The role of template superhelicity in the initiation of bacteriophage λ DNA replication

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ABSTRACT

The prepriming steps in the initiation of bacteriophage λ DNA replication depend on the action of the λ O and P proteins and on the DnaB helicase, single-stranded DNA binding protein (SSB), and DnaJ and DnaK heat shock proteins of the *E. coli* host. The binding of multiple copies of the λ O protein to the phage replication origin (*ori λ*) initiates the ordered assembly of a series of nucleoprotein structures that form at *ori λ* prior to DNA unwinding, priming and DNA synthesis steps. Since the initiation of λ DNA replication is known to occur only on supercoiled templates *in vivo* and *in vitro*, we examined how the early steps in λ DNA replication are influenced by superhelical tension. All initiation complexes formed prior to helicase-mediated DNA-unwinding form with high efficiency on relaxed *ori λ* DNA. Nonetheless, the DNA templates in these structures must be negatively super-twisted before they can be replicated. Once DNA helicase unwinding is initiated at *ori λ* , however, later steps in λ DNA replication proceed efficiently in the absence of superhelical tension. We conclude that supercoiling is required during the initiation of λ DNA replication to facilitate entry of a DNA helicase, presumably the DnaB protein, between the DNA strands.

INTRODUCTION

Studies of bacteriophage λ DNA replication *in vivo* and *in vitro* have yielded new insights into the fundamental biochemical mechanisms involved in the initiation of chromosomal DNA replication and have aided our understanding of the molecular events involved in the viral recruitment of cellular replication machinery. Two phage encoded proteins, the products of the λ O and P genes, join with multiple *Escherichia coli* proteins to bring about replication of the λ genome. The required host-encoded proteins include a group of proteins that participate in propagation of replication forks along the bacterial chromosome and a set of proteins that are known to function in the *E. coli* heat shock response (for a review of λ DNA replication see reference 1). Crude soluble systems that support the initiation of λ DNA replication have been developed (2-4) and recently we have established this reaction with a mixture of 9 purified proteins (5; K. Mensa-Wilmoth *et al.*, manuscript submitted for publication).

Mechanistic studies of the initiation of λ DNA replication *in vitro* indicate that a series of nucleoprotein structures are assembled at the λ origin (*ori λ*) prior to priming and DNA synthesis (6-10). This assembly process is triggered by the binding of multiple copies of the λ O initiator to

ori λ , which contains an array of O protein recognition sites (Fig. 1). Electron microscopic analysis reveals the presence at *ori* λ of an "O-some" (6), a nucleosome-like structure in which the origin DNA is bent (11) or wrapped in a specific configuration (6). The O-some provides a foundation for the assembly of more complex nucleoprotein structures. The λ P protein interacts with the *E. coli* DnaB helicase to form a P-DnaB complex (12-14), which in turn binds to the O-some (6). In the presence of ATP, the *E. coli* DnaJ and DnaK heat shock proteins subsequently function to activate the latent helicase activity of the DnaB protein in the *ori* λ :O-P-DnaB complex, culminating in the localized unwinding of 500 to 1000 base pairs of duplex DNA adjacent to the λ origin (7). This nucleoprotein "unwound complex" apparently directs additional protein interactions that ultimately result in priming and DNA synthesis.

At least one or more of these steps must depend on the presence of superhelical tension in the template DNA, since the initiation of λ DNA replication both *in vivo* (15) and *in vitro* (3) occurs only on negatively supercoiled DNA templates. Recently, Schnos *et al.* (16) have demonstrated that the A/T-rich region on the right side of *ori* λ (Fig. 1) undergoes a conformational change upon formation of the O-some. This alteration, which is presumed to be a limited unwinding of the DNA duplex in the A/T-rich region, requires both O protein and supercoiled DNA. Deletion analysis indicates that the A/T-rich region of *ori* λ is required both for DNA helicase-mediated unwinding of *ori* λ plasmid DNA to generate the unwound complex (7) and for initiation of λ DNA replication in the crude *in vitro* system (17). In view of the fact that A/T-rich segments are essential structural elements of replication origins from diverse prokaryotic and eukaryotic chromosomes, e.g., *E. coli* (18,19), *Bacillus subtilis* (20), bacteriophage T7 (21), yeast (22-24) simian virus 40 (25) and adenovirus (26,27), it is widely believed that these A/T-rich motifs may facilitate opening of the DNA duplex to permit the entry of proteins that participate in the propagation of replication forks.

The investigation reported here was designed to examine the influence of template superhelicity on the various steps involved in the initiation of bacteriophage λ DNA replication. Our data demonstrate that superhelical tension is not required for the efficient assembly of any of the prepriming complexes that are initially formed at *ori* λ . These complexes include the O-some, the O-P-DnaB complex and a newly identified 6-protein complex containing O, P, DnaB, DnaJ, DnaK and SSB. Supercoil tension is, however, required in order to initiate DNA helicase-mediated unwinding of an *ori* λ template. Later steps in λ DNA replication can proceed efficiently in the absence of superhelical tension.

MATERIALS AND METHODS

Materials

Sources of materials were: unlabeled ribonucleotides and deoxyribonucleotides, P-L Biochemicals; adenylyl-imidodiphosphate (AMP-PNP), Boehringer Mannheim; Hepes, Schwarz/Mann; bovine serum albumin (BSA), Miles; Bio-Gel A-15m agarose (100-200 mesh), Bio-Rad; n-octylglucoside, Sigma; EM-grade glutaraldehyde, Polysciences; Bicine, Calbiochem; nitrocellulose, Schleicher and Schuell; En^hance, DuPont; nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates, and alkaline phosphatase-conjugated

Figure 1. Structures of *oriλ* plasmid pRLM4 and the λ replication origin. Plasmid pRLM4 (3), a λ dv-like plasmid that specifies resistance to kanamycin, contains single *EcoRI* and *BamHI* restriction sites. An enlarged diagram of the λ origin region is presented on the right. The 19 bp O protein recognition sites (iterons) are represented as large cross-hatched arrows. The smaller solid arrows indicate partial dyad symmetry. A 40 bp A/T-rich region is located in the right segment of the λ origin.

goat anti-rabbit gamma globulin, Promega Biotech; [methyl- ^3H]dITP (67 Ci/mmole) and [methyl- ^3H]thymidine (73 Ci/mmole), ICN.

