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Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: Localized unwinding of duplex DNA by a six-protein reaction

(initiation of DNA replication/DNA–protein interaction/electron microscopy)

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ABSTRACT **The O protein of bacteriophage λ localizes the initiation of DNA replication to a unique site on the λ genome, *ori*λ. By means of electron microscopy, we infer that the binding of O to *ori*λ initiates a series of protein addition and transfer reactions that culminate in localized unwinding of the origin DNA, generating a prepriming structure for the initiation of DNA replication. We can define three stages to this prepriming reaction, the first two of which we have characterized previously. First, dimeric O protein binds to multiple DNA binding sites and self-associates to form a nucleoprotein structure, the O-some. Second, λ P and host DnaB proteins interact with the O-some to generate a larger complex that includes additional DNA from an A+T-rich region adjacent to the O binding sites. Third, the addition of the DnaJ, DnaK, and Ssb proteins and ATP results in an origin-specific unwinding reaction, probably catalyzed by the helicase activity of DnaB. The unwinding reaction is unidirectional, proceeding "rightward" from the origin. The minimal DNA sequence competent for unwinding consists of two O binding sites and the adjacent A+T-rich region to the right of the binding sites. We conclude that the λ O protein localizes and initiates a six-protein sequential reaction responsible for but preceding the precise initiation of DNA replication. Specialized nucleoprotein structures similar to the O-some may be a general feature of DNA transactions requiring extraordinary precision in localization and control.**

Bacteriophage λ initiates DNA replication at a single replication origin, *ori*λ (1–3). The DNA sequence of the *ori*λ region has two major characteristics: four direct repeats of 18 base pairs (bp), each of which is an inverted repeat; and an adjacent region to the right of the repeats that is extremely rich in A+T (4–6). Two λ proteins, O and P, are required for viral DNA replication (7–9). The O protein recognizes the origin, and P localizes the essential replication enzymes of *Escherichia coli* at this site by initiating a series of protein–protein interactions (3). The O protein binds to the four repeats (10, 11), probably recognizing each local inverted repeat as a dimer (ref. 12; unpublished work). P interacts with O and with *E. coli* DnaB protein (12–18). Localized initiation of DNA replication depends on these interactions and on the host DnaJ and DnaK proteins (18–24). Recently, replication systems dependent on these purified proteins have been developed (ref. 24; unpublished work). In a reaction with purified O, P, DnaB, DnaJ, DnaK, and single-strand DNA-binding protein (Ssb), a DNA–protein complex is formed that is competent to initiate DNA replication after the addition of DnaG primase and DNA polymerase III holoenzyme (ref. 24; unpublished work). A knowledge of the pathway to this "prepriming" complex is clearly central to understanding localized initiation of DNA replication.

Recent electron microscopic experiments have indicated that the formation and modification of specialized nucleoprotein structures at *ori*λ are responsible for localizing the initiation of DNA replication (25). We can infer three stages for the prepriming reaction (25). First, dimeric O protein binds to the direct repeats and self-associates to form a specific nucleoprotein structure, the O-some. Second, P and DnaB proteins interact with the O-some to generate a larger complex that includes additional DNA from the adjacent A+T-rich region. Third, DnaJ and DnaK provide for transition of the second-stage structure into a form competent for localized activity of the DnaG primase and DNA polymerase III holoenzyme (ref. 24; unpublished data).

In this study, we show that the third stage of the prepriming reaction yields origin-specific unwinding of duplex DNA, presumably catalyzed by the helicase activity of DnaB (26). We infer that a locally unwound DNA structure associated with DnaB becomes the specific substrate for localized priming by DnaG. Thus, the O protein builds a nucleoprotein structure at the replication origin of λ and initiates a series of protein assembly events culminating in the localized initiation of DNA replication. The DnaA protein of *E. coli* appears to act in a similar fashion to localize the initiation of bacterial DNA replication to the single site, *oriC* (27, 28). Below, we compare the two pathways and consider the possible general use of specialized nucleoprotein structures in high-precision DNA transactions.

