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Assembly of the Primosome of DNA Replication in *Escherichia coli**

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Assembly of the *Escherichia coli* primosome requires six proteins, PriA, PriB, PriC, DnaB, DnaC, and DnaT, acting at a primosome assembly site (*pas*) on an SSB-coated single-stranded (ss) DNA. Assembly is initiated by interactions of PriA and PriB with ssDNA and the *pas*. PriC, DnaB, DnaC, and DnaT then act on the PriA-PriB-DNA complex to yield the primosome. In the primosome, the dATPase (ATPase) of PriA becomes hyperactivated. In addition, the assembled primosome appears to block the *pas*, preventing it from activating additional PriA molecules. Either ATP alone or dATP in combination with GTP is sufficient for primosome assembly, while ATP or GTP provides for its maintenance during isolation. These nucleotide requirements can be reconciled with the need for ATP or dATP for DnaB-DnaC complex formation and hydrolysis of ATP or GTP by DnaB when it binds ssDNA. Such isolated primosomes contain a dATPase, the hallmark of PriA, and a GTPase indicative of DnaB. Further studies indicate that the isolated primosome contains the PriB replication activity in addition to PriA and DnaB.

Replication of the single-stranded DNA of phage φX174 to its duplex replicative form has been reconstituted using host-encoded proteins (1, 2). This replication process begins with PriA, PriB, PriC, DnaB, DnaC, and DnaT assembling a primosome on the SSB-coated[1] ssDNA. A special hairpin structure on the DNA called a primosome assembly site or *pas* is also required (3, 4). The primosome can then be recognized by primase to lay down RNA primers for complete replication of the template to the duplex RF by DNA polymerase III holoenzyme. (As originally coined, the term "primosome" defined a multiprotein complex containing a primase sufficient to generate a primer (5). The protein complex formed prior to the addition of primase was called a preprimosome. Lack of a stable association of primase with the primosome has diminished this distinction. To reflect this fact and the variety of primosomal forms in other systems (6, 7), we use the term "primosome" for the multiprotein complex, knowing that primase must act at this complex, however transiently, to synthesize a primer.) This ss to RF system was established as a model for lagging strand replication during which multiple primers must be provided for discontinuous synthesis (see Refs. 6 and 7 for recent reviews). Indeed, the primosome replication system has been implicated in lagging strand synthesis of the pBR322 and ColE1 plasmids both *in vivo* (8) and as reconstituted *in vitro* (9).

As their names imply, the products of the *dna* genes used in φX replication are also known from genetic studies to be required for chromosomal replication (10–12). These requirements have been largely borne out in the *in vitro oriC* initiation system (13), which utilizes DnaB, DnaC, primase (DnaG), SSB, and DNA polymerase III holoenzyme in common with the φX system. One notable exception is the *dnaT* gene product, which has been implicated in chromosomal replication *in vivo* (11, 12), but for which the only *in vitro* involvement to date has been in primosome assembly.

The remaining components, PriA, PriB, and PriC, are primosome-specific. Preceding the recent isolation of the genes encoding these proteins (14–17), their role in chromosomal replication was difficult to determine. Disruption of the *priA* gene induces the SOS response (18) and decreases the strain's viability 10–100-fold (18, 19); both findings are consistent with a role in DNA replication.

Historically, PriA, PriB, and PriC were identified as a single fraction, protein n (1, 2), which was only subsequently resolved into proteins n′, n, and n″ (now termed PriA, PriB, and PriC, respectively). Identification of these genes has allowed the construction of efficient overproducers and the isolation of large quantities of each individual protein (14–17).[2] Moreover, several of these components are more active when isolated from the overproducing source than from wild-type cells, probably due to the high degree of overexpression and the brevity of the purification (16).[2]

With these newly obtained, highly active, homogeneous components, the early steps of primosome assembly have now been investigated. The interactions of PriA and PriB with both φX174 and M13 DNA have been studied. In addition, the requirements for primosome assembly and maintenance have been characterized and the enzymatic activities in an isolated primosome identified.

MATERIALS AND METHODS

Reagents—Sources were as follows: ribonucleoside triphosphates, DNA, and Tris base, Sigma; deoxyribonucleoside triphosphates, Pharmacia LKB Biotechnology Inc.; [α-^{32}P]dTTP, [α-^{32}P]dATP, [α-^{32}P]ATP, [α-^{32}P]GTP, Amersham Corp.; Bio-Gel A-15m and Bio-Rad protein assay reagent, Bio-Rad; polyethyleneimine-cellulose (Polygram CEL300 PEI), Brinkmann; and M13 mp18 ssDNA and T4 gp32 protein, U. S. Biochemical Corp. φX174 ssDNA was prepared as described (20). Purified replication components were essentially as described (16, 20, 21) with protein concentration determined using the Bio-Rad protein assay reagent and ovalbumin as a standard, according to the manufacturer's specifications. For consistency all protein amounts are reported using these Bio-Rad determinations. In addition, the concentration of PriA was determined by the method of Gill and von Hippel (22) under native

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[1] The abbreviations used are: SSB, *E. coli* single-stranded DNA binding protein; RF, replicative form; ss, single-stranded; *pas*, primosome assembly site.