Enzymes, Proteins and Antibodies

Calf thymus topoisomerase I was a gift of Dr. Leroy F. Liu (Johns Hopkins University School of Medicine, Baltimore, Maryland). *BamHI* and *EcoRI* were from New England Biolabs. Anti-DnaG protein gamma globulin was a gift of Dr. Arthur Kornberg (Stanford University School of Medicine, Stanford, California). The purification and specific activities of λ O and P proteins and *E. coli* replication proteins SSB, DnaB, DnaJ, DnaK, primase (DnaG protein), and DNA polymerase III holoenzyme have been described previously (7, 28). *E. coli* DNA gyrase subunits A and B were purified by a modification of a published procedure (29). Specific rabbit immunoglobulin preparations were prepared as described (30-32).

DNA Substrates

The λ dv-like plasmid, pRLM4, which contains the replication origin of bacteriophage λ , has been described (3). A diagram of this *oriλ* plasmid, with relevant restriction sites, is shown in Figure 1. Relaxed, covalently closed (Form IV) pRLM4 DNA was prepared by treatment of negatively supercoiled (Form I) pRLM4 DNA with calf thymus DNA topoisomerase I. pRLM4 DNA (100 μg) was incubated with DNA topoisomerase I (8 μg) in a buffer (750 μl) composed of: 40 mM Tris/HCl, pH 7.5, 100 mM potassium chloride, 10 mM magnesium chloride, 0.5 mM dithiothreitol, 0.5 mM EDTA, and BSA (30 $\mu\text{g}/\text{ml}$). The mixture was incubated for 60 min at 37°C, followed by 5 min at 30°C, and the reaction was terminated by addition of sodium dodecyl sulfate to 0.5 %. The sample was extracted twice with phenol, twice with isobutanol, and once with ether. The relaxed DNA was precipitated with isopropanol and resuspended in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA. Linear (Form III) DNA was prepared by digestion of pRLM4 plasmid DNA at its *BamHI* site, and was purified as described for relaxed DNA. pRLM4 DNA labeled with tritium was made by addition of 25 mCi of [methyl- ^3H]thymidine to *E. coli* RIM439 (3) growing exponentially (0.5×10^8 cells/ml) at 30°C in one liter of M9 medium (33) containing deoxyadenosine (250 $\mu\text{g}/\text{ml}$). Cells were harvested five hours later and plasmid DNA was

purified according to standard procedures (33). The final specific radioactivity of purified plasmid DNA was 58,000 cpm/ μ g.

Assays for DNA Replication

The standard replication reaction was modified from that used in the crude soluble *in vitro* replication system (3). A standard mixture (30 μ l) contained the following components: Hepes/KOH, pH 7.6, 40 mM; magnesium acetate, 11 mM; ATP, 4 mM; dATP, dCTP, and dGTP, each 160 μ M; dTTP, 60 μ M, with [methyl-³H]dTTP at 100–150 cpm per pmol of total deoxynucleotide; pRIM4 DNA (negatively supercoiled, unless stated otherwise), 215 ng; λ O protein, 130 ng; λ P protein, 120 ng; SSB, 710 ng; DnaB, 175 ng; DnaJ, 60 ng; DnaK, 3.7 μ g; primase, 220 ng; DNA polymerase III holoenzyme, 310 ng; DNA gyrase A subunit, 230 ng; DNA gyrase B subunit, 240 ng. Reaction mixtures were assembled at 0°C and incubated for 30 min at 30°C, unless stated otherwise. DNA synthesis (total dNMP) was measured by determining the level of incorporation of labeled deoxynucleotide into acid-insoluble material, which was collected on glass fiber filters (Whatman 934-AH) and counted in a liquid scintillation spectrometer.

Assays of filtered nucleoprotein complexes for replication activity were carried out as follows. A first stage reaction mixture, containing HM buffer (40 mM Hepes/KOH, 11 mM magnesium acetate) with 4 mM ATP plus 7.5-fold amounts (see replication assay) of DNA and replication proteins, was assembled in 37.5 μ l and incubated for 10 min at 30°C. A 7.5 μ l portion was reserved, and 30 μ l was applied at room temperature to a 1 ml Bio-Gel A-15m column equilibrated with HM buffer containing 4 mM ATP and 0.0075% n-octylglucoside. The void volume (120 μ l), containing plasmid DNA and associated proteins, was collected. Recovery of DNA was typically 90%. Portions of the filtered and unfiltered first-stage incubation mixtures (20 μ l and 5 μ l, respectively), containing approximately a standard assay amount of plasmid DNA and associated proteins, were next supplemented with each component of the standard replication reaction that had been omitted from the first-stage incubation. These complete reaction mixtures were then incubated and processed as in the standard replication reaction.

In certain experiments nucleoprotein structures were formed on supercoiled DNA and the effect of subsequent linearization of the DNA on the replicative potential of the complex was assessed. First-stage reaction mixtures (18 μ l), containing negatively supercoiled *ori* λ DNA (215 ng), and standard replication assay amounts of nucleotides and replication proteins (as specified), were assembled in duplicate in HM buffer containing 50 μ g/ml BSA. Each mixture was incubated for 20 min at 30°C and then supplemented with 2 μ l of 100 mM dithiothreitol/500 mM sodium chloride. *Bam*HI (5 units) was added to one of each duplicate pair of mixtures and all mixtures were incubated further for 15 min at 30°C. Each mixture was then supplemented with all components of the standard replication reaction which were not present in the first-stage incubation, and the mixtures were subsequently incubated and processed as described for a standard assay.

