The ATP-activated Hexameric Helicase of Bacteriophage T4 (gp41) Forms a Stable Primosome with a Single Subunit of T4-coded Primase (gp61)*

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We have examined the formation of the primosome subassembly of the bacteriophage T4-coded DNA replication (elongation) complex from its helicase, primase, and DNA components. Previously, we had shown that the T4 helicase (gp41) exists in solution in a stable monomer ↔ dimer equilibrium at physiological protein (and salt) concentrations and forms a hexamer upon activation by ATP (or GTP) by Feng (Dong, F., Gogol, E. P., and von Hippel, P. H. (1995) J. Biol. Chem. 270, 7462–7473). Here we report that the T4 primase (gp61) is a monomer in solution under the same conditions, and that the ATP-activated helicase binds to a single gp61 primosome molecule on appropriate DNA templates to reconstitute a stable primosome. We show that: (i) the gp41 helicase alone does not form a stable complex with DNA templates, although this helicase by itself can carry out moderately processive ATP-driven translocation along single-stranded DNA (Young, M. C., Schultz, D. E., Ring, D., and von Hippel, P. H. (1994) J. Mol. Biol. 235, 1447–1458); (ii) the primase alone does form a stable complex with DNA; (iii) the helicase can bind to the primase-DNA complex in the presence of ATP or GTP to form a stable ternary complex; (iv) this complex consists of six helicase subunits and one primase subunit; and (v) the reconstituted primosome is stable for at least 10 to 20 min after NTP cleavage and dissociation of the hydrolysis products. These results strongly suggest that the functional T4 DNA replication primosome consists of an integrated 6:1 helicase-primase complex bound to DNA, and that the ATP-activated helicase hexamer remains intact throughout the processive DNA replication process.

The elongation phase of DNA replication in many organisms is characterized by similar patterns of molecular organization and mechanism (see Kornberg and Baker (1992)). The replication complex typically contains three functional subassemblies that operate at the replication fork. These are: (i) the central DNA polymerase that catalyzes template-directed 5′ → 3′ leading and lagging strand synthesis of the nascent DNA and often carries a 3′ → 5′ exonuclease (editing) activity as well; (ii) a set of polymerase accessory proteins that stabilize the polymerase on the DNA template by forming a “holoenzyme” complex to permit processive synthesis, especially of the leading strand within the DNA replication fork; and (iii) a primosome complex (composed of helicase and primase) that unwinds the double-stranded template DNA ahead of the replication fork and lays down small RNA primers for the multiplicity initiation events required for the discontinuous synthesis of lagging strand DNA. These subassemblies combine to form an integrated DNA replication complex that is capable of carrying out coordinated leading and lagging strand synthesis and drive the overall movement of the DNA replication fork.

The bacteriophage T4 utilizes seven discrete T4-coded proteins to carry out the elongation phase of DNA replication (Alberts, 1987; Cha and Alberts, 1988; Nossal, 1994). These proteins comprise a DNA-dependent DNA polymerase (gp43),* three accessory proteins (gp45, which forms the processivity “clamp” that binds the polymerase to the template DNA, and a gp44-gp62 complex, that uses ATP to drive the loading of the clamp onto the DNA and polymerase), and the T4-coded single-stranded DNA binding protein (gp32). In concert, these components form a polymerase holoenzyme that can carry out processive DNA replication on primed single-stranded DNA templates under physiological conditions. Efficient DNA replication on double-stranded DNA templates also requires the participation of a primosome subassembly, consisting of the helicase (gp41) and primase (gp61) components. The primosome catalyzes the ATP- (or GTP-) driven unwinding of the double-stranded DNA template and synthesizes the pentameric RNA primers required for coordinate lagging-strand DNA replication. In this paper we focus on the assembly and properties of the functional primosome.