MATERIALS AND METHODS

Proteins. Highly purified proteins, all of which were 90% or greater in purity, were used; their specific activities were as follows: λ O protein, 1×10^5 units/mg (29); λ P protein, 2.5×10^5 units/mg (18); DnaB protein, 2.2×10^5 units/mg (30); Ssb protein, 4×10^4 units/mg (ref. 31; unpublished data); DnaJ protein, 4×10^5 units/mg (32); DnaK protein, 4.1×10^3 units/mg (ref. 33; unpublished data); GyrA protein subunit, 4.4×10^5 units/mg (34); GyrB protein subunit, 3.5×10^4 units/mg (34). One unit of replication activity catalyzes the incorporation of 1 pmol of deoxyribonucleotides per min into trichloroacetic acid-insoluble material under standard assay conditions (18, 30, 32, 33). Restriction enzymes were from New England Biolabs.

DNA. The structure and preparation of plasmid pRLM4, which contains the replication origin of phage λ, have been

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Abbreviations: bp, base pair(s); Ssb, single-strand DNA-binding protein.

described (25). A detailed description of the construction of M13mp8*ori*λ deletions will be published elsewhere. Briefly, a *Bgl* II–*Eco*RI restriction fragment containing the λ origin was cloned in either orientation into the *Sma* site of M13mp8 to yield the two recombinants, M13*ori*λleft and M13*ori*λright. Deletions extending into the region of *ori* containing the direct repeats or into the region containing the A+T-rich sequence were created by BAL-31 exonuclease digestion after cleavage by *Eco*RI of M13*ori*λleft or M13*ori*λright, respectively. The endpoints of the M13*ori*λleft deletions are at positions 39049, 39072, 39089, and 39101 on the λ map (35). The endpoints of deletions similarly constructed from M13*ori*λright are at positions 39160 and 39131 on the λ map. A schematic diagram of these constructions is provided in Table 1 of the *Results* section. The supercoiled DNAs used in the binding reactions were purified by extraction with phenol and passage over Sepharose 4B, followed by two cycles of CsCl density gradient centrifugation.

Binding of Proteins to DNA and Electron Microscopy. The reaction mixtures (20 μl) contained the following: Hepes/KOH at pH 7.6, 25 mM; magnesium acetate, 7 mM; KCl, 50 mM; NaCl, 30 mM; ATP, 2 mM; supercoiled pRLM4 or M13*ori*λ DNA, 215 ng; λ O protein, 51 ng; λ P protein, 108 ng; DnaB protein, 103 ng; Ssb protein, 617 ng; DnaJ protein, 60 ng; DnaK protein, 5 μg. Some reaction mixtures (where noted) contained in addition: GyrA protein subunit, 34 ng; GyrB protein subunit, 200 ng. The protein concentrations and DNA-to-protein stoichiometry are sufficient for the initiation of *in vitro* replication (ref. 24; unpublished data). All reaction mixtures were assembled on ice. Mixtures were incubated for 10 min at 30°C, followed by the addition of glutaraldehyde to 0.1% and incubation for an additional 15 min at 30°C. The mixtures were then passed through a Sepharose 4B column (4 × 0.5 cm) that had been previously equilibrated with column buffer (Hepes/KOH at pH 7.6, 40 mM; magnesium acetate, 11 mM). The peak fraction was diluted 1:4 in column buffer (final DNA concentration, ≈1 μg/ml), and electron microscopy was carried out by the polylysine technique of Williams (36). Samples that were to be cut with *Nsi* I restriction enzyme after binding of protein to DNA were fixed in glutaraldehyde as above and passed through a Sepharose 4B column that had previously been equilibrated with *Nsi* I restriction buffer. Peak fractions were pooled (50 μl) and treated with 5 units of *Nsi* I restriction enzyme for 15 min at 37°C. The reactants were then passed again over a Sepharose 4B column and examined by electron microscopy as above without further dilution.

RESULTS

DNA Unwinding by a Six-Protein Reaction. Previous experiments have characterized the first two stages of the prepriming reaction at *ori*λ. First, O protein associates with *ori*λ DNA to generate a nucleoprotein structure containing all of the four binding sites for dimeric O protein, the O-some (25) (Fig. 1*a*). Second, the P and DnaB proteins interact with the O-some in an ATP-independent reaction, yielding a larger nucleoprotein complex including the binding region for O and more of the adjacent A+T-rich region (25) (Fig. 1*b*). As noted above, correctly initiated DNA replication requires in addition the DnaJ, DnaK, and Ssb proteins, which can be presumed to play a role in the generation of the third stage prepriming complex (ref. 24; unpublished work). To investigate the function of these proteins, we have studied in detail the six-protein reaction at *ori*λ involving O, P, DnaB, DnaJ, DnaK, and Ssb.