Immunoblot for Detection of Nucleoprotein Complexes

The specific details of the immunoblotting procedure will be published elsewhere (Alfano and McMacken, in preparation). Briefly, 6-fold amounts (see replication assay description above) of *ori* λ DNA and specified replication proteins, were assembled in HM buffer (29 μ l, final volume) and nucleotides (as indicated). Each mixture was incubated for 10 min at 30°C. Glu-

Table 1. Replication of Various Topological Forms of *ori* λ Plasmid DNA

	Template	DNA gyrase	synthesis (pmol)
1	Linear	-	3
2	Linear	+	5
3	Relaxed, covalently closed	-	4
4	Relaxed, covalently closed	+	503
5	Negatively supercoiled	-	84
6	Negatively supercoiled	+	536

Various forms of pRIM4 DNA were made as described in Materials and Methods and were assayed using the standard replication reaction (650 pmol of template DNA). DNA gyrase was omitted where indicated. Each value represents the average of three determinations.

taraldehyde was added to 0.1% final concentration and incubation was continued for 15 min at 30°C. The 30 μ l sample was then filtered through a 1 ml Bio-Gel A-15m agarose column equilibrated with HM buffer and 0.0075% n-octylglucoside to remove glutaraldehyde and proteins not associated with DNA. The void volume (120 μ l) was divided into 6 equal portions, each of which was applied to a separate agarose gel (0.8% agarose, 40 mM Bicine/KOH, pH 8.35), and electrophoresed for 3.5 hours at 4 V/cm. Nucleoprotein complexes were electrophoretically transferred from the gels to nitrocellulose membranes in a Hoefer Transphor Electrophoresis Unit for 3 hours at 0.5 A. Each of the replicate nitrocellulose blots was incubated with a rabbit antibody preparation specific for a particular replication protein, followed by treatment with alkaline phosphatase-conjugated anti-rabbit secondary antibody and color development.

Other Procedures

In some experiments nucleoprotein structures were formed on ^{3} H-labeled pRIM4 plasmid DNA. The positions of DNA bands, following electrophoresis of the complexes in agarose gels, were identified using fluorography with En^3 -hance. Autoradiography was performed using Kodak X-Omat AR film and a DuPont Cronex Lightning-Plus intensifying screen.

RESULTS

Replication of an *ori* λ Plasmid Requires a Supercoiled Template

We have recently developed a system, composed of nine purified λ and *E. coli* proteins, that specifically initiates and carries out a single round of DNA replication of an *ori* λ plasmid, yielding nicked-circular plasmid daughter molecules (K. Mensa-Wilmot *et al.*, manuscript submitted for publication). Replication of plasmid DNA in this system requires the introduction of negative supercoils into the template DNA (Table 1). In the absence of DNA gyrase, both linear and relaxed circular forms of *ori* λ DNA are inert as templates. Although initiation of λ DNA replication does not depend on the presence of DNA gyrase when the template DNA is negatively supercoiled (Table 1, line 5), DNA synthesis ceases prematurely in this case. Apparently, in the absence of a DNA topoisomerase, further fork propagation is blocked as a

Figure 2. Effect of DNA topology on the assembly of prepriming and unwound complexes at *oriλ*. A mixture containing SSB, O, P, DnaB, DnaJ, DnaK and primase was incubated with various topological forms of pRLM4 DNA as follows: negatively supercoiled DNA, lanes 1 and 2; relaxed, covalently closed circular DNA, lanes 3 and 4; linear DNA, lanes 5 and 6. ATP was added to lanes 2, 4, and 6 to a final concentration of 4 mM. Nucleoprotein complexes were formed, isolated, and characterized as described in Materials and Methods. The panel labeled "DNA" is a photograph of a gel stained with ethidium bromide. All other panels are nitrocellulose blots which were incubated with antibody preparations that are specific for the indicated replication protein. The bracket labeled U indicates the region where the unwound complex migrates. The various topological forms of template used are labeled in the DNA panel. Negatively supercoiled plasmid DNA also contains small amounts of nicked circle, linear, and dimer forms. The symbols used are: R, relaxed, covalently closed circular DNA; L, linear DNA; SC, negatively supercoiled DNA. Other symbols are referred to in the text.

consequence of the accumulation of positive supercoils in the unreplicated portion of the closed circular template (unpublished data). If the replication system is supplemented with DNA gyrase, only those forms of *oriλ* DNA that can be enzymatically converted to a negatively supercoiled form serve as effective templates for the initiation and propagation of *λ* DNA replication (Table 1).

Supercoiling Is Not Required for Assembly of Prepriming Complexes

The finding that initiation of *λ* DNA replication is blocked when relaxed *oriλ* DNA is used as the template in the *in vitro* system indicates that at least one step in the initiation reaction depends on supercoiled DNA. Previous studies have demonstrated that a series of nucleoprotein structures are assembled at *oriλ* prior to the initiation of DNA synthesis. We used an immunoblotting technique to determine if supercoiling influences the formation of these structures (Fig. 2). In this procedure, nucleoprotein structures formed at *oriλ* were stabilized by glutaraldehyde cross-linking and were subsequently resolved by electrophoresis in agarose. The replication proteins present in each structure were electrophoretically transferred to nitrocellulose and identified by their reaction with specific antibodies.

If the λ O and P proteins and the *E. coli* DnaB, DnaJ, DnaK, SSB and primase proteins are incubated with *ori* λ plasmid DNA in the absence of ATP, nucleoprotein structures containing all seven proteins are formed (Fig. 2) on supercoiled (band x, lane 1), relaxed circular (lane 3) or linear (lane 5) forms of the template. The formation of these complex structures depends on the capacity of the λ O protein to specifically recognize and bind to *ori* λ . If O protein is omitted from the incubation mixtures, no detectable nucleoprotein structures are formed (9; Alfano and McMacken, manuscript in preparation). With the relaxed circular and linear templates, little or no change in the electrophoretic mobilities of the nucleoprotein structures is observed when ATP is included in the incubation mixture. However, significantly reduced amounts of P, DnaB, DnaJ and primase remain associated with the structure. This ATP-dependent disassembly of nucleoprotein structures formed at *ori* λ is catalyzed by the DnaJ and DnaK heat shock proteins and will be discussed in depth elsewhere (Alfano and McMacken, manuscript in preparation; Dodson, McMacken and Echols, manuscript in preparation).