Many studies have shown that while the helicase and primase alone can each carry out its specialized tasks to some extent, the full physiological properties and activities of these enzymes are only displayed when they function within an integrated primosome complex. Thus, the full primosome is required to permit the helicase to unwind double-stranded DNA at the high rates (up to 500 base pairs per s) characteristic of DNA replication in vivo and to permit efficient synthesis by the primase of the physiologically relevant pentanucleotide RNA primers (Cha and Alberts, 1986; Hinton and Nossal, 1987; Liu and Alberts, 1980, 1981). In addition, both proteins are needed to coordinate leading and lagging strand DNA synthesis at the moving DNA replication fork (Nossal and Hinton, 1984).

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The T4 Primosome Is a 6:1 Helicase-Primase Complex

As an initial step in approaching the molecular properties of the T4 primosome, we examined the association states and the structures of the helicase itself (Dong et al., 1995) and showed that the subunits of the T4 DNA replication helicase assemble into a toroidal (ring-shaped) hexamer from its monomer and dimer solution constituents upon activation by binding of the nucleoside triphosphate substrate (ATP or GTP) of the helicase. This study also showed that the NTP-activated gp41 helicase exists as a hexagonal trimer of dimers and suggested that the hexamer may be characterized by pseudo-C3 or pseudo-D3 symmetry.

Here, we build on that study to present a physical biochemical description of the formation of the T4 primosome complex on DNA templates. We show that the ATP-activated gp41 helicase hexamer binds to a single subunit of gp61 primase on a DNA template to form the primosome subassembly of the T4 DNA replication complex. Our results also show that the primosome formed by the hexamer and the primase is very stable and suggest that the primosome remains associated during the entire cycle of ATP hydrolysis, DNA template unwinding, and presumably lagging-strand primer formation.

EXPERIMENTAL PROCEDURES

Reagents and Buffers—The chemicals used for buffers, for gel electrophoresis (including acrylamide, bisacrylamide, TEMED, and ammonium persulfate), and for the silver staining of protein gels, were all Gel Electrophoresis or Analytical Reagent grade. Purinenucleosidetriphosphates and their nonhydrolyzable analogs (ATP, GTP, ATP-S, and GTP-S) were obtained from Boehringer Mannheim. The homobifunctional protein-protein cross-linking reagent diithiobis(succinimidyl propionate), DSP, was purchased from Sigma.

Preparation of Proteins—The T4-coded DNA replication helicase (gp41) was overexpressed using Escherichia coli strain OR1265/pDH518 (Hinton et al., 1985) and purified as described previously (Dong et al., 1995). The primase (gp61) was prepared as described (Hinton and Nossal, 1985). The E. coli strains used to overexpress both proteins were generously provided by Drs. Deborah Hinton and Nancy Nossal (NIH). Both purified proteins migrated as single bands in SDS-polyacrylamide gel electrophoresis when stained with Coomassie Blue G-250. Protein concentrations were determined by UV absorbance at 280 nm, using molar extinction coefficients (\(\epsilon_{280}\)) of 7.6 × 10³ M⁻¹ cm⁻¹ (gp41) and of 6.9 × 10³ M⁻¹ cm⁻¹ (gp61) that were calculated as described (Odell et al., 1979).

Preparation of Reconstituted Primosome Samples—Single-stranded M13mp18 DNA was prepared as described previously (Lechner and Richardson, 1983) and was used as the initial DNA template on which to reconstitute primosome complexes. At room temperature this material contains multiple DNA hairpins, each of which has the essential geometry of a small DNA replication fork. Reconstituted primosomes were usually prepared in the same buffer that we had used previously to form ATP-activated helicase hexamers (Dong et al., 1995). This buffer (TAMK) contained 33 mM Tris-OAc, 50 mM KOAc, 6 mM Mg(OAc)₂, (pH 7.8) and was made 1 mM (sometimes 2 mM) in ATP or GTP (or their nonhydrolyzable analogs, ATP-S and GTP-S) before the single-stranded M13 DNA template was added (usually to a final concentration of 5 or 10ng/ml of DNA nucleotides). The primase was then added, followed by the helicase. The final concentration of the primase was usually set at 0.5 or 1 μM, and that of the helicase ranged from 0.5 to 6 μM (in protein monomers). Due to carryover from the protein storage buffer, the experimental samples also generally contained 10 to 20% glycerol (by volume). Control experiments (data not shown) indicated that glycerol concentrations in this range had little or no effect on the behavior of the reconstituted primosomes in the experiments described in this study. Samples were generally incubated at room temperature for 1 to 2 min after addition of all components (the helicase was added last) prior to being used for further characterization.