[2] G. C. Allen, Jr. and A. Kornberg, unpublished results.

conditions. The concentration of PriA by the Bio-Rad assay was 1.84 mg/ml compared with 1.31 mg/ml by the method of Gill and von Hippel (22).

The ϕX ss to RF Replication Assay—Reconstituted replication was as described previously (20) with the following modifications. The standard reaction contained 220 pmol of ϕX174 ssDNA (as nucleotide), 20 mM Tris-HCl (pH 7.5), 0.1 mg/ml bovine serum albumin, 8 mM dithiothreitol, 8 mM magnesium acetate, 0.01% Brij-58, 125 mM potassium glutamate, 13.2 pmol of *E. coli* SSB (as tetramer), 220 fmol of DnaB (as hexamer), 875 fmol of DnaC, 367 fmol of primase, 131 fmol of DNA polymerase III*, 634 fmol of DNA polymerase III β subunit (as dimer), 40 fmol of PriB (as dimer), 97.4 fmol of PriA, 217 fmol of PriC, 288 fmol of DnaT (as trimer), 1 mM ATP, 50 μM each of dATP, dGTP, dCTP, and 50 μM [α-^{32}P]dTTP (100 cpm/pmol) in a 25-μl reaction volume. Reactions were incubated for 10 min at 30 °C; nucleotide incorporation was measured in a liquid scintillation counter after trichloroacetic acid precipitation onto Whatman GF/C glass-fiber filters. In general, duplicate assays vary by 10% or less in the amount of nucleotide incorporated.

The ϕX ss to RF reaction was staged to allow primosome isolation. The standard reaction was performed in 20 μl except with 1 mM magnesium acetate (rather than 8 mM) and with the omission of the DNA synthesis components, primase, DNA polymerase III holoenzyme, and the dNTPs. Primosomes were formed by incubation at 30 °C for 15 min; the reactions were then cooled to room temperature for 2 min, and the protein-DNA complexes isolated as described below. Prior to analysis of replication activity, the fractions loaded onto the gel filtration column were diluted 2-fold with column buffer; 20 μl of this fraction (containing 110 pmol of ϕX DNA) and 20 μl of the excluded fraction ("isolated complex") were then assayed. The presence of primosomal replication activity was detected following addition of primase, DNA polymerase III holoenzyme, labeled dNTPs, and supplementation of magnesium acetate to 8 mM, all provided in a 5-μl volume. DNA synthesis was measured after 10 min at 30 °C. The amount of synthesis in the "isolated complex" fractions was adjusted to account for the dilution that occurred during gel filtration.

Isolation of Protein-DNA Complexes by Gel Filtration—Various protein DNA complexes were isolated by gel filtration at 22 °C. The reaction mixtures (100 μl) were applied to a 1-ml Bio-Gel A-15m column equilibrated with 20 mM Tris-HCl (pH 7.5), 0.1 mg/ml bovine serum albumin, 8 mM dithiothreitol, 8 mM magnesium acetate, 0.01% Brij-58, 125 mM potassium glutamate, and 1 mM ATP, with modifications as noted. The void fraction (typically ~320 μl) was collected as a pool and referred to as the "isolated complex." The isolation typically took ~10 min. When these fractions were to be assayed for replication activity, the load fraction was diluted 2-fold with column buffer; then this fraction (20 μl) and the "isolated complex" fraction (20 μl) were supplemented with the remaining components in a volume of 5–10 μl and processed accordingly.

Assay of rNTP and dNTP Hydrolysis—The assay measured the production of nucleoside diphosphates from the input triphosphates. The standard reaction was performed under the same conditions as in the ϕX ss to RF assay with the following modifications. The reaction was scaled down proportionately to 15 μl omitting the synthesis components; the α-^{32}P-labeled rNTP or dNTP substrate was added to a final concentration of 1 mM from a 15 mM stock (40 Ci/mol). The reaction was incubated at 30 °C for the indicated time; 2-μl samples were applied to polyethyleneimine-cellulose TLC plates (prespotted with ADP and ATP as markers). The plate was developed with 1 M formic acid and 0.5 M LiCl, and then dried. The locations of diphosphates and triphosphates were determined by UV absorption; the plate was cut and individual spots were counted in a liquid scintillation counter. When column fractions were assayed for nucleotide hydrolysis, the particular nucleotide was added to a final concentration of 0.7 mM from a 15 mM stock with the remaining procedures as described above. In general, duplicate assays vary by 10% or less in the amount of nucleotide hydrolyzed.

RESULTS

PriA/DNA Interactions Prior to Primosome Assembly—ϕX174 ssDNA was used as a natural template, containing a *pas*, and M13 ssDNA as a generic ssDNA, without a *pas*. All reactions were performed at the same concentration of proteins and DNA under which primosome assembly and replication occur. An interaction was identified as stable by the isolation of protein-DNA complexes away from free protein by gel filtration. The isolated complexes were then assayed for the presence of a given replication activity.