If the *ori* λ template was supertwisted, at least three new nucleoprotein species were generated when ATP was included in the incubation mixture with the replication proteins (Fig. 2, lane 2). A significant portion of each added protein was present in a broad band (labeled "U" in the SSB blot of Fig. 2) that migrated just ahead of the nucleoprotein complex formed on linear DNA (lane 5). This new nucleoprotein species formed on supercoiled DNA is almost certainly identical to the unwound structure, first detected by electron microscopy (7), that is formed near *ori* λ under similar conditions. Its diagnostic features are its enhanced reactivity with antibodies directed against SSB and its strongly reduced electrophoretic mobility. Densitometric analysis indicated that the ratio of SSB to DNA increased at least 2.5-fold in this species, presumably a reflection of the binding of SSB to the 500 to 1000 bp of single-stranded DNA present in each unwound template molecule. The presence or absence of primase (either with or without CTP, GTP and UTP) did not affect the level of unwound complex formed, in sharp contrast to an absolute requirement for the presence of each of the other six proteins (7).

The remaining two new nucleoprotein structures formed on a supercoiled template in the presence of ATP migrate slightly slower or slightly faster (Fig. 2, lane 2, bands y and z, respectively) than the structure formed in the absence of ATP (Fig. 2, lane 1, band x). All seven replication proteins were associated with the DNA in band y, whereas only O, SSB and DnaK were detected in band z. Two of the replication proteins present in the 7 protein mixture, DnaB and DnaK (31,34,35), contain intrinsic ATPase activity. Either or both could contribute to the ATP-dependent modifications of nucleoprotein structures reported here (see below).

We investigated if the nucleoprotein structures formed on nonsupercoiled DNA were functional by determining the replicative potential of various complexes. As discussed above (Table 1), relaxed circular *ori* λ DNA can be converted into an active template in the presence of DNA gyrase, whereas linear DNA remains inert. Thus, if a complex which is assembled on a relaxed template is potentially active for replication, then, after removal of free protein by gel filtration, it should be possible to reactivate the isolated complex for replication by supertwisting its DNA with DNA gyrase. Alternatively, if incomplete or incorrect structures are formed at *ori* λ on a relaxed template, then one should observe a requirement for DNA gyrase action prior to the removal of free protein by gel filtration. The results (Table 2) in-

Table 2. Efficiencies of Assembly of Initiation Complexes on Various Topological Forms of *oriλ* Plasmid DNA

First Stage	Assay Sample	Form I DNA		Form IV DNA	
		Synthesis (pmol)	Ratio ¹ (a/b)	Synthesis (pmol)	Ratio ¹ (a/b)
1	O	338	0.96	299	0.90
	O	352		331	
2	O,P,B	197	0.56	225	0.79
	O,P,B	349		286	
3	O,P,B,gyr	166	0.54	175	0.78
	O,P,B,gyr	310		224	
4	O,P,B,J	123	0.76	162	0.80
	O,P,B,J	148		202	
5	O,P,B,J,gyr	133	0.78	128	0.68
	O,P,B,J,gyr	171		188	

Reactions were carried out as described in Materials and Methods for assays of filtered complexes. All first-stage reactions contained pRIM4 DNA, ATP (4 mM) and SSB in addition to the proteins indicated. Each filtered reaction mixture (indicated as "F") or unfiltered reaction mixture was supplemented with the proteins indicated in the table as well as with all components of the standard λ replication assay that were not present in the first-stage mixture. Form I is negatively supercoiled DNA and Form IV is relaxed, covalently closed circular DNA. Protein abbreviations used: O, λ O protein; P, λ P protein; B, DnaB; J, DnaJ; gyr, DNA gyrase.

¹"Ratio" is an expression of the efficiency of formation of a particular nucleoprotein complex. It is the amount of filtered template which can be replicated in the absence of further addition of a particular protein (or group of proteins) divided by the amount of replication obtained when that protein is also added during the replication stage.

indicate that O protein forms an active complex on each supercoiled and relaxed *oriλ* DNA with an efficiency of at least 90% (see ratio columns for experiment 1). Likewise, the conversion of an O-some to an *oriλ*:O-P-DnaB structure and the conversion of the latter complex to an *oriλ*:O-P-DnaB-DnaJ structure is just as efficient on a relaxed template as on a supercoiled template (Table 2, experiments 2 and 4). These assembly reactions are not stimulated by the presence of DNA gyrase (experiments 3 and 5). Neither DnaK nor primase remains stably associated with nucleoprotein complexes during gel filtration and such complexes, therefore, could not be analyzed for their replicative potential.

We conclude from the results presented in Fig. 2 and Table 2 that superhelical tension does not play a role in the assembly of prepriming nucleoprotein structures at the λ origin. Nevertheless, we cannot rule out the possibility that some rearrangement of these structures, dependent on supercoiling, is required before the complexes function in DNA replication.

ATP-Dependent Formation of Prepriming Structures on Supercoiled *oriλ* DNA

As assayed by agarose gel electrophoresis and immunoblotting, three dif-

Figure 3. Nucleotide and protein requirements for the assembly of prepriming and unwound complexes at *oriλ*. All samples contained negatively supercoiled pRIM4 DNA and proteins SSB, O, P and DnaB. DnaJ and DnaK proteins and nucleotides were added as indicated. Each nucleotide was present at a final concentration of 4 mM. The single nitrocellulose blot presented was incubated with antibody specific for DnaB. The panel labeled "DNA" is a photograph of a gel stained with ethidium bromide. The bracket labeled "U" indicates the position of the unwound complex formed on monomeric supercoiled plasmid DNA. The second bracket labeled "UD" indicates the position of a similar complex formed on a supercoiled *oriλ* plasmid dimer.

ferent nucleoprotein structures are formed when supercoiled *oriλ* DNA is incubated with ATP and the seven proteins required for the prepriming and priming stages of λ DNA replication (Fig. 2, bands U, y, and z). Two of these structures (Fig. 2, bands U and y) contain each of the seven proteins. The slower migrating of these structures constitutes the unwound prepriming complex (7), but the other structure (structure y) has not previously been reported.

In order to further characterize structure y and contrast its properties with those of the unwound complex, we have used the immunoblotting approach utilized in Fig. 2 to determine the protein and nucleotide requirements for the formation of these large nucleoprotein structures on supercoiled DNA templates (Fig. 3). Primase was not included in this experiment, since structures U and y are both assembled with equal efficiency in the absence of this enzyme (Fig. 3, lane 5). Furthermore, only the DnaB immunoblot is presented in this analysis, since antibodies against DnaB reveal the presence of structure y but do not detect the proteins of the *oriλ*:SSB-O-DnaK species (structure z), which is only marginally resolved from structure y. Neither the unwound complex nor structure y is formed if DnaJ, DnaK or ATP is omitted from the reaction mixture. GTP, but not ADP or AMP-PNP, could substitute for ATP as a nucleotide cofactor in the formation of both of these nucleoprotein complexes.