Aagarose and Polyacrylamide Gel Electrophoresis—Native agarose gels were prepared using 0.5% agarose dissolved (with heating in a microwave oven) in a gel-running buffer containing 33 mM Tris-OAc, 50 mM KOAc, 0.1 mM Mg(OAc)₂, (pH 7.8). A minigel 10 cm in length and 6.5 cm wide was usually poured with a comb to form 12 sample wells. After the samples had been prepared and incubated at room temperature for 1 to 2 min, they were loaded onto the gel and were electrophoresed at 2 V/cm (~40 V for the minigel apparatus) at room temperature. One or two separate lanes were usually loaded with an indicator dye to monitor the progress of the electrophoresis. Gels were run until the bromophenol blue indicator dye migrated for about 3 cm, after which the gel was removed and stained with either ethidium bromide or with SYBR-Green I (Molecular Probes Inc.) to visualize the free DNA and the protein-DNA complex bands.

Discontinuous SDS-polyacrylamide gels for quantitative determination of the protein constituents of protein-DNA complexes were prepared using the method of Laemmli (Laemmli, 1970). A 12.5% (110:1 acrylamide:bisacrylamide) separating gel with a 6% (110:1 acrylamide:bisacrylamide) stacking gel system was usually used. This system provided good separation between denatured helicase and primase subunits. After electrophoresis, the gels were stained with silver and were analyzed by soft laser scanning densitometry.

Quantitation of Protein Constituents of the Primosome Complexes—Reconstituted primosomes were subjected to native gel electrophoresis to remove unbound protein, after which the protein-DNA complexes were excised from the native agarose gels and were analyzed by SDS-polyacrylamide gel electrophoresis to determine the amounts of helicase and primase present. A Zêneph SL-504 XL soft laser scanning densitometer was used for this quantitation, and data acquisition and analysis were performed using the Videophoresis II computer program (BioMed Instruments Inc.). Due to the shallow linear-response (dynamic) range of the silver-stained gels and the densitometry scanning technique, various amounts of each of the two proteins were also loaded onto gels to which sample proteins of known concentration had been added for calibration. The amount of each protein was then determined by measuring peak areas in densitometer scanning.

Protein-Protein Chemical Cross-linking—The bifunctional chemical cross-linker DSP, diithiobis(succinimidyl propionate), was used to analyze the association state(s) of the gp61 primase by cross-linking protein complexes, as described previously for the gp41 helicase (Dong et al., 1995). Cross-linking experiments were carried out in the same buffer used for the preparation of the primosome samples, either in the presence or in the absence of 1 mM purine nucleoside triphosphate cofactor. Samples were prepared by incubating the gp61 primase (1 μM) in the buffer for 1 to 2 min at room temperature, after which cross-linking was initiated by adding small amounts (2% of the total reaction volume) of freshly prepared DSP stock solutions in dimethylformamide (final DSP concentrations in cross-linking reactions were 0.1% or 0.01%, w/v, depending on experiments). Cross-linking reactions were allowed to proceed at room temperature for 20 minutes and were then quenched by adding a one-tenth volume of 1.4 m ethanediamine-HCl (pH 8.0). After further incubation at room temperature for 20 min, the samples were made 1% in SDS and 10% in glycerol (by volume), incubated at 37 °C for 30 min, and analyzed by SDS-polyacrylamide gel electrophoresis using the phosphate-SDS gel system (Weber and Osborn, 1977). After electrophoresis, the protein bands were observed by silver staining.

RESULTS

Formation of the Primosome Complex—A native gel bandshift assay was used to measure the interactions between the components of the primosome. Previous functional and enzymatic studies had shown that the gp41 helicase can interact with single-stranded or forked DNA templates (Young et al., 1994; Dong et al., 1995). However, our native gel bandshift experiments, using single-stranded M13 DNA as a putative binding and translocation (and perhaps helicase) substrate, show that the gp41 helicase by itself does not form complexes with these single-stranded M13 DNA templates that are stable enough to be visualized by this technique (see Fig. 1A, lanes 2–5). Similar observations had been made previously with ϕX174 single-stranded DNA (Richardson and Nossal, 1989a, 1989b).