The addition of all six proteins to supercoiled plasmid DNA carrying *ori*λ produced nucleoprotein structures similar to those found with O, P, and DnaB alone (data not shown). However, if ATP was also added, a notably different nucleoprotein structure was observed (Fig. 1 *c, e, f, g*). The duplex was locally unwound to yield single strands, apparently coated by Ssb. Typically, 20–30% of the substrate DNA molecules exhibited ring-like structures similar to those shown in Fig. 1; the average unwound DNA segment was about 800 bp (see below). At least 60% of the unwound structures had a large knob at one end (as in Fig. 1 *e, f, g*). Omission of any of the six proteins or ATP abolished the unwinding reaction. For unwinding to occur, the minimum molar ratio of monomer O protein to *ori*λ DNA was 20:1; the standard reaction employed 30:1.

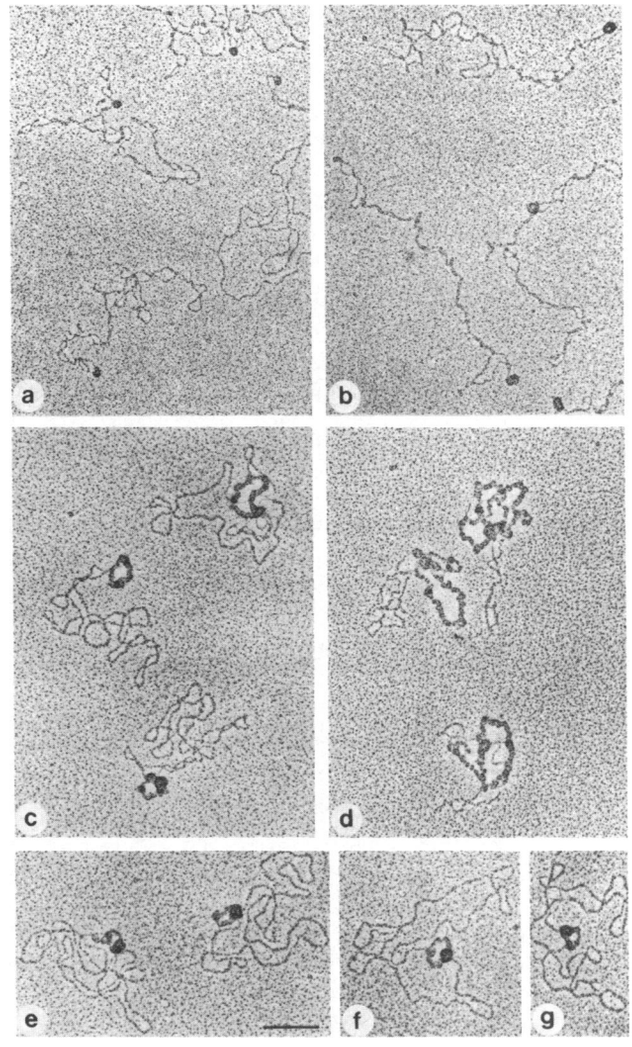

FIG. 1. Electron micrographs of nucleoprotein structures at *ori*λ. (*a*) O protein alone and *ori*λ plasmid DNA. (*b*) *ori*λ DNA with O, P, and DnaB proteins. (*c*) *ori*λ DNA with O, P, DnaB, Ssb, DnaJ, and DnaK proteins and ATP. (*d*) *ori*λ DNA with O, P, DnaB, Ssb, DnaJ, DnaK, and DNA gyrase proteins and ATP. (*e–g*) *ori*λ DNA and the six-protein reaction of *c*. (Bar = 0.1 μm.)

The role of the individual proteins in the unwinding reaction is of course not revealed by the microscopy. Because DnaB is known to possess helicase activity (26), we conclude that the ATP-dependent DNA unwinding is probably carried out by DnaB. We presume that DnaJ and DnaK release DnaB from the O/P/DnaB/*ori*λ structure to allow DnaB to function as a helicase. The Ssb protein facilitates the reaction and stabilizes the product by binding to the single-stranded DNA produced by the unwinding reaction. We presume that the early stage of the DnaB-mediated unwinding reaction generates an appropriate substrate for the DnaG primase and DNA polymerase III holoenzyme, thus localizing the initiation of DNA replication.