The substitution of dTTP for ATP was especially enlightening. In the presence of dTTP the production of the unwound complex, as measured by densitometry of ethidium bromide stained-DNA species, was reduced four fold relative to the amount made in the presence of ATP. Moreover, the length of unwound DNA formed in the presence of dTTP was quite limited, judging from the relatively high mobility of those nucleoprotein structures that contained DnaB protein (Fig. 3, lane 7). This result is consistent with our previous finding that dTTP is a poor effector of the helicase activity of DnaB (36). In contrast, dTTP could support the conversion of a high proportion of the initiation complex formed on superhelical template DNA into species y. While

it is evident that nucleotide hydrolysis is required for the formation of both structure y and the unwound complex, it was not possible to determine with certainty if the required hydrolysis is performed by DnaB protein, DnaK protein, or both. The observed nucleotide specificities for complex formation closely parallel the known specificities of each DnaB and DnaK (36-38). It is notable, however, that DnaK, but not DnaB, can hydrolyze ADP (38). Yet, the presence of this nucleotide did not permit formation of the various rearranged nucleoprotein structures.

Replication of Linear *ori* λ Template DNA

As demonstrated above, the linear form of *ori* λ DNA is not a functional template for λ DNA replication *in vitro*. Nor can linear DNA be reactivated by addition of DNA gyrase to the replication system. We have utilized these facts to perform an experiment that defines the last step in the initiation of λ DNA replication that depends on superhelical tension. Various prepriming nucleoprotein complexes were formed in a first-stage reaction on supercoiled *ori* λ DNA and the template in each structure was subsequently linearized by enzymatic cleavage at a site outside of the origin region. Each reaction mixture was then supplemented with all components of the replication reaction that had been omitted from the first stage. The amount of replication of the linearized DNA was determined and compared to the amount of replication obtained in an equivalent reaction in which the template was left intact (Table 3). The requirement of superhelical tension for λ DNA replication is not bypassed by assembling any of those prepriming nucleoprotein structures that are formed prior to activation of the DnaB helicase and the ensuing generation of the unwound complex. These early structures include the O-some, the *ori* λ :O-P-DnaB complex (6), the *ori* λ :O-P-DnaB-DnaJ structure (Table 3, lines 3-5, respectively) and various complexes that contain both O and DnaK, which have been detected by immunoblot or electron microscopic analysis (Alfano and McMacken, manuscript in preparation; Dodson, McMacken and Echols, manuscript in preparation), such as *ori* λ :O-DnaK-SSB and *ori* λ :O-P-

Table 3. Replication of Linearized *ori* λ Plasmid DNA

	<u>Proteins in first stage</u>	<u>Replication Potential¹</u> (%)
1	None	0
2	SSB	1
3	SSB, O	1
4	SSB, O, P, DnaB	1
5	SSB, O, P, DnaB, DnaJ	1
6	SSB, O, DnaK	1
7	SSB, O, P, DnaB, DnaK	1
8	SSB, O, P, DnaB, DnaJ, DnaK	31

Reactions were carried out as described in Materials and Methods for replication of linearized DNA. All first stage reactions contained negatively supercoiled pRIM4 DNA and ATP (4 mM). Proteins were added as indicated.

¹Replication Potential is expressed as the percentage of replication on a linearized template as compared to that on undigested negatively supercoiled template. Typically, 275 pmol of DNA synthesis was obtained if the DNA was not linearized. Each value represents the average of at least three determinations.

DnaB-DnaK-SSB structures.

Only when supercoiled *oriλ* DNA is incubated in the presence of ATP with all six prepriming proteins can a nucleoprotein intermediate be formed that permits DNA replication to be initiated in the absence of superhelical tension (Table 3, line 8). The available data suggest that this active intermediate is the unwound complex. Electron microscopic analysis of the products of this reaction detected only full-length linear *oriλ* plasmid DNA. Thus, the percentage of replication defines the percentage of active complexes formed. This number is in good agreement with the percentage of template DNA molecules that are converted into unwound molecules in the six protein reaction (7).

Formation of the active intermediate requires hydrolysis of a nucleoside triphosphate (Table 4). As found for the formation of the unwound complex, dTTP proved to be a relatively weak substitute for ATP. The presence of primase during the assembly of the active complex produced a two-fold increase in the amount of DNA synthesis obtained on linearized DNA (Table 4, line 7). The stimulation engendered by primase, however, depended on the presence of all 4 ribonucleoside triphosphates. This suggests that RNA primers synthesized by primase are responsible for the sharp increase in the replication of linearized DNA. We present the following explanation of these results. The unwound structure formed when the six prepriming proteins are incubated in the presence of ATP with supercoiled DNA contains active DnaB helicase. Unwinding of the supercoiled *oriλ* template is limited in the absence of topoisomerase action to approximately 1000 bp (7), due to the presence of positive supercoils which accumulate as helicase unwinding proceeds. Linearization of the template DNA at this point eliminates this obstruction and the DnaB helicase completes unwinding the template DNA, perhaps even running off

Table 4. Effect of Nucleotides and RNA Priming in the Replication of Linearized DNA

First Stage		Replication Potential ¹ (%)
Proteins	Nucleotides	
1 Prepriming mixture ²	None	1
2 Prepriming mixture	ATP	31
3 Prepriming mixture	ATP + rNTPs	34
4 Prepriming mixture	rNTPs	37
5 Prepriming mixture + primase	None	3
6 Prepriming mixture + primase	ATP	40
7 Prepriming mixture + primase	ATP + rNTPs	86
8 Prepriming mixture + primase	AMP-PNP	1
9 Prepriming mixture + primase	dTTP	12

Reactions were carried out as described in Materials and Methods and Table 3. ATP and dTTP were added to a final concentration of 4 mM. rNTPs is CTP, GTP, and UTP; each was added to a final concentration of 0.5 mM. AMP-PNP was added to a final concentration of 1 mM. Each value represents the average of at least three determinations.