PriA interacts with DNA as indicated by its DNA-dependent ATPase (dATPase) activity (3, 22). The DNA interaction was demonstrated in its stable binding to both ϕX174 and M13 ssDNAs in the absence of SSB (Table I). When these DNAs were coated with *E. coli* SSB, both DNAs still interacted with PriA (Table I), although the M13 lost its ability to activate the PriA ATPase (data not shown) (22). DNA synthesis in reactions containing this PriA-M13-SSB complex was still dependent on added ϕX template (data not shown). To discern whether PriA was interacting with the M13 DNA or whether it might be influenced by the *E. coli* SSB, gp32, the ssDNA-binding protein of bacteriophage T4, was used in place of *E. coli* SSB. As with *E. coli* SSB, gp32 prevented M13 from activating the PriA ATPase but was without effect on the activation by ϕX DNA (data not shown). Accordingly, PriA formed a stable complex with gp32-coated ϕX DNA but not with gp32-coated M13 DNA. Thus, PriA can bind stably to naked ssDNA and a specific site on SSB-coated ϕX DNA. In addition, these findings suggest that a specific interaction with *E. coli* SSB may assist this DNA binding. Studies of PriA-DNA complexes in the absence of ATP during initial binding and filtration gave results identical to those above (data not shown).

PriB/DNA Interactions Prior to Primosome Assembly—Interactions of PriB with ssDNAs were investigated as with PriA (Table II). In the absence of any ssDNA-binding protein, PriB bound to both ϕX and M13 ssDNAs. When these DNAs were coated with *E. coli* SSB, PriB still complexed with both. Furthermore, even when coated with gp32, PriB bound stably to M13 and ϕX174 ssDNAs. These findings are in contrast to previous studies that failed to find ssDNA binding by PriB and instead identified a direct interaction with SSB (23). Possibly, the PriB of lower specific activity purified from a wild-type strain lacks the DNA binding capability now observed in the PriB purified from an overproducing strain. Furthermore, the reductive methylation used to label PriB for the previous SSB interaction studies may have introduced a feature not inherent in the untreated PriB protein.

The influence of PriB DNA binding on PriA binding was also investigated (Table III). PriB bound to both *E. coli* SSB- and gp32-coated M13, whereas PriA bound only to SSB-coated M13. On SSB-coated M13, both PriA and PriB activities were recovered, as expected. On gp32-coated M13, only PriB was recovered in a DNA complex, as implied by restoration of full activity with PriA supplementation. Thus, interactions between PriA and PriB are insufficient to induce PriA to bind to the gp32-

TABLE I
PriA interactions with DNA

PriA was mixed with the indicated DNA and SSB in 20 μl of the standard reaction buffer and incubated at 30 °C for 15 min. This initial mixture was cooled to room temperature for 2 min, and then protein-DNA complexes were isolated by gel filtration. The PriA replication activity in these fractions was assayed as described under "Materials and Methods"; the remaining replication components were added in 10 μl and the reaction incubated for 10 min at 30 °C to allow DNA synthesis. When not added in the first stage, ϕX DNA was included in the second stage. The ϕX DNA was present at 110 pmol (as nucleotide) in both sets of assays. Each of these experiments was performed a total of three times with less than 10% variation in percentage recovery of activity. Data from a representative experiment are given.

ssDNA	SSB	DNA synthesis	
		Initial	Complex
		pmol	
ϕX174	None	98	91
	E. coli	94	95
	T4 gp32	87	99
M13	None	82	92
	E. coli	78	134
	T4 gp32	83	6

TABLE II
PriB interactions with DNA

The experimental procedures were identical to those in Table I except that PriB was used in place of PriA.

ssDNA	SSB	DNA synthesis	
		Initial	Complex
		pmol	
φX174	None	87	73
	E. coli	117	98
	T4 gp32	97	95
M13	None	72	71
	E. coli	88	123
	T4 gp32	82	97

TABLE III
Interactions of PriA, PriB, and PriC with DNA

The experimental procedures were identical to those in Table I except that the indicated primosomal proteins were included in the first stage incubation prior to isolation. Each of these experiments was performed twice with less than 10% variation in percentage recovery of activity. Data from one such experiment are given.

Primosomal proteins	ssDNA	SSB	DNA syntheis	
			Initial	Complex
			pmol	
PriA, PriB	M13	*E. coli*	83	99
		T4 gp32	87	0[a]
PriA, PriB, PriC	φX174	None	107	7[b]

[a] When supplemented with PriA in addition to φX DNA and the omitted replication components, this fraction was restored to full activity (89-pmol synthesis).

[b] When supplemented with PriC in addition to the omitted replication components, this fraction was restored to full activity (110-pmol synthesis).

coated M13-PriB complex.

PriC Interactions Prior to Primosome Assembly—A complex including PriA, PriB, and PriC was also sought (Table III). When all three were incubated with naked φX DNA, no complex containing all activities was found. The only component lacking in the isolated complexes was PriC, inasmuch as its addition restored full activity.