Role of Supercoiling in Initiating and Limiting the Unwinding Reaction. The unwinding reaction is absolutely dependent on a supercoiled *ori*λ substrate; no unwinding was observed with plasmid DNA linearized with a restriction enzyme. The formation of O/P/DnaB/*ori* structures is favored by a supercoiled substrate (2.5-fold) (25). Thus, a major component of the superhelicity requirement for unwinding is attributable to formation of the appropriate precursor structure. There may be also an additional boost to release of DnaB from the precursor structure provided by negatively superhelical DNA.

With the standard negatively superhelical substrate, the unwinding reaction is limited in extent (Fig. 1 *c, e, f, g*). The limiting factor might be the energetic barrier generated by positive supercoiling of the residual duplex DNA (the negative supercoils will be removed after about 400 bp of unwinding). If so, the unwinding reaction should proceed farther in the presence of DNA gyrase. After the addition of DNA gyrase to the standard six-protein reaction mixture, much more extensive unwinding was typically observed; about ⅔ of the unwound DNA molecules exhibited the extensively unwound structures shown in Fig. 1*d*. The fraction of the total DNA molecules unwound also increased from 30% to nearly 50%.

Unidirectional Unwinding from *ori*λ. To examine the specificity and the direction of the six-protein unwinding reaction, we cleaved the DNA with the restriction enzyme *Nsi* I, which cuts asymmetrically with respect to *ori*λ (Fig. 3*c*). The distance from the DNA ends to the edge of the nucleoprotein structure was measured for two types of molecules: the structures formed with no visibly unwound region (Fig. 2*a* and Fig. 3*a*) and the partially unwound molecules (Fig. 2*b* and Fig. 3*b*). The data of Fig. 3*a* localize a structure that appears to be identical to the O/P/DnaB/*ori* structure analyzed previously (25) (Fig. 3*c*). The data of Fig. 3*b* show that the unwinding reaction proceeds rightward from the origin but not leftward. Addition of DNA gyrase to the reaction mixtures did not induce bidirectional unwinding (Fig. 2 *c, d, e* and unpublished data). Thus, the data of Fig. 3 suggest that the prepriming event that localizes the initiation of DNA syn-

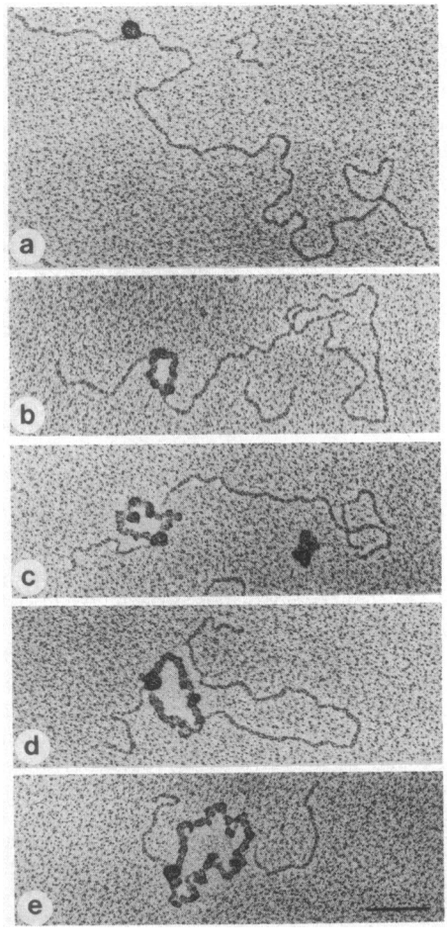

FIG. 2. Electron micrographs of nucleoprotein structures generated on supercoiled *ori*λ DNA, followed by cleavage by *Nsi* I. The *ori*λ plasmid DNA was incubated with O, P, DnaB, Ssb, DnaJ, and DnaK proteins and ATP, and then cut with *Nsi* I restriction enzyme. (*a*) Presumed *ori*λ/O/P/DnaB complex. (*b*) Partially unwound nucleoprotein complex formed by the six-protein unwinding reaction. (*c–e*) More extensively unwound nucleoprotein structures formed by the six-protein unwinding reaction in the presence of DNA gyrase. (Bar = 0.1 μm.)

