Initiation of Bidirectional Replication at the Chromosomal Origin Is Directed by the Interaction between Helicase and Primase*

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Hiroshi Hiasa‡ and Kenneth J. Marians
From the Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Several protein-protein interactions have been shown to be critical for proper replication fork function in *Escherichia coli*. These include interactions between the polymerase and the helicase, the helicase and the primase, and the primase and the polymerase. We have studied the influence of these interactions on proper initiation at *oriC* by using mutant primases defective in their interaction with the helicase and DNA polymerase III holoenzyme lacking the τ subunit so that it will not interact with the helicase. We show here that accurate initiation of bidirectional DNA replication from *oriC* is dependent on proper placement of the primers for leading strand synthesis and is thus governed primarily by the interaction between the helicase and primase.

Chromosomal DNA replication in *Escherichia coli* initiates at a unique site, *oriC*, proceeds bidirectionally around the circular chromosome, and ends in the terminus region, 180° away from *oriC* (1, 2). Initiation and termination of DNA replication are tightly regulated to ensure coupling of chromosomal replication to the cell cycle (3).

Kornberg and co-workers have reconstituted *oriC* DNA replication *in vitro* with purified proteins and small plasmid templates, defining the molecular mechanisms of initiation of DNA replication (4–6). The initiator protein, DnaA, binds to four binding sites (DnaA boxes) forming a large nucleoprotein complex. This leads to the unwinding of an A+T-rich region at the origin. HU protein plays a critical role during this local unwinding step. The replicative helicase, DnaB, is introduced to the interaction between DnaA and DnaB (7). Subsequent additions of pol III holoenzyme (pol III HE),1 completes formation of the replication fork.

Key protein-protein interactions have also been shown to be necessary for proper replication fork propagation. An interaction between DnaB and the τ subunit of the pol III HE is required for rapid replication fork movement (8). The molecular basis of this step, however, was unclear. The high concentrations of primase involved implied the need to ensure occupancy of a binding site at a specified time, but binding could have been mediated by DnaB or a subunit of the HE (11).

In this report we have continued our investigation of this effect. The role of these protein-protein interactions during initiation at *oriC* was investigated by using mutant primases defective in their interaction with DnaB and HE lacking the τ subunit. We show that proper bidirectional initiation depended only on accurate placement of the primers for leading strand synthesis that, in turn, depended only on the interaction between DnaB and DnaG.

MATERIALS AND METHODS

Replication Proteins and Plasmid DNAs—*E. coli* DNA replication proteins have been described previously. The preparations of mutant primases were described by Tougu and Marians (16). The subunits of pol III were generous gifts of Charles McHenry (University of Colorado, Denver, CO), and pol III HE was reconstituted as described previously (8).

An *oriC* plasmid, pBROT535 type I, was prepared according to Marians et al. (17) with slight modifications. Briefly, pBROT535 type I DNA (14) was prepared from *E. coli* K38 tus::kan by the alkaline lysis procedure (18). The plasmid DNA was then banded in CsCl and then purified by sedimentation through 5–20% sucrose gradients containing 1 M NaCl. Form I DNA was dialyzed against TEN buffer extensively, concentrated by extraction with sec-butanol, dialyzed again, and then ethanol-precipitated.

*oriC* DNA Replication—Standard *oriC* DNA replication reaction mixtures (12.5 μl) were as described by Hiasa and Marians (14). Tus protein, mutant primases, and reconstituted pol III HEs (RHEs) were as indicated in the figure legends. Gel electrophoretic analyses of replication products were performed as described previously (15).

Pulse-Chase Analysis of Replication Products—Pulse-chase analysis of *oriC* DNA replication products was performed according to Hiasa et al. (19) except that pol III and DnaN were replaced with RHEs. Any other changes in reaction conditions are indicated in the figure legends.

RESULTS

The Interaction between Helicase and Primase Modulates the Mode of *oriC* DNA Replication—In our previous studies (14), we found that varying the primase concentration had a striking effect on the DNA products generated during *oriC* DNA replication *in vitro*. At low concentrations of primase, the dominate

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1 The abbreviations used are: pol III, *E. coli* DNA polymerase III; HE, holoenzyme; form II, nicked or gapped circular DNA molecule; RHE, reconstituted pol III HE; nt, nucleotide(s); kb, kilobase(s); SSB, single-stranded binding protein.

‡ Present address: Dept. of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455.

H. Hiasa and K. J. Marians, unpublished observations.
mode of replication was asymmetric, where synthesis of each strand was initiated at random positions both at and outside of oriC and synthesized continuously. This led to rolling circle DNA replication at longer incubation times. As a result, the majority of the products formed were either unit length or greater in size. In contrast, at high concentrations of primase, rolling circle type DNA replication was completely inhibited and the replication products appeared as two populations, one half unit length and the other centered about 400 nucleotides (nt). These populations were shown to be nascent leading and lagging strands, respectively. Under these conditions, DNA replication was initiated at and only at oriC and proceeded bidirectionally.