Protein/DNA Interactions Prior to Primosome Assembly—The foregoing data indicate that PriB binds any ssDNA whether naked or SSB-coated. For PriA to bind to an SSB-coated DNA lacking a *pas*, only the *E. coli* protein and not gp32 is effective, implying an interaction with *E. coli* SSB. To date, no other complexes on DNA have been detected short of the addition of PriC, DnaB, DnaC, and DnaT to complete primosome assembly. For example, the φX ss to RF assay shows only a 3–6-fold dependence on PriC due to a high level of synthesis in its absence. This background suggests that an intermediate complex may form on the DNA in the absence of PriC. When all of the primosome components except PriC were incubated with φX DNA, no intermediate complexes capable of replication with the subsequent addition of PriC, primase, and DNA polymerase III were found (data not shown). The only activities identified in the complexes formed without PriC were PriA and PriB, as expected from their individual binding activities. In view of the weak dependence of the φX ss to RF reaction on PriC and the lack of formation of a stable primosomal intermediate, it may be more accurate to view PriC as a specificity factor that favors the *pas*-directed over nonspecific replication pathways.

PriA Interactions with the pas during Primosome Assembly—Two activities of PriA are dependent on the *pas*: its ATPase (dATPase) in the presence of SSB (3) and primosome assembly and replication (4). The relationship between these two functions was examined by investigating the PriA dependence of these activities. PriA saturated the φX ss to RF replication assay at a stoichiometry of one PriA molecule per φX circle

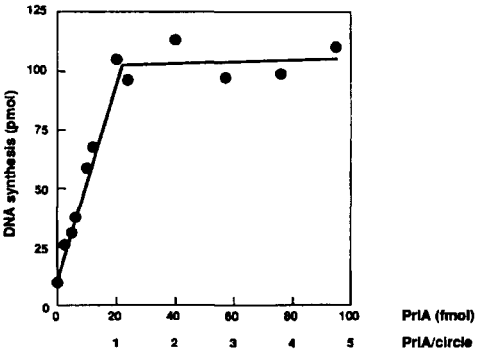

FIG. 1. **Replication of φX174 DNA requires one PriA molecule per circle.** PriA was titrated into the standard reaction on ice prior to incubation for 10 min at 30 °C. The φX template DNA was present at 110 pmol (as nucleotide) so that all other components would be saturating with respect to DNA.

TABLE IV
Several PriA molecules can bind to a single φX circle

PriA at the indicated level (1-, 2-, and 3-fold the standard amount) was incubated in the φX ss to RF reaction buffer with 220 pmol of φX DNA (as nucleotide) coated with SSB (13.2 pmol) for 15 min at 30 °C. The reactions were cooled to room temperature for 2 min; the protein-DNA complexes were then isolated. Other procedures were as described under "Materials and Methods" with DNA synthesis being determined for 10 min at 30 °C and dATP hydrolysis determined after 30 min at 30 °C. At the time of synthesis, the φX DNA was present at 110 pmol (as nucleotide) in both sets of assays.

PriA/φX174 circle	DNA synthesis		dATP hydrolysis	
	Initial	Complex	Initial	Complex
	pmol		*nmol*	
1.2	130	122	1.9	1.4
2.4	116	115	2.6	2.9
3.6	107	105	3.9	4.3

added and replicated (Fig. 1). This saturation at a stoichiometry of one PriA per circle was not seen, however, in the ssDNA binding and dATPase activities of PriA (Table IV). In these experiments, various concentrations of PriA were incubated with SSB-coated φX ssDNA and the protein-DNA complexes isolated. As in the above titration, replication was saturated with one PriA added per circle; additional PriA did not stimulate further synthesis. In contrast, additional PriA did result in increased dATP hydrolysis in the initial mixtures (*i.e.* before filtration). Moreover, this additional hydrolysis activity was recovered as a complex of PriA with the ssDNA implying that more than one PriA can be bound to and activated by a single φX circle (Table IV).

This conclusion was confirmed in a titration of the PriA dATPase (Fig. 2). Addition of PriA in excess of activating template yielded increasing dATP hydrolysis. In this assay, all components required for primosome assembly except for DnaC were included (*–Primosome*) such that if any of these other proteins limited the activation of the PriA dATPase they might do so. No such limitation was seen. However, when all primosome components were included (*+Primosome*), the titration of PriA for dATP hydrolysis paralleled that for replication, saturating at a molar ratio of PriA to template of about 1. This experiment also revealed that primosome assembly activates the PriA dATPase 2-fold compared with PriA in the absence of the primosome. To exclude that DnaC is the primosomal component that hyperactivates PriA, a similar titration, but with DnaB omitted to prevent primosome formation, showed identical results to those when DnaC was omitted (data not shown).