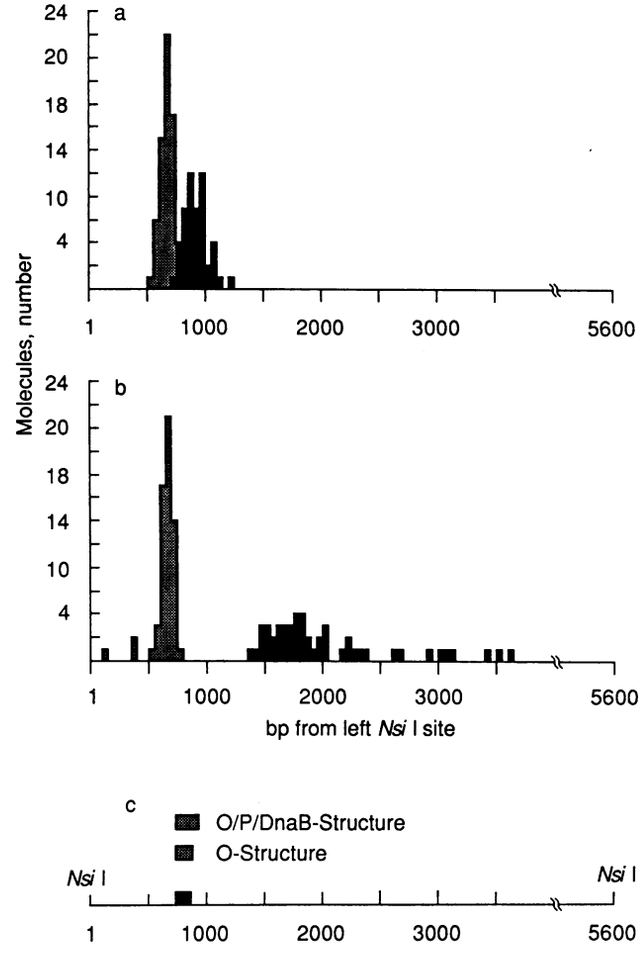

FIG. 3. Length distribution of the unwound DNA with respect to *ori*λ. After the six-protein unwinding reaction, the nucleoprotein complexes were cleaved with *Nsi* I to produce a 5600-bp fragment with *ori*λ and a 538-bp fragment. Molecules similar to those shown in Fig. 2*a* or 2*b* were photographed, and the distances from the DNA ends to the edge of the rotary-shadowed complex were measured from the projected electron micrographs. The shorter DNA tails extend from the left side of *ori*λ; the long tails extend from the right side of *ori*λ. The distances from the free ends to the edge of the complex are plotted relative to distance along the 5600-bp fragment. The position of the 18-bp repeats of *ori*λ is indicated by the black rectangle in *c*. The stippled bars represent distance from the shorter (left) end; the solid bars represent distance from the longer (right) end (a few molecules are not explicitly shown because solid and stippled overlap). The smaller, 538-bp-long, *Nsi* I restriction fragment of the plasmid DNA served as an internal standard. (*a*) Presumed *ori*λ/O/P/DnaB complex. (*b*) Unwound nucleoprotein complex. (*c*) Comparison of the DNA regions contained in the O-some and the *ori*/O/P/DnaB structure (from ref. 25).

Table 1. Effect of deletions in *ori*λ on the formation of unwound nucleoprotein complexes

Region of *ori*λ remaining intact		Unwound structures,
18-bp repeats	A+T-rich	%
▬▬▬▬	———	18
▬▬▬▬	———	15
▬▬▬	———	6
▬▬	———	0
▬	———	0
▬▬▬▬	———	20
▬▬▬▬	———	24
▬▬▬▬	———	0

Binding assays for each M13*ori*λ construct were performed as described in *Materials and Methods*. The constructs from top to bottom are: M13*ori*λleft, M13*ori*λΔ39049, M13*ori*λΔ39072, M13*ori*λΔ-39089, M13*ori*λΔ39101, M13*ori*λright, M13*ori*λΔ39160, and M13*ori*λΔ-39131. At least 500 DNA molecules were counted for each construct.

thesis is an origin-specific unwinding reaction proceeding rightward from the origin.