The observation that the binding of gp41 with its DNA cofactors cannot be observed in standard gel-shift experiments is not necessarily at odds with earlier demonstrations that these DNA cofactors, in similar buffers and at comparable concentrations, can stimulate the ATPase activity of the helicase and primase.
The T4 Primosome Is a 6:1 Helicase-Primase Complex

Fig. 1. Association of prinosome proteins with DNA. Native (0.5%) agarose gel-shift electrophoresis was used to analyze the association with single-stranded (hairpin-containing) M13 DNA of gp41 helicase or gp61 primase alone (A) and gp41 helicase and gp61 primase together (B). All reactions were carried out in TAMK buffer (see “Experimental Procedures”), and samples were loaded for gel electrophoresis after 2 min of incubation at room temperature. The concentration of ss M13 DNA in all reactions was 5 nM. A: lanes 2–5, 2 μM helicase and ss M13 DNA in buffer only, plus 2 mM ATP, 2 mM GTP, S, and 2 mM ADP, respectively, as indicated; lanes 6–9, 1 μM primase and ss M13 DNA in buffer only, plus 2 mM ATP, 2 mM GTP, S, and 2 mM ADP, respectively, as indicated. Lanes 1 and 10 contained free single-stranded M13mp18 DNA only. B, titration of various concentrations of helicase to a constant concentration of primase and ss M13 DNA. The concentrations of the ss M13 DNA and of the primase were 5 nM and 0.5 μM, respectively, in all reactions. The concentration of the helicase was 0, 0.5, 1, 1.5, 2, 3, 4, and 6 μM in lanes 1 through 8. Lanes labeled D contained free SSM13 DNA only.

Perhaps serve as relatively inefficient helicase substrates. An important difference between these experiments is that while the samples for the band-shift experiments were prepared in the presence of ATP or GTP (or their analogs), they were typically run without nucleotide cofactors in the native agarose gel electrophoresis experiment itself. Thus, the interactions between gp41 and single-stranded or forked DNA that we have observed by ATPase stimulation (Young et al., 1994), by native polyacrylamide gel electrophoresis (with ATP present in the gels and the running buffer, Young et al. (1994)), and by direct cryoelectron microscopic imaging (Dong et al., 1995), were all dependent on the continuous presence of purine nucleoside triphosphates and were shown to be relatively weak or transient in the absence of these cofactors.

On the other hand, we now find that gp61 primase can be shown by standard gel-shift experiments to bind and remain stably associated with ss M13 DNA under all buffer conditions tested (Fig. 1A, lanes 6–9). No nucleotide cofactors are required for this association (lane 6). However, the complexes formed by the primase and ss M13 DNA have a very strong tendency to aggregate, especially when the primase is present at high concentrations such as those used in Fig. 1A. At lower primase concentrations (representing also a lower primase:DNA ratio), aggregation is less severe (Fig. 1B, lane 1). This aggregation of primase on ss M13 DNA is completely alleviated by the presence of proper amounts of the gp41 helicase, as shown in the experiments presented in Fig. 2A, which were performed at the same primase and ss M13 DNA concentrations (and under the same experimental conditions) as those in Fig. 1A.

Fig. 2B also demonstrates that gp41 binds stably to a primase-DNA complex in the presence of PuTP as indicated by the further retardation of the complex in the native agarose gels. (A similar increase in protein association on adding gp41 to primase-DNA complexes was observed previously in similar experiments using single-stranded φX174 DNA as cofactor (Ri-
subunits was determined by densitometry of the component gel electrophoresis, and the ratio of bound helicase to primase plexes were then freed of excess components by native agarose was clearly present in excess. The resultant DNA-bound com-
stituted complexes, increasing amounts of gp41 were titrated into fixed concentrations of ss M13 DNA and gp61 until gp41 was clearly present in excess. The resultant DNA-bound complexes were then freed of excess components by native agarose gel electrophoresis, and the ratio of bound helicase to primase subunits was determined by densitometry of the component gp41 and gp61 bands on SDS-polyacrylamide gels (Fig. 2).