The titration of the PriA dATPase during primosome assembly suggested that once assembled, the primosome could pre-

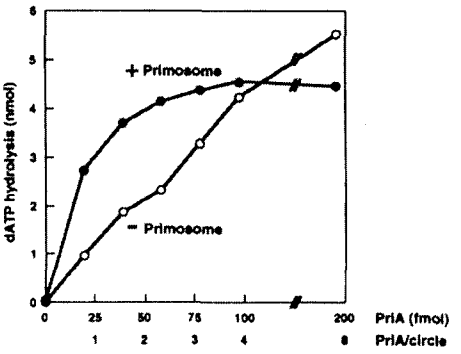

FIG. 2. **Primosome assembly hyperactivates a limited number of PriA molecules for dATPase.** The standard hydrolysis reaction was performed except that GTP was provided at 0.5 mM in place of ATP along with [α-^{32}P]dATP as substrate for hydrolysis. Primosome formation was precluded by the omission of DnaC (*–Primosome*). PriA was titrated into the reaction on ice prior to incubation at 30 °C for 30 min. No nucleotide hydrolysis was seen in the absence of PriA.

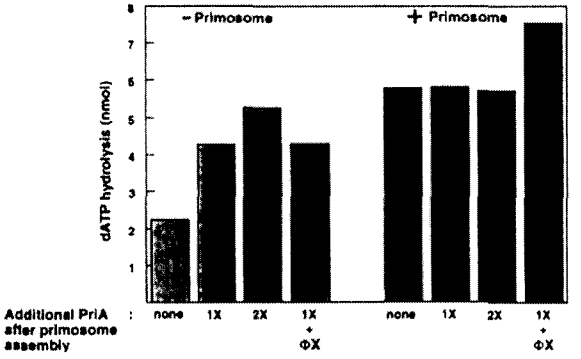

FIG. 3. **Primosome assembly blocks the *pas* to access by additional PriA.** Primosomes were preformed in the staged ϕX ss to RF reaction during a 15-min incubation at 30 °C and then chilled on ice. The dATPase activity in these reactions was detected after the addition of [α-^{32}P]dATP and a second incubation at 30 °C for 30 min. Primosome formation was precluded by the omission of DnaC (*–Primosome*). Where indicated, additional PriA was provided in the second stage after primosome assembly. The level of PriA supplement is indicated with respect to that present during the first incubation, *e.g.* 1× indicates the addition of 97.4 fmol of PriA. Additional ϕX DNA was also provided as an SSB-DNA complex (110 pmol of ϕX DNA plus 6.61 pmol of SSB) (*+ϕX*).

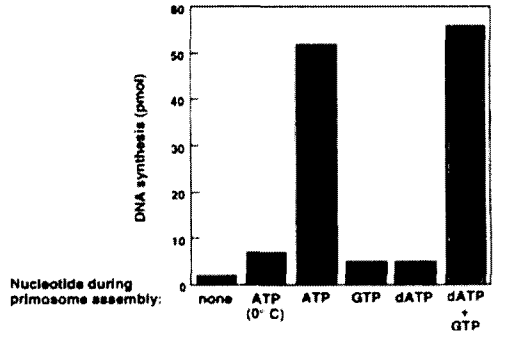

FIG. 4. **ATP alone or dATP plus GTP support primosome assembly.** The staged ϕX ss to RF reaction was assembled on ice with each nucleotide present at 100 μM in place of 1 mM ATP, as indicated. The reactions were incubated for 15 min at 30 °C to allow primosome formation and then chilled on ice. The synthesis components were added along with ATP (to 1 mM final concentration), and DNA synthesis was allowed for only 1 min at 30 °C. The ϕX DNA was present at 220 *pmol* (as nucleotide).

TABLE V
Nucleotide required for primosome isolation

The standard staged ϕX ss to RF reaction was performed with the indicated nucleotide used during gel filtration. The nucleotide was present at 100 μM final concentration (A) or at 1 mM (B). Where indicated, the "isolated complexes" were supplemented with various proteins prior to addition of the replication components along with ATP (to 1 mM, final concentration) and incubation at 30 °C for 10 min. The amount of supplemented protein was one-half that provided in the first stage, *e.g.* 438 fmol of DnaC. The ϕX DNA was present at 110 pmol (as nucleotide) in both sets of assays. Each of these experiments was performed twice with less than 10% variation in percentage recovery of activity. Data from one such experiment are given.

Nucleotide during filtration		Additions	DNA synthesis[a]
			pmol
A.	None		5
	ATP		56
	dATP		14
	GTP		50
B.	None		5
	ATP		63
	GTP		66
	None	DnaC, PriC, DnaT	3
		DnaC, PriC, DnaT, DnaB	101
		DnaC, PriC, DnaT, PriA	3
		DnaC, PriC, DnaT, DnaB, PriA	91

[a] Values given are for replication using the isolated complex. The complete system yielded 74 pmol of DNA synthesis in A and 84 pmol in B.

vent activation of additional PriA by the *pas*. This prediction was tested directly by preforming primosomes with equimolar PriA and then challenging them with more PriA (Fig. 3). The accessibility of the *pas* was monitored by its activation of the PriA dATPase. A reaction containing all primosomal components but for DnaC served as a control for activation in the absence of assembled primosomes. Without the primosome, both 1- and 2-fold additional PriA gave increased dATP hydrolysis. When primosomes were assembled, however, additional PriA failed to be activated for dATP hydrolysis. This limitation could not be attributed to saturation of the assay, inasmuch as an addition of SSB-coated ϕX DNA (lacking a primosome) along with the additional PriA showed increased hydrolysis over that of the preassembled primosome.