DNA replication by phage λ *in vivo* usually proceeds bidirectionally, although there is a substantial fraction of molecules exhibiting unidirectional rightward replication (1, 3, 37). Bidirectional molecules also show a rightward bias (37). Although there is not complete agreement between *in vivo* and *in vitro* data, the overall picture is consistent with an early rightward unwinding event that usually leads to subsequent bidirectional replication.

Minimal Sequence Requirements for the Unwinding Reaction. To investigate the limits of *ori*λ required for the unwinding reaction, we analyzed nucleoprotein structures formed on a series of plasmid DNAs containing deletions into the O-binding region from the left and into the A+T-rich region from the right (Table 1). The six-protein unwinding reaction was markedly reduced if only the two right-side 18-bp repeats remained and was totally blocked for less than two (Table 1, lines 1–5). A deletion of most of the A+T-rich region also completely blocked the unwinding reaction (Table 1, lines 6–8). These unwinding data are in close quantitative agreement with DNA replication assays performed on the same DNA substrates (unpublished results). We conclude that the minimum *ori*λ sequence competent for origin-specific unwinding consists of the two right-side binding sites for dimeric O protein plus a major portion of the A+T-rich region.

DISCUSSION

Prepriming Pathway for λ. For phage λ, we have inferred a three-stage prepriming pathway that localizes the initiation of DNA replication to a single origin selected from the 5×10^4 bp of the viral DNA. Our proposed pathway depends on the formation and modification of specialized nucleoprotein structures, as depicted schematically in Fig. 4.

In the initial stage, dimeric O protein binds to the four 18-bp direct repeats and generates a localized nucleoprotein complex in which the DNA is wound or bent (11, 25, 38). There are two plausible mechanisms for the assembly of the O-some: (*i*) self-association of only the DNA-bound O molecules (eight monomers); (*ii*) nucleation by the DNA-bound O molecules of a larger O protein assembly, in which other O molecules add solely by protein–protein interactions. On the basis of the size of the nucleoprotein structure, we have previously estimated a rough molecular weight of ≈500,000, which places eight O monomers (M_r = 34,000) at the low end, even allowing for DNA (25). There is some additional suggestive evidence to favor slightly the second possibility: (*i*) the minimal molar ratio of O protein to *ori* DNA competent for substantial unwinding is 20:1; (*ii*) DNA carrying only two of the repeats is capable of some (though reduced) unwinding; (*iii*) DNA carrying only two repeats can form an O-some with appearance similar to the structure formed on the DNA with four repeats (unpublished work).

The accurate recognition of the initiation site provided by the O-some must be converted into specific reactivity. We believe that the crucial step is the localization of DnaB in a state competent for helicase activity. In the second stage of the prepriming pathway, P and DnaB are added to the O-some through protein association reactions (Fig. 4). The larger nucleoprotein complex includes additional DNA from the A+T-rich region and is highly favored by a superhelical substrate. Thus, the initial target for DnaB may be this A+T-rich region in a strained state favorable for unwinding. However, DnaB is probably initially in an inactive conformation because of its tight association with P (16–18).

In the third stage of the prepriming pathway, we presume that DnaJ and DnaK act to free DnaB, thus allowing the initiation of an origin-specific unwinding reaction, stabilized by Ssb (Fig. 4). The locally unwound structure containing the DnaB helicase is now competent to add the DnaG primase and polymerase III holoenzyme. Thus, the O protein locates precisely the replication origin and initiates an ordered series of protein–protein interactions culminating in the localized initiation of DNA replication. The route to bidirectional replication remains to be established. Other cellular protein(s) or the process of DNA replication or transcription might be required to free another DnaB for leftward unwinding. A unidirectional rightward replication fork that collides with an undissociated O-some (after a round of DNA syn-