The results presented in Fig. 2, A and B, clearly show that in the presence of excess gp41, the amount of gp41 bound within the complex is limited by the amount of gp61 available. In support of this interpretation, we show (in Fig. 2A, lanes 4–6) that reducing the amount of gp61 by 50% or 75% results in the loss of a corresponding amount of gp41 from the complexes. This shows that gp41 interacts stochiometrically with gp61 and that the primase is not just acting as a "loading" factor to facilitate the interaction of DNA with gp41. Two-fold higher concentrations of proteins (relative to those used in Fig. 1) were used in this experiment to permit more accurate measurement of protein concentrations in SDS-polyacrylamide gels by silver staining.

The ratio of gp41 to gp61 subunits in the complexes was determined by laser densitometric scanning of the silver-stained SDS gels, using known amounts of each protein loaded onto the same gel for calibration, and was found to reach a limit ratio of 5:1 to 7:1 when excess of gp41 was present (see Table I). This subunit stoichiometry ratio is in accord with the titration experiment shown in Fig. 1B. A completely accurate stoichiometry ratio cannot be determined using this technique because of the very narrow dynamic quantitation range available with protein silver-stained SDS-polyacrylamide gels. However, since gp41 itself forms a hexamer upon activation by the binding of its PuTP substrate (Dong et al., 1995), these data strongly support a 6:1 stoichiometry of gp41:gp61 subunits within the primosome. This ratio is also in good accord with previous reports on the stimulation of gp41 ATPase and heli-
case activities by added gp61 (Hinton and Nossal, 1987; Richardson and Nossal, 1989b).

To check for the possibility of additional subunit interactions within the primosome, we also used protein-protein cross-linking to see whether primase alone forms dimers or higher associated forms in solution. We used the protein-protein chemical cross-linking procedure that we had previously employed to demonstrate the various association forms of the gp41 helicase (Dong et al., 1995). Cross-linking experiments were performed with gp61 in both the presence and absence of purine nucleo-
side triphosphates. Our results (see Fig. 3) suggest that, unlike the helicase (lane S), the primase alone exists as a monomer under all solution conditions tested and does not associate to higher forms. This result, together with the fact that gp41 hexamers also do not associate further in solution (Dong et al., 1995), strongly suggests that the functional primosome subas-
sembly contains six helicase subunits together with one mon-
omer of primase.

The T4 Primosome Is a 6:1 Helicase-Primase Complex

**Composition and Stoichiometry of the Primosome**—To determine directly the protein subunit stoichiometry of these reconstituted complexes, increasing amounts of gp41 were titrated into fixed concentrations of ss M13 DNA and gp61 until gp41 was clearly present in excess. The resultant DNA-bound complexes were then freed of excess components by native agarose gel electrophoresis, and the ratio of bound helicase to primase subunits was determined by densitometry of the component gp41 and gp61 bands on SDS-polyacrylamide gels (Fig. 2). The results presented in Fig. 2A and B, clearly show that in the presence of excess gp41, the amount of gp41 bound within the complex is limited by the amount of gp61 available. In support of this interpretation, we show (in Fig. 2A, lanes 4–6) that reducing the amount of gp61 by 50% or 75% results in the loss of a corresponding amount of gp41 from the complexes. This shows that gp41 interacts stochiometrically with gp61 and that the primase is not just acting as a "loading" factor to facilitate the interaction of DNA with gp41. Two-fold higher concentrations of proteins (relative to those used in Fig. 1) were used in this experiment to permit more accurate measurement of protein concentrations in SDS-polyacrylamide gels by silver staining.