¹See Table 3 for definition.

²Prepriming mixture contains negatively supercoiled pRLM4 DNA, SSB, O, P, DnaB, DnaJ, and DnaK.

the ends of a portion of the molecules. Only those linearized unwound molecules that still contain bound DnaB protein are primed by primase and converted to duplex DNA. In contrast, if RNA priming is permitted during DNA unwinding, all unwound molecules, whether or not they contain DnaB protein, are replicated.

The data reported here confirm that six proteins, nucleotide hydrolysis and superhelical tension are the minimal requirements for the formation of a nucleoprotein complex whose replicative potential is insensitive to linearization of the template DNA. Precisely the same array of proteins and nucleotide cofactors participate in the formation of the structure γ and unwound nucleoprotein complexes (Fig. 2). The exact nature of the structure γ complex is uncertain, but it apparently represents a rearranged form of the *ori λ* :SSB-O-P-DnaB-DnaJ-DnaK initiation complex. This rearrangement could arise from an ATP-dependent conformational change, such as insertion of the DnaB helicase between the DNA strands at the origin. An alternative possibility is that the rearrangement represents the binding of additional protein molecules to the origin initiation complex when ATP hydrolysis is permitted.

Our discovery that the type of nucleotide present in the *in vitro* system influenced the relative amounts of structure γ and the unwound complex formed (Fig. 3) made it possible to compare the efficiency of formation of these structures with the level of replication obtained when the *ori λ* DNA templates of these structures were cleaved. This type of analysis indicated that the amount of replication obtained on linearized DNA was closely correlated with the amount of unwound complex formed prior to digestion of the template. There was no correlation between the amount of structure γ formed and the level of replication of linearized DNA. For example, under conditions where 31% of the starting supercoiled template DNA was replicated following linearization (Table 4, line 2), analysis by densitometry indicated that approximately 30% of the supercoiled template had been converted into an unwound complex prior to linearization, whereas about 45% of the input DNA had been converted to the structure γ form (Fig. 3, lane 5). If dTTP was substituted for ATP in this reaction, about 90% of the supercoiled *ori λ* was converted into structure γ , with the remainder transformed into unwound complex. The reduced efficiency of forming the unwound complex in the presence of dTTP correlates well with the low efficiency of replication of a linearized template in this situation (Table 4, line 9).

Initiation of DNA Unwinding at *ori λ* Depends on λ O Protein and SSB

It has recently been discovered that the conformation of the A/T-rich region on the right side of *ori λ* (Fig. 1) is altered upon binding of the O protein initiator to its recognition sites in the origin (16). This conformational change was detected by the increased sensitivity of the A/T-rich region to single-strand specific nucleases and was shown to depend on superhelical tension in the template DNA. This alteration, which is presumed to be a localized unwinding of the DNA helix, extends even beyond the A/T-rich region on a small percentage of the template molecules, as evidenced by O protein induced protection of the *EcoRI* site, located 49 bp from the nearest O recognition site (Fig. 1).

We have used a similar assay to characterize the protein and superhelicity requirements for localized unwinding under standard conditions for the initiation of λ DNA replication. In this experiment nucleoprotein structures assembled on supercoiled or linear *ori λ* plasmid DNA (both with and without

Figure 4. A localized unwinding reaction induced by O protein binding to *oriλ* requires a supercoiled template and SSB. Nucleoprotein complexes were formed as described in Materials and Methods with the following modifications. Proteins were incubated with either negatively supercoiled, ³H-labeled pRLM4 DNA (FI) or linear ³H-labeled pRLM4 DNA (FIII) for 10 min at 30°C. Glutaraldehyde was added to samples where indicated, and incubation was continued for 15 min at 30°C. After filtration, dithiothreitol, sodium chloride, and BSA were added to the void volume samples to final concentrations of 10 mM, 50 mM, and 100 μg/ml, respectively. *Eco*RI (60 units) was added to the samples applied to lanes 3-11, and all mixtures were incubated for 60 min at 30°C. *Bam*HI (75 units) was then added to all samples, and incubation was continued for 5 min at 30°C. Samples were then electrophoresed and processed as described in Materials and Methods. The panel labeled DNA is an autoradiogram of a gel enhanced by fluorography. The other panels are nitrocellulose blots incubated with antibodies raised against either SSB or O. The lengths of DNA species were estimated from the mobilities of unlabeled linear standards visualized by ethidium bromide staining.

stabilization by glutaraldehyde cross-linking) were digested to completion with both *Eco*RI and *Bam*HI to determine the level of protection of the *Eco*RI site. Digestion of protein-free *oriλ* plasmid with both *Eco*RI and *Bam*HI yields a 3.6 kb fragment bearing *oriλ* and a 2.6 kb fragment (Figure 4, lane 3). An O-SSB nucleoprotein complex is formed at *oriλ* on both supercoiled and linear templates (Fig. 4, lanes 5 and 9, respectively, of O and SSB immunoblots). However, only on the supercoiled template does the formation of this complex impede complete cleavage of the *Eco*RI site, as demonstrated by the presence of a small quantity of a nucleoprotein complex (Fig 4, lane 5) that comigrates with an *oriλ*:O-SSB complex present on full-length linear DNA (Fig. 4, lane 2). If O protein, SSB or glutaraldehyde cross-linking was omitted, no protection of the *Eco*RI site was detected.

Two additional features of this experiment are notable. First, the presence of SSB in a nucleoprotein complex depended on the presence of λ O protein, regardless of the conformation of the template DNA (Fig. 4, lanes 5, 6,

9 and 10). This suggests that the assembly of SSB into an *ori* λ :O-SSB complex depends on direct interactions between the two proteins and does not arise simply from SSB-coating of an unwound single-stranded region generated by O protein binding. Second, in the presence of the O initiator and glutaraldehyde, a new nucleoprotein species was formed (band D in Fig. 4, lanes 4, 5, 8 and 9). This complex migrated slightly slower than the full-length *ori* λ DNA containing bound O protein and SSB. Our analysis indicates that this species contains two 3.6 kb *Eco*RI - *Bam*HI *ori* λ fragments that are joined together via intermolecular cross-links between individual O-somes. Interactions between O protein molecules bound to separate λ origins have also been detected by electron microscopy (16). Using probes specific for the 3.6 kb and 2.6 kb *Eco*RI - *Bam*HI fragments in a Southern blotting analysis, we have confirmed that band D (Fig. 4) contains only sequences from the 3.6 kb *ori* λ fragment. As expected, the 6.15 kb band in lane 5 hybridizes to both probes, confirming that it is a full-length linear fragment of the *ori* λ plasmid template (data not shown).