Primase-catalyzed primer synthesis during DNA replication in the presence of SSB requires DnaB (20). The enzyme does have a low intrinsic affinity for DNA that allows it to catalyze primer synthesis on naked single-stranded DNA, but this activity is one three-hundredth that observed in the presence of DnaB (12). In addition, it is completely inhibited in the presence of SSB (12). Thus, during oriC replication, access of primase to the DNA is directed by its interaction with DnaB.

To determine whether the effect of varying the primase concentration on the mode of oriC DNA replication was a result of the interaction between primase and DnaB, oriC DNA replication was reconstituted with purified replication proteins using either the wild-type or mutant primases, DnaG K580A and DnaG Q576A. In previous studies, we identified a C-terminal domain of primase that was required for functional interaction with DnaB (12). Subsequently, we defined the region on primase involved as the last 16 C-terminal amino acids (16). Mutant primases lacking this domain are unable to prime DNA synthesis in any DNA replication reaction requiring DnaB (13, 16). However, using the specialized bacteriophage G4 origin, where primase binds to a region of secondary structure on the phage DNA in the presence of SSB to synthesize a primer (21, 22), it could be demonstrated that the ability of the mutant proteins to catalyze oligoribonucleotide synthesis was completely unaffected (13, 16).

The K580A and Q576A mutant proteins contain amino acid substitutions that were constructed in the C-terminal interaction domain (13, 16). Using a rolling circle DNA replication system, where we showed previously that the size of Okazaki fragments was directed by the primase-DnaB interaction and varied inversely with primase concentration (23–25), DnaG K580A exhibits a slightly lower affinity for DnaB, whereas that of DnaG Q576A is severely reduced, as measured by the variation in Okazaki fragment size as a function of primase concentration (13). This has been confirmed by the demonstration that the strength of the physical interaction between primase and DnaB is reduced in the presence of the Q576A mutant primase (15).

Variation of the concentration of either the wild-type or DnaG K580A primase in the oriC replication system produced an identical pattern of DNA products (Fig. 1). On the other hand, about 15-fold higher concentrations of DnaG Q576A were required to observe similar patterns of DNA products (Fig. 1). In each case, however, the same shift in replication products from those characteristic of rolling circle replication to those characteristic of bidirectional replication was observed inversely correlated to primase concentration.

To confirm the mode of replication extent using high concentrations of the mutant primases, a direct test, which we had developed previously (14), was applied. This test utilizes a blocking template that carries oriC and two TerB sequences that are located 2 and 3 kb in opposite directions away from oriC, with roughly 1 kb of sequence between them. The TerB sequences in the blocking template are oriented so as to exclude passage of replication forks between them when they are bound by Tus. Thus, this template allows for a rapid determination of the mode of DNA replication. If DNA replication from oriC is bidirectional, denaturing gel analysis of the DNA products generated in the presence of Tus protein will show two distinct leading strands, 2 and 3 kb in length, and a population of small Okazaki fragments. In contrast, if DNA replication is continuous from random positions, the product analysis will show a maximum length of 5 kb with a smear of smaller products.

Replication reaction mixtures containing the blocking template were therefore incubated in the presence of Tus, and either low or high concentrations of both the wild-type and mutant primases and the DNA products were analyzed by electrophoresis through denaturing alkaline agarose gels (Fig. 2). As expected, at high concentrations of primases, two prominent bands corresponding to 2 and 3 kb and a discrete Okazaki fragment population were observed in all cases. On the other hand, one band corresponding to 5 kb with an associated smear of smaller products was observed in all cases when the primase concentration was low. In the absence of Tus, the pattern of DNA products observed are identical to the ones displayed in Fig. 1.

Thus, the same effect on the mode of DNA replication was observed for both the wild-type and mutant enzymes. In addition, the same qualitative dependence on primase concentration was also observed, although higher concentrations of DnaG Q576A were required compared with wild type. About 15-fold higher concentrations of DnaGQ576A were required to give the pattern typical of saturation of primase concentration in the reaction. This is identical to the shift in concentration of DnaGQ576A required to saturate the primase-directed variation in Okazaki fragment size during rolling circle DNA replication and thus parallels the reduction in affinity of the mutant primase for DnaB (13, 16). We therefore conclude that the mode of replication from oriC is directed by the primase-helicase interaction. The question that we addressed next was whether this reflected a requirement for the first leading strand primer to be placed close enough to DnaB that when it was bound to
markers were as in the legend to Fig. 1.

Replicative intermediate; II:II dimer synthesis in the 15-min reactions (as nucleotide) was as follows: lane 1, 153 pmol; lane 2, 177 pmol; lane 3, 114 pmol; lane 4, 185 pmol; lane 5, 106 pmol; lane 6, 178 pmol. Abbreviations and size markers were as in the legend to Fig. 1.