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**Effects of ADP on the Formation and Stability of the Primosome**—ADP is produced by the ATPase activity of gp41; thus, it is important to ask whether ADP has any effect on the stability of the primosome. The experiments shown in Fig. 4 test the effects of this nucleotide on the stability of the primosome and show that ADP does seem to affect the formation or the stability of the primosome. When primosome assembly experiments were performed in the presence of ADP and analyzed by native agarose gel electrophoresis (using the same conditions as the previous native gels, i.e., no nucleotide cofactors in the running buffer), some complexes larger than the primase-DNA complexes seen in Fig. 1A (lanes 5–9) were detected. However, these complexes were much smaller than those formed in primosome assembly experiments carried out in the presence of ATP or GTP-γ-S. On the basis of these findings alone, we cannot know whether these intermediate-sized complexes correspond to "regular" (6:1) primosomes that have partially dissociated during gel electrophoresis or to complexes that were incompletely assembled prior to loading onto the gels. As we will show below, primosomes assembled by ATP binding are stable for long periods of time, even after the nucleoside triphosphate substrates have been completely removed. Such results strongly argue that the intermediate-sized complexes we observe here...
Primosome Complexes Remain Stable after ATP Hydrolysis Is Complete—Previously (Dong et al., 1995), we had shown that the gp41 helicase alone undergoes reversible hexamer formation that is dependent on the binding of its PuTP substrate. Therefore, it was important to determine whether the helicase within the primosome remains hexameric during the entire translocation/helicase/replication cycle or whether it dissociates and reassociates during each cycle of ATP binding and hydrolysis. We have approached this question by determining whether helicase hexamers within the reconstituted primosome complexes bound to ss M13 DNA remain hexameric after ATP removal. (We note that the persistence of such complexes in native gel-shift experiments, as in Figs. 1A and 2A, do not speak to this point since complexes that appear stable within gels may be held together artificially by gel "caging" effects.) Fig. 5 shows that primosomes formed as 6:1 helicase-primase complexes on ss M13 DNA and incubated for 10 min (Fig. 5) or 20 min (data not shown) at room temperature after ATP removal remain intact.

Stable Primosomes after ATP Depletion Do Not Contain ATP, ADP, or Pi—We have shown above that primosomes formed by the interaction of helicase and primase are stable for prolonged periods, even after ATP depletion. We ask here whether these stable primosomes contain bound ATP or its hydrolysis products. To test this, experiments were performed using radioactively labeled ATP carrying $^{32}$P at either the $\gamma$ or the $\alpha$-phospho...

The T4 Primosome Is a 6:1 Helicase-Primase Complex

The amounts of radioactivity in lanes 5 and 6 were chosen so that if all six subunits of the helicase bind $^{32}$P, the radioactivity in the primosome complexes (loaded in lanes 1 and 2) would be equal to that seen in these lanes. We find, however, that no detectable radioactivity is found at the position at which the primosome complexes run on the native agarose gel. (The position is defined in the control experiment using SYBR-green I nucleic acid stains in Fig. 6B, lane 2). Therefore, the primosome complex remains intact as a 6:1 helicase:primase complex even after all nucleoside triphosphate and its hydrolyzed products have been released.

Taking these results together, it is clear that the PuTP substrate of the helicase is required to form the primosome. However, once formed, the primosome is very stable by itself. Thus, even though the ATP-activated hexamerization of the helicase is reversible (Dong et al., 1995), the helicase hexamer is stabilized against dissociation by the primase within the primosome. Hence, the primosome remains stable over a much longer period than the milliseconds required to complete the ATPase and helicase cycles, which strongly suggests that once the primosome is formed the gp41 helicase component remains hexameric during the full functional cycle and does not alternate between different oligomeric forms.
The T4 Primosome Is a 6:1 Helicase-Primase Complex

Formation and Stabilization of the Primosome of the T4 DNA Replication Complex—In this study we have shown that the gp61 primase binds stably by itself to appropriate DNA templates, while the gp41 helicase hexamer in the absence of strong DNA cofactors relatively weakly and transiently. These observations support an assembly pathway like that outlined in Fig. 7. However, the order of addition of the components shown here may not be invariant, since we know that gp41 helicase alone can interact functionally with the DNA substrate as shown in previous studies (Young et al., 1994; Dong et al., 1995) and that primase and helicase in the absence of all other components do not interact. We have shown that the stable primosome complex, containing 1 primase and 6 helicase subunits, is likely to remain assembled through the entire replication cycle on the T4 genome.