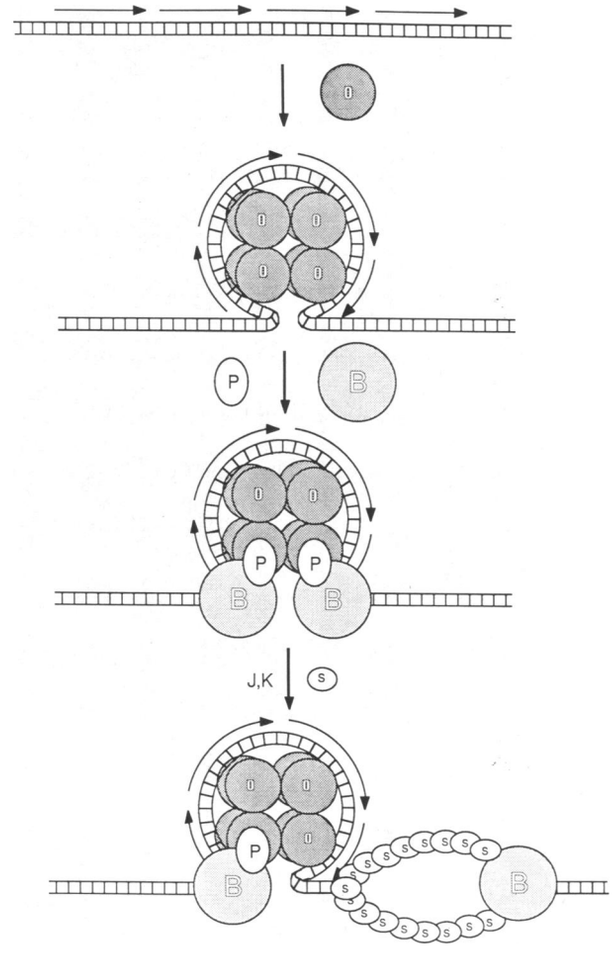

FIG. 4. Proposed prepriming pathway for localized initiation of DNA replication by phage λ. See text for details. Reprinted with permission (42).

thesis) might in fact be the event that initiates the late, rolling-circle form of λ DNA replication.

Comparison of the Prepriming Reactions for λ and *E. coli*. There appears to be a striking similarity between the prepriming reaction at *ori*λ and the sequence of events responsible for localized initiation of DNA replication at the *E. coli* replication origin, *oriC*. In the first stage of the *E. coli* prepriming reaction, DnaA protein locates the initiation region by binding to four DNA sites and generating a nucleoprotein structure containing 240 bp of *ori*C DNA and 20–30 molecules of DnaA (27). With the addition of DnaC and DnaB, a larger, second-stage nucleoprotein complex is produced (B. E. Funnell, T. A. Baker, and A. Kornberg, personal communication). In the presence of Ssb and DNA gyrase, an extensive ATP-dependent unwinding reaction occurs, presumably a result of helicase activity by DnaB (28). A more limited unwinding reaction is found in the absence of DNA gyrase (T. A. Baker, B. E. Funnell, and A. Kornberg, personal communication). Thus, *E. coli* also carries out a three-stage prepriming pathway resulting in localized unwinding of duplex DNA by the helicase activity of DnaB. The major functional difference appears to be the capacity of DnaC to localize DnaB at *oriC* in an active conformation, obviating the need for the additional *ori*λ reaction involving DnaJ and and DnaK. The role of DnaJ and DnaK for *E. coli* is unclear. DnaJ and DnaK are "heat shock" proteins (19, 22); possibly these two proteins function in other protein disassembly reactions crucial for stress responses.

Role of Specialized Nucleoprotein Structures ("Snups") in High-Precision DNA Transactions. For the λ and *E. coli* replication systems, there is strong evidence that specialized nucleoprotein structures are used to locate and activate the initiation sites for chromosomal DNA replication. For site-specific recombination by phage λ, specialized nucleoprotein structures are also used to localize and control the directionality of the recombination reaction (39–41). These DNA transactions have in common the need for very high precision in site location and regulation. For example, the *E. coli* origin must be selected from 5×10^6 bp and used once per cell generation. The use of multiple specific binding interactions and a higher-order nucleoprotein structure can ensure a precisely defined site and highly directed reactivity. Thus, specialized nucleoprotein structures (snups) may be a general mechanism for conferring very high precision on DNA transactions in which even a rare error in localization and control is intolerable (40–42). This point of view is encouraged by the fact that multiple DNA–protein interactions are widely found at other origins of DNA replication in prokaryotes and eukaryotes and in additional examples of site-specific recombination (reviewed in refs. 41 and 42). The multiple DNA–protein interactions responsible for localization and control of eukaryotic transcription may be another example of snups (41, 42).

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