DISCUSSION

In order to define the requirement for superhelical tension in λ DNA replication, we have investigated which of the early steps in the *in vitro* initiation pathway depend on negative superhelicity. These early steps include an ordered series of protein assembly and disassembly events that occur at *ori* λ prior to priming of DNA replication. Binding of multiple copies of the λ O protein to *ori* λ to form the O-some (6) not only initiates formation of these nucleoprotein structures, but this interaction also serves to localize subsequent assembly events to the origin, since the O-some establishes the foundation for the assembly of additional replication proteins. The λ P protein, the other λ -encoded replication protein, forms a tight complex with the host DnaB helicase (12-14), thereby enhancing the affinity of the P protein for the carboxy terminal domains of the O protein exposed in the O-some (10) (Alfano and McMacken, manuscript in preparation; Dodson, McMacken and Echols, manuscript in preparation). Interaction of the P-DnaB complex with the O-some generates an *ori* λ :O-P-DnaB complex (6), completing delivery of this key bacterial replicative helicase to the λ origin. Both DnaJ and DnaK can bind to the *ori* λ :O-P-DnaB complex to form an *ori* λ :O-P-DnaB-DnaJ-DnaK initiation complex (Fig. 2; Alfano and McMacken, manuscript in preparation; Dodson, McMacken and Echols, manuscript in preparation). This structure is poised to initiate unwinding of the template in preparation for priming and DNA synthesis.

We have demonstrated in this report that each of these complexes can readily assemble on linear and relaxed circular templates. Furthermore, we have shown that the O-some, O-P-DnaB and O-P-DnaB-DnaJ structures are assembled on a relaxed template with comparable efficiencies and activities as the equivalent complexes formed on a supercoiled template (Table 2). Thus, superhelical tension is not required for the assembly of any of the prepriming complexes discussed above.

It is evident from both immunoblotting (this paper) and direct visualization by electron microscopy (6) that the first step in the initiation pathway, formation of the O-some, proceeds with high efficiency on linear fragments containing the λ origin of replication. Footprinting (39,40) and electron microscopic studies indicate that the O-some encompasses the four iter-

ons, but does not include the adjacent A/T-rich region (Fig. 1). Binding of O induces an ATP-independent unwinding of this A/T-rich segment, as inferred from the enhanced sensitivity of the region to single-strand specific nucleases and from partial protection of the EcoRI site at the right border of the AT-rich region (16) (Fig. 4). This unwinding reaction is the first step in the initiation of λ DNA replication that is strictly dependent on the presence of superhelical tension. It is possible that a portion of the free energy of binding and bending the origin DNA is harnessed by the O-some and is utilized, together with the free energy available from negative superhelical tension, to drive separation of the DNA strands in the adjacent A/T-rich region (11,16).

SSB is able to bind to the template only after the O-some has formed. This interaction is not dependent on supercoiling (Fig. 4). Since O binding to a linear template does not induce unwinding of the A/T-rich segment in *ori λ* (16) (Fig. 4), we infer that SSB binding to an O-some formed on supercoiled DNA requires direct O-SSB protein interactions and is not simply due to coating of single-stranded DNA in the unwound A/T-rich region. Schnos *et al.* (16) demonstrated that binding of O protein alone to *ori λ* elicited some protection of the nearby EcoRI site, provided that the putative single-stranded character of the unwound region was preserved with formaldehyde treatment. In contrast, when we utilized the EcoRI protection assay for O protein-induced DNA unwinding, performed under the conditions of the standard λ replication reaction, we discovered that protection of the EcoRI site depended also on the presence of SSB. Unlike formaldehyde, the glutaraldehyde used in our assay apparently was incapable of fixing the unwound A/T-rich DNA in the single-stranded state. Instead, it seems likely that SSB, following an initial protein-protein interaction with the O-some, also binds to and stabilizes the unwound DNA generated when O binds to a supercoiled *ori λ* template. In our view glutaraldehyde cross-linking of the SSB to the unwound DNA is required to maintain the DNA in an unwound configuration. Taken together, these results indicate that torsional strain functions at an early stage in the initiation of λ DNA replication to lower the energy required to destabilize the helix adjacent to the site of assembly of the prepriming initiation complex.

Formation of the *ori λ* :O-SSB structure, however, does not satisfy the requirement for a supercoiled template for the initiation of λ DNA replication, as judged by the inability of a linearized template containing an O-SSB structure to be replicated in the *in vitro* system (Table 3). Following O binding, an *ori λ* :O-P-DnaB-DnaJ-DnaK initiation complex is ultimately formed. Assembly of this sizable complex does not require either ATP hydrolysis or template superhelicity. In the absence of nucleoside triphosphate hydrolysis, the O-P-DnaB-DnaJ-DnaK-SSB nucleoprotein complex formed at *ori λ* is apparently inert, whether or not the template DNA is negatively supercoiled. If nucleotide hydrolysis is permitted, however, several events occur, as monitored by immunoblot analysis of the prepriming nucleoprotein structures (Fig. 2). These events include (i) formation of an unwound complex (7) in which 500 to 1000 bp of the duplex DNA to the right of *ori λ* is unwound; (ii) formation of a second nucleoprotein structure (structure y), containing all six prepriming proteins (band y, Fig. 2); and (iii) disassembly of nucleoprotein structures. The protein disassembly reactions are catalyzed by the *E. coli* DnaK heat shock protein and will be discussed in detail elsewhere

(Alfano and McMacken, manuscript in preparation; Dodson, McMacken and Echols, manuscript in preparation). Of these three events, however, only the protein disassembly reactions can proceed in the absence of superhelical tension (Fig. 2).