The Role of Nucleotides in Assembly of a Stable Primosome—Assembly of the primosome requires ATP and incubation at an adequate temperature (Fig. 4) (2, 20, 24). There is a 1-min time lag in assembly of the primosome, which was utilized to determined the precise nucleotide requirements. Assembly was attempted at 30 °C with the indicated nucleotide, after which the reaction mixture was chilled on ice. The presence of primosomes was scored by adding DNA polymerase III and primase and following DNA synthesis at 30 °C for only 1 min (the length of the lag). After the first incubation, ATP was added to all reactions to provide for any requirements during the synthesis stage. The effectiveness of this staging was demonstrated in the reaction lacking nucleotide in the first incubation. Even when supplemented with ATP for the synthesis stage, no replication was seen in this experiment. When ATP was provided in the first incubation, it promoted efficient primosome formation, as expected. By the same measure, neither dATP nor GTP alone allowed primosome formation. When both were present, primosome assembly occurred to the same extent as with ATP.

The assembled primosome is a stable complex on ϕX DNA and can be isolated by gel filtration (Table V) (24). As with primosome formation, its isolation required the presence of a nucleotide. Both ATP and GTP allowed isolation of a stable complex capable of supporting DNA replication; dATP was poor at stabilizing the complex during filtration. The deficiencies in the complexes isolated without nucleotide were determined by

supplementing them with primosomal components. The addition of DnaC, PriC, DnaT, and DnaB restored full activity. Additional PriA was without effect in combination with DnaC, PriC, and DnaT nor did it stimulate when present in this combination with DnaB. These findings imply that PriA and PriB remained in the protein-DNA complex even when isolated without nucleotide.

Contents of the Isolated Primosome—The components of the primosome present in a stable protein-DNA complex were identified after isolation by gel filtration. To a first approximation, the presence of several enzymatic activities in the complex was determined (Table VI). The complete primosome assembly reaction mixture, as loaded on the column, contained both the dATPase activity of PriA and the GTPase activity of DnaB. Both of these hydrolytic activities were recovered as components of the isolated primosome. Under the conditions used for these assays, only PriA hydrolyzed dATP and only DnaB hydrolyzed GTP (data not shown).

To obtain a full inventory of the components in the isolated primosome, a variation on the isolation experiment was performed. The primosome, as initially isolated, could support priming and DNA synthesis, yet these complexes were not indefinitely stable and lost this capacity after an overnight incubation at 0 °C. Supplementation of the replication deficiency then restored full activity and indicated which primosomal components were present in the isolated complexes (Fig. 5). Addition of PriC, DnaC, and DnaT to the decayed complexes restored full replication activity. No pair of these components nor a single component was sufficient for restoration. Furthermore, addition of PriA or PriB did not stimulate synthesis above that obtained with PriC, DnaC, and DnaT alone. The loss of replication activity of the isolated primosome was not caused by inactivation of an individual component as the complete reaction mixture showed no loss of activity following the same overnight incubation (data not shown). Given that only PriC, DnaC, and DnaT need to be supplemented to the decayed primosome, the remaining components, PriA, PriB, and DnaB, are likely the only components of the isolated primosome.

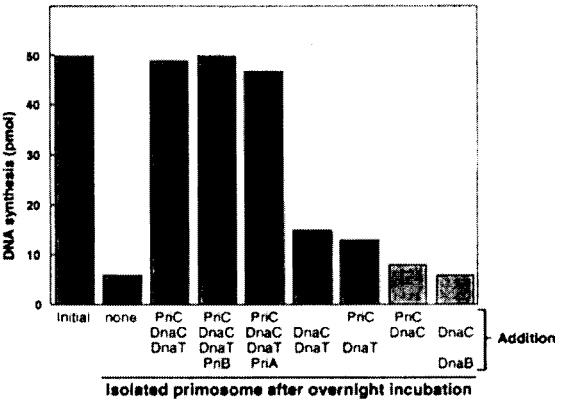

FIG. 5. **The isolated primosome contains PriA, PriB, and DnaB.** The staged ϕX ss to RF reaction was performed, and the primosomes were isolated and assayed for replication activity ("initial"). These isolated primosomes were then incubated overnight on ice to allow decay of their replication activity. Where indicated, various primosomal components were added to the decayed, isolated primosome fraction to restore replication activity. The proteins were added at one-half the amount provided during primosome assembly, *e.g.* 438 fmol of DnaC. The synthesis components were then added and the reactions incubated for 10 min at 30 °C. The ϕX DNA was present at 110 pmol (as nucleotide) in all assays.