Implications of the Hexagonal Structure of the Active gp41 for the Structure of the Primosome—Determination of subunit stoichiometry and composition of the primosome is the first step to establishing the structure of the complex. The 6 to 1 stoichiometry established here, in combination with our previous demonstration that the helicase itself is a ring-shaped hexamer (Dong et al., 1995), seems to suggest that the helicase hexamer is likely to remain in this general conformation within the complex. However, this result does not define the position of the primase within the primosome. We know that the primase must interact with the DNA and, at least transiently, with all six helicase subunits. This raises the question of the location of gp61 inside the primosome complex. Considering that the helicase hexamer itself is a ring-shaped structure with pseudo-C3 or D3 symmetry, the primase is most likely to reside in a position close to the C3 rotational axis of the helicase hexamer. However, a primase subunit that shuttles from one helicase monomer (or dimer) binding site to another during primosome function is also possible. Such behavior has been seen for the subunits within the bovine heart mitochondrial F1-ATPase (Abrahams et al., 1994). In addition, we note that the bacteriophage T7 primosome complex is also hexameric, but that each subunit carries both a helicase and a primase domain (Mendelman et al., 1993; Patel and Hingorani, 1995). At present, there is no structural evidence to discriminate between a centrally located primase within the primosome and a primase that shuttles from one helicase subunit to another as the primosome functions.

Functional Integration of the Primosome within the T4 DNA Replication Complex—Formation of separate DNA polymerase holoenzyme and primosome subassemblies seems to be general in the DNA replication systems of many different organisms (Kornberg and Baker, 1992). In addition, many (most?) DNA helicases that are involved in genomic DNA replication seem to exist and function as hexamers (see Table II in Dong et al., 1995) upon activation either by their PuTP substrates or by the DNA template. Furthermore, those hexameric helicases that have been investigated in detail all seem to adopt a toroi-

DISCUSSION

The association pathway proposed for the in vitro assembly of the T4 DNA replication primosome from the gp41 helicase, the gp61 primase, and the DNA template in the presence of nucleoside triphosphate substrates. Note that this is not the only pathway that is consistent with our data (see text).

somemains remain stable for 20 min or more under the conditions tested even if the supply of unhydrolyzed PuTP is depleted. Physiological conditions, under which ample PuTP is available, certainly favor stable primosome formation, and we have shown that the stable primosome complex, containing 1 primase and 6 helicase subunits, is likely to remain assembled through the entire replication cycle on the T4 genome.

A

![Fig. 6. Primosome complexes remain stable after release of bound ATP and its hydrolyzed products.](Image)

B

![Fig. 7. The association pathway proposed for the in vitro assembly of the T4 DNA replication primosome from the gp41 helicase, the gp61 primase, and the DNA template in the presence of nucleoside triphosphate substrates. Note that this is not the only pathway that is consistent with our data (see text).](Image)
(ring-shaped) subunit arrangement. In spite of these large scale structural similarities, no atomic level structure has been established for any helicase or primosome. Nevertheless, it is likely that DNA replication primosomes from different organisms will be found to share a certain degree of structural similarity, and it is reasonable to speculate that the hexameric structure of these helicases (and the resulting common structural features that may characterize primosomes) will turn out to have general mechanistic significance.

Recent studies (Spacciapoli and Nossal, 1994; Barry and Alberts, 1994; Morrical et al., 1994) suggest that the T4-coded gp59 protein (the gp41 helicase assembly factor) is important under some conditions for the loading of the gp41 helicase onto DNA to form a functional complex with other replication proteins. Our finding here that the primosome can be formed on ssM13 DNA in the absence of the gp59 protein supports the notion that this protein may only be required to assemble the helicase onto single-stranded DNA-binding protein (gp32)-coated DNA templates, in agreement with previous results (Barry and Alberts, 1994; Morrical et al., 1994; Spacciapoli and Nossal, 1994). Whether this assembly factor is required to load the activated helicase hexamer onto a primase that is already present on a gp32-coated DNA template is not yet known.

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