Both the unwound complex and structure γ depend on nucleoside triphosphate hydrolysis and superhelical tension for their formation in the complete six-protein prepriming reaction (Fig. 3). After the formation of these two structures, later steps in the λ replication pathway can proceed in the absence of superhelical tension. Since the amount of replication of linearized DNA observed correlates well with the amount of unwound complex present at the time of linearization, but does not correlate with the amount of structure γ present, we infer that the DNA in structure γ can not be replicated in the absence of superhelical tension. Moreover, our data suggest that the formation of the unwound complex is the final step in the initiation of λ DNA replication that requires a supercoiled template. Once DnaB successfully initiates the enzymatic unwinding of the duplex DNA near the λ origin, the superhelical tension of the template can be completely eliminated and subsequent priming and DNA synthesis steps in the replication pathway proceed normally (Tables 3 and 4).

We have tentatively concluded that structure γ is an intermediate in either the protein disassembly reaction or in the formation of the unwound complex or both. One simple possibility is that structure γ represents a protein rearrangement of the *ori* λ :O-P-DnaB-DnaJ-DnaK-SSB initiation complex such that the DnaB helicase has been inserted between the strands at *ori* λ . It seems likely that the binding of DnaB protein to the unwound DNA formed in the A/T-rich region may depend on removal of a fraction of the six λ P protein molecules that are believed to be bound to each DnaB hexamer that is present in the initiation complex (14). This suggestion is based on the finding that the capacity of the *E. coli* DnaB protein to bind to single-stranded DNA is suppressed when it is in a protein complex with λ P protein (Mallory and McMacken, manuscript in preparation). Removal of one or two P molecules from the initiation complex might permit a like number of DnaB subunits to bind to DNA. Yet, such DnaB molecules would not be capable of unwinding lengthy stretches of duplex DNA by helicase action, since they would at this point in the initiation reaction still be tethered to the O-some through bridging interactions of additional P molecules bound to the other subunits of the DnaB hexamer. The minimal amounts of SSB present in structure γ (Fig. 2) confirms that the template DNA is not extensively unwound.

Based on these results and the findings of others, we suggest the following model for the role of superhelical tension in the initiation of λ DNA replication. The proposed scheme is depicted in Fig. 5. The unwound A/T-rich segment, induced by O and SSB binding to *ori* λ on a negatively supercoiled template, is maintained after P, DnaB, DnaJ, and DnaK proteins are added to form the initiation complex. When ATP hydrolysis is permitted, two reactions occur (represented by the circled numbers in Fig. 5). DnaB, positioned near the denatured A/T-rich region (6), binds to the single-stranded DNA and is inserted between the strands. The helicase is now poised to unwind the template, but is held at the origin by contacts with other proteins of the initiation complex. The binding of the helicase to DNA, as discussed above, may depend on one or more steps of the second reaction, which is the DnaK

Figure 5. A model for the role of superhelical tension in the initiation of λ DNA replication. See text for details. The two steps dependent on superhelical tension are indicated by asterisks; the two steps requiring ATP hydrolysis are labeled with circled numbers. The altered conformation of the A/T-rich region induced by O protein binding is shown here as an unwound region. Only a single initiation complex is depicted here. However, in order for replication to proceed bidirectionally, a second initiation complex may need to be assembled to deliver a second DnaB helicase molecule that will create a fork that proceeds in the opposite direction. Proteins are symbolized by single shapes and letters for simplicity. The symbols used are: O, λ O protein; P, λ P protein; S, SSB; B, DnaB; J, DnaJ; K, DnaK.

protein-catalyzed disassembly of the initiation complex. In order for DnaB protein to remain stably inserted between the strands, superhelical tension must be maintained in the template DNA as the protein disassembly process proceeds. On approximately one-third of the supercoiled template molecules, at least one of the DnaB molecules contained in the initiation complex is liberated during the disassembly reactions, whereupon it proceeds to actively translocate away from the origin, unwinding the duplex template DNA as it moves. This active DnaB helicase is free to interact with primase and generate the primers needed for DNA synthesis. On relaxed or linearized DNA, the absence of superhelical tension precludes insertion of the DnaB helicase between the strands and the initiation complex is partially disassembled by the action of the heat shock proteins.

In order to initiate replication of a double-stranded DNA template, a mechanism must be developed to promote insertion of a helicase between the

strands. Both torsional strain and θ protein binding play pivotal roles in induction of unwinding of the A/T-rich sequences of *ori* λ , a critical step in the initiation of λ DNA replication (16). Localized unwinding of A/T-rich segments of chromosomal replication origins, detected by enhanced sensitivity to single-strand nucleases, has also been demonstrated at the replication origins of the *E. coli* (19) and yeast 2μ plasmid (41) chromosomes. Just as for λ , unwinding of A/T-rich DNA at *ori*C, the *E. coli* origin, depends both on superhelical tension in the template DNA and on the binding of multiple copies of an initiator protein (DnaA) to a set of repeating sequences present at the origin. This origin unwinding is believed to promote the entry of two DnaB helicase molecules between the strands, ultimately resulting in bidirectional unwinding of the template DNA (19,42). In contrast, nuclease sensitivity of the yeast 2μ origin is observed even in the absence of an initiator protein, provided that the template is negatively supercoiled. It is not known, however, if torsional stress plays a role in replication of the yeast 2μ plasmid or the yeast genome *in vivo*.

A/T-rich sequences are commonly associated with replication origins, both prokaryotic and eukaryotic, especially those that are used to initiate bidirectional DNA replication. We suggest that such A/T-rich sequences facilitate the entry of replication machinery, specifically DNA helicases, between the strands of origin DNA. There are indications, however, that it is not simply A/T-richness that is the critical structural feature of these segments (16,19,24,43). It is possible that the initiator protein interacts directly with sequences in the A/T-rich region to promote localized unwinding (D. Bramhill and A. Kornberg, personal communication). Nevertheless, direct interactions of θ protein or DnaA protein with the A/T-rich regions associated with *ori* λ or *ori*C have not been documented to date. On the other hand, it is possible that a particular DNA structure, other than single-stranded DNA, is created in the A/T-rich region by superhelical tension and that this structure is required for the initiation of DNA replication. It is interesting to note that sequences associated with static DNA bends (44-46) are generally present in the A/T-rich segments of replication origins (47).

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