DISCUSSION

The *E. coli* primosome is a replication complex of host proteins originally identified for its role in the conversion of phage ϕX174 ssDNA to the duplex replicative form (1, 2). Six proteins, PriA, PriB, PriC, DnaB, DnaC, and DnaT, are required to form the primosome, which can then be acted on by primase and DNA polymerase III holoenzyme to replicate the template. Early studies of the ϕX ss to RF replication system utilized a single fraction, protein n, containing PriA, PriB, and PriC. Even after resolution, these three components were most often provided as PriA (protein n′) and a fraction containing both PriB and PriC (proteins n and n″), so the role of individual components in overall primosome assembly was difficult to address. With the recent isolation of the *priA*, *priB*, and *priC* genes (14–17), their overproduced products have now been obtained in homogeneous form and, in several cases, with higher specificity activities than previously available. In addition, the ϕX ss to RF reaction is now highly efficient; 90% or more of the input template is replicated in the standard assay (Fig. 1). These features, in conjunction with new information on the properties of individual components, have prompted further investigation of the early stages of primosome assembly.

Prior to primosome formation, both PriA and PriB displayed stable interactions with DNA. PriB did so independent of the specific *pas* or the presence and source of ssDNA-binding protein. PriA interacted with naked ssDNA, both ϕX containing a *pas* and M13 lacking one, as measured by the activation of its dATPase and by its stable binding to those DNAs during gel filtration. Coating the M13 DNA with either *E. coli* SSB or T4 gp32 prevented activation of the dATPase. Interestingly, PriA still formed isolable complexes with SSB-coated M13, suggesting that it may also interact directly with SSB. In contrast, both SSB- and gp32-coated ϕX DNA retained their activation capacity due to the presence of the *pas*.

In the absence of the primosome, more than one PriA can bind to a single ϕX circle and become activated (Table IV and Fig. 2). Upon primosome assembly, the dATPase becomes hyperactivated, and only a limited number of PriA molecules could apparently bind and be activated. Additional PriA could not be activated, suggesting that access to the *pas* was blocked. (In the experiments in Fig. 2, dATP hydrolysis was followed during assembly of the primosome, and the amount of hydrolysis reflects that occurring both before and after assembly. Under these circumstances, the saturation for hydrolysis at 2 PriA/circle likely results from the combination of these two effects, since when primosomes are preassembled at 1 PriA/circle, no additional hydrolysis is seen with added PriA (Fig. 3).) One explanation to encompass these findings is as follows: PriA gains access to SSB-coated DNA through the *pas* that activates its dATPase. PriA is also a helicase (25, 26) and can translocate on ssDNA. Powered by these activities, PriA departs the *pas* for other DNA, which activates its dATPase less well. Upon pri-

TABLE VI
Activities present in the isolated primosome

Primosomes were formed and isolated in the staged ϕX ss to RF reaction as described under "Materials and Methods." The activities present in these complexes were determined: DNA synthesis for 10 min at 30 °C, dATP hydrolysis after 30 min at 30 °C, and GTP hydrolysis after 15 min at 30 °C. At the time of synthesis, the ϕX DNA was present at 110 pmol (as nucleotide) in both sets of assays. This experiment was performed three times with data from a representative experiment given.

Fraction	Activity	
Complete	DNA synthesis (pmol)	80
	dATP hydrolysis (nmol)	1.5
	GTP hydrolysis (nmol)	3.1
Isolated primosome	DNA synthesis (pmol)	49
	dATP hydrolysis (nmol)	0.9
	GTP hydrolysis (nmol)	4.0

mosome formation, PriA movement is restricted; thus it remains at the *pas*, where it is hyperactivated for dATP hydrolysis and blocks other PriA molecules from access to the site. Although assembled primosomes lacked several components, restriction of PriA to the *pas* required complete primosome assembly, inasmuch as no single component or subset of components was sufficient to engender this property. This restriction of the *pas* has the added benefit of limiting a template to containing a single primosome. The presence of multiple PriA molecules on a single template provided no apparent advantage for DNA replication (Table IV). By preventing multiple PriAs from binding, the primosome may circumvent competition for what may be a limiting reagent in primosome assembly and replication.

Nucleotides play a role during both primosome assembly and maintenance. For assembly, either ATP alone or dATP in combination with GTP was sufficient (Fig. 4), whereas either ATP or GTP alone provided for stable maintenance (Table V). The DnaB and DnaC proteins form a complex in solution which is stabilized by ATP or dATP but not by GTP (27–29). The adenine nucleotide used in primosome assembly could provide for formation of this DnaB-DnaC complex. DnaB is a DNA-dependent rNTPase but will not hydrolyze the deoxy-NTPs (30). Studies of DnaB interactions with naked poly(dT) found that DNA binding was stimulated by rNTPs (30). In the context of the primosome, this nucleotide dependence became absolute (Table V) (20); without a ribonucleotide, DnaB activity was not recovered in the complex. Either ATP or GTP could provide this stability during assembly and isolation.

Several lines of evidence indicate that the ATP requirement was not likely due to hydrolysis by PriA. First, PriA protein binds stably to DNA in the absence of a nucleotide, both on template alone and in the primosome when isolated without a nucleotide present (Table V). Second, mutant versions of PriA protein that fail to hydrolyze nucleotides still support primosome assembly and replication (31).

