate observed at this locus for gp61 alone, while at the C terminus the phosphorylation rate was reduced to only 64% of its control value (Fig. 7). This result is in good accord with earlier findings (29–31) that the N-terminal domain, including the zinc finger motif of gp61, is significantly involved in the binding and recognition of the DNA cofactor of the primosome. Our results also support previous findings (7) that the overall stability of the ternary primosome is much higher than the individual stabilities of the constituent binary complexes.

The results of our protease digestion assays, which serve to define the positions of specific binary and ternary protein–protein and protein–DNA interactions along the gp61 polypeptide chain, are also consistent with the kinase protection results. The tryptic protection footprinting data showed that the residues near the N terminus of the gp61 chain are strongly protected against proteolytic digestion upon binding of the primase to single-stranded DNA. Other discrete sites of both protection and hypersensitivity were manifested when the gp41 was included in the proteolytic footprinting reactions. More protection sites, located close to the C terminus of the gp61 chain, were observed when both ssDNA and gp41 hexamer were included in the proteolytic probing reaction. The most striking feature of the tryptic digestion studies is the finding that, in the presence of gp41 hexamer and either in the presence or absence of ssDNA, two major bands reflecting cleavage at residues 34 and 51 are significantly enhanced, and one new band, reflecting cleavage around residue 116, appears. These enhanced bands can also be observed, to a lesser degree, in the presence of gp41 dimer (i.e. in the absence of GTP–Y–S). This fact suggests that gp61 can interact with either the gp41 hexamer or the dimer and that the interaction with the hexamer is significantly stronger. The appearance and enhancement of these bands is probably due to a conformational change, localized near the N terminus of the primase, which is induced in gp61 by binding of the gp41 helicase. The most straightforward interpretation of these findings is that gp61 undergoes one or more structural transitions during primosome assembly.

The above findings, based on these incisive (but basically nonequilibrium) enzymic “probing” assays, have been complemented and confirmed by steady-state fluorescence measurements.2 The fluorescent intensity of gp61, extrinsically labeled and confirmed by steady-state fluorescence measurements, is significantly enhanced by the binding of both the gp41 helicase and single-stranded DNA, suggesting that under these conditions the fluorophore may be shifted into a significantly more nonpolar environment. The fact that this shift is relevant to “real” ternary interactions within the primosome is supported by the observation that interaction of the primase with gp41 or ssDNA alone only modestly increases this signal. We note that the concentrations of the protein components used in the fluorescence studies were only about one-third of those used in the kinase protection and footprinting assays, yet evidence of specific protein–protein and protein–DNA interactions is clear with all of the “probing” methods that we have employed. Thus, the specific interactions observed within the assembled primosome in these *in vitro* studies are likely also to exist within the functional primosome subassemblies operating within the replication complex at the T4 DNA replication fork.

Considering all of the existing evidence, we conclude that all three components of the primosome interact with one another via weak but specific pairwise interactions and that these interactions are stabilized by the presence of all three components. Our study suggests that the presence of the helicase at the replication fork not only stabilizes the primase onto the DNA to form a primosome but may also change the way in which the primase interacts with the DNA of the lagging strand in primer formation. Based on our experimental results, it is tempting to speculate that binding of the helicase hexamer to the primase monomer induces a structural change near the N terminus of the latter, perhaps resulting in further exposure of the recognition site of the primase. This effect could then increase the effective dwell time of the primase on the template and perhaps increase the synthesis of the physiologically active pentamer primers by prolonging interaction with the DNA template. Furthermore, this stabilized interaction of primase with the DNA template could then also be important in facilitating the interaction of the nascent primers with the replication polymerase (gp43) in lagging strand DNA synthesis.

Finally, it is satisfying to obtain significant evidence, using a variety of techniques, for local conformational change and multiple protein–protein and protein–DNA contacts involving the gp61 monomer in the course of primosome formation, since the T4 primase subunit is clearly the “central player” in regulating the interactions of the six gp41 subunits with the DNA fork within the active primosome. The results presented here provide the beginnings of a low resolution “interaction map” of the gp61 primase, which future work can extend to include functionally important contacts with other protein components of the T4 replisome that operate at the replication fork during DNA synthesis.

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