**Fig. 2.** The interaction between DnaB and DnaG modulates the mode of oriC DNA replication. DNA products generated in the presence of Tus (Tus:DNA = 20:1) at either low (lanes 1, 3, and 5) or high (lanes 2, 4, and 6) concentrations of either the wild-type or mutant primases were analyzed by electrophoresis through 0.7% alkaline agarose gels as described under “Materials and Methods.” Total DNA primases were analyzed by electrophoresis through 0.7% alkaline agarose gels as described under “Materials and Methods.” Total DNA replication reactions incubated with pol III HE (0.1 pmol) reconstituted in either the presence or absence of Tus (Tus:DNA = 20:1) at either low (lanes 1, 3, and 5) or high (lanes 2, 4, and 6) concentrations of either the wild-type or mutant primases were analyzed by electrophoresis through 0.7% alkaline agarose gels as described under “Materials and Methods.” Abbreviations were as in the legend to Fig. 1.

**Fig. 3.** Replication forks assembled in the absence of the τ subunit of the holoenzyme can support oriC DNA replication in vitro. Aliquots of oriC DNA replication reactions incubated with pol III HE (0.1 pmol) reconstituted in either the presence or absence of the τ subunit were analyzed by either neutral agarose gel electrophoresis (panel A) or alkaline agarose gel electrophoresis (panel B). All reactions contained high concentrations of the wild-type primase. Total DNA synthesis in the 15-min reactions (as nucleotide) was as follows: lane 1 (both panels), 261 pmol; lane 2 (both panels), 322 pmol. LRI, late replicative intermediate; II:II di, form II-form II DNA dimers. Size markers were as in the legend to Fig. 1.

oriC DNA Replication in Vitro—The DNA products from oriC replication reactions incubated in the presence of high concentrations of wild-type primase and HE reconstituted either in the presence (RHE (+τ)) or absence of τ (RHE (−τ)) were analyzed by agaroose gel electrophoresis (Fig. 3). Under these conditions, both RHE (+τ) and RHE (−τ) were capable of supporting oriC DNA replication in vitro, although the efficiency of the RHE (−τ)-supported oriC DNA replication was slightly (10–20%) lower than that of the RHE (+τ)-supported reaction. Analysis by native agarose gel electrophoresis showed that the major products were highly linked DNA dimers and late Cairn’s-type replication intermediates (Fig. 3A). These were the DNA products expected because these reactions contained DNA gyrase, which can not decatenate the linked daughter molecules (26), as the only topoisomerase present. Two distinct populations of DNA products were observed when replication products were analyzed through denaturing alkaline agarose gels (Fig. 3B). The larger population, which centered around half-unit length, represented nascent leading strands, and the shorter population represented nascent lagging strands. The lagging strands generated in the RHE (−τ)-supported reaction were slightly shorter than those generated in the RHE (+τ)-supported reaction. This may be because of the uncoupling of the pol III cores at the fork and/or the absence of the HE-DnaB interaction.

The τ subunit of the pol III HE interacts directly with DnaB and this interaction is required for rapid replication fork progression (8). Thus, the oriC pulse-chase protocol was employed to compare the rate of progression of replication forks formed in either the presence or absence ofτ (Fig. 4). Early replication intermediates were formed and 32P-labeled by incubating the replication system in the presence of [α-32P]dATP but in the absence of any topoisomerase. Under these conditions, replication forks form at oriC on the supercoiled template and elongation can proceed in the absence of a topoisomerase until positive supercoils accumulate. At this point (the early intermediate, where the nascent leading strands are about 600–800...
phenomenon could therefore be explained by two models: 1) the primase simply becomes random. We determined that a high concentration of primase was required to ensure that replication was, in fact, bidirectional, initiating at or very near oriC.

Even in the Absence of \( \tau \), the Helicase-Primase Interaction Governs the Mode of Replication from oriC—To determine whether the \( \tau \)-DnaB interaction had any effect on the mode of replication from oriC, we asked whether the primase concentration (Fig. 5). In each case, the rate of replication fork progression was at least 180 nt/s. Because digestion of the DNA template by \( SmaI \) with \( RHE \) (0.1 pmol) was observed in the absence of Tus, we examined replication on the blocking origin in an ATP-dependent manner. As we have shown previously, these events require extensive oligomerization of DnaA at the origin. The manner in which activities are assigned to individual promotors (or groups of them) in this DnaA aggregate is not yet appreciated. The single-stranded DNA in the resultant denaturation bubble is presumably rapidly coated with SSB. The key to subsequent replication fork assembly is the introduction of the replication fork helicase, DnaB, to the DNA. Avoidance of the promiscuous introduction of this protein to any single-stranded DNA in the cell comes about because DnaB cannot bind to SSB-coated DNA. This creates a demand for another specific interaction, this time between DnaA and DnaB (7), that allows DnaB to bind the denaturation bubble.

This point in the temporal sequence of events during initiation is crucial for the determination of the mode of replication. DnaB is a motor protein that can rapidly move away from the origin in an ATP-dependent manner. As we have shown previously for replication of small plasmid templates (14), if it does so before replication forks are established, synthesis of each strand becomes continuous and the location of initiation becomes random. We determined that a high concentration of primase was required to ensure that replication was, in fact, bidirectional, initiating at or very near oriC.

Replication fork formation is driven by the obligatory develop-
opment of an interaction between the $\tau$ subunit of the pol III HE and DnaB that acts to couple DNA synthesis to unwinding of the parental template (8). We sought to determine in this report whether the required high concentration of primase reflected a requirement for primer placement to facilitate establishment of the $