The requirement for both DnaB and DnaC in several replication systems has been interpreted as the need for a DnaB-DnaC-ATP complex. This necessity has been difficult to demonstrate discretely, however, because most of these systems require a nucleotide for other aspects of the initiation process. The observation here that dATP in combination with GTP supports primosome assembly when neither alone can suffice has separated these various aspects. These findings represent the first clear demonstration of the requirement for formation of a DnaB-DnaC-(d)ATP complex in an initiation process. In addition, a significant level of dATPase activity was detected when both DnaB and DnaC were incubated with naked ssDNA suggesting that nucleotide turnover may also be involved in the mobilization of DnaB from the complex with DnaC.[2]

The primosome has now been redefined in terms of the components present in a stable protein-DNA complex sufficient to allow replication by primase and DNA polymerase III holoenzyme. Isolated as a complex by gel filtration, the primosome contains the PriA, PriB, and DnaB proteins. These proteins were identified by their characteristic nucleotide hydrolysis activities (Table VI) as well as by their activity in primosome assembly (Fig. 5). The presence of both DnaB and PriA has also been suggested by the finding that the primosome can move in both the 5′ to 3′ and 3′ to 5′ directions powered by one or the other of these two helicases (32). PriC, DnaC, and DnaT were required for formation of the primosome yet were not stably associated with it. Other methods of investigation or undiscovered factors might yield a primosome that contains components in addition to those observed in this study. The isolated primosome was competent to allow priming and replication of the complementary strand, yet this finding does not exclude the action of PriC, DnaC, and DnaT in another aspect replication. In fact, such a possibility has been realized in rolling-circle replication, which extends beyond the amount of input template (33).

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REFERENCES

1. Schekman, R., Weiner, J., Weiner, A., and Kornberg, A. (1975) *J. Biol. Chem.* **250,** 5859–5865
2. Wickner, S., and Hurwitz, J. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 4120–4124
3. Shlomai, J., and Kornberg, A. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 799–803
4. Arai, K.-I., and Kornberg, A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 69–73
5. Arai, K.-I., and Kornberg, A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 69–73
6. Kornberg, A., and Baker, T. (1992) *DNA Replication*, 2nd Ed., W. H. Freeman and Co., New York
7. Marians, K. J. (1992) *Annu. Rev. Biochem.* **61,** 673–719
8. Masai, H., and Arai, K.-I. (1989) *J. Bacteriol.* **171,** 2975–2980
9. Minden, J., and Marians, K. J. (1985) *J. Biol. Chem.* **260,** 9316–9325
10. Wechsler, J. A., and Gross, J. D. (1971) *Mol. Gen. Genet.* **113,** 273–284
11. Lark, K. G., and Lark, C. A. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 537–549
12. Lark, C. A., Riazi, J., and Lark, K. G. (1978) *J. Bacteriol.* **136,** 1008–1017
13. Kaguni, J. M., and Kornberg, A. (1984) *Cell* **38,** 183–190
14. Lee, E. H., Masai, H., Allen, G. C., Jr., and Kornberg, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4620–4624
15. Nurse, P., DiGate, R. J., Zavitz, K. H., and Marians, K. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4615–4619
16. Allen, G. C., Jr., and Kornberg, A. (1991) *J. Biol. Chem.* **266,** 11610–11613
17. Zavitz, K. H., DiGate, M. J., and Marians, K. J. (1991) *J. Biol. Chem.* **266,** 13988–13995
18. Nurse, P., Zavitz, K. H., and Marians, K. J. (1991) *J. Bacteriol.* **173,** 6686–6693
19. Lee, E. H., and Kornberg, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3029–3032
20. Arai, K.-I., Low, R. Kobori, J., Shlomai, J., and Kornberg, A. (1981) *J. Biol. Chem.* **256,** 5273–5280
21. Stamford, N. P. J., Lilley, P. E., and Dixon, N. E. (1992) *Biochim. Biophys. Acta* **1132,** 17–25
22. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326
23. Shlomai, J., and Kornberg, A. (1980) *J. Biol. Chem.* **255,** 6794–6798
24. Low, R. L., Shlomai, J., and Kornberg, A. (1982) *J. Biol. Chem.* **257,** 6242–6250
25. Weiner, J. H., McMacken, R., and Kornberg, A. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 752–756
26. Lee, M. S., and Marians, K. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8345–8349
27. Lasken, R., and Kornberg, A. (1988) *J. Biol. Chem.* **263,** 5512–5518
28. Wickner, S., and Hurwitz, J. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 921–925
29. Kobori, J., and Kornberg, A. (1982) *J. Biol. Chem.* **257,** 13770–13775
30. Wahle, E., Lasken, R. S., and Kornberg, A. (1989) *J. Biol. Chem.* **264,** 2463–2468
31. Arai, K.-I., and Kornberg, A. (1981) *J. Biol. Chem.* **256,** 5252–5259
32. Zavitz, K. H., and Marians, K. J. (1992) *J. Biol. Chem.* **267,** 6933–6940
33. Lee, M. S., and Marians, K. J. (1989) *J. Biol. Chem.* **264,** 14531–14542
34. Allen, G. C., Jr., Dixon, N. E., and Kornberg, A. (1993) *Cell* **74,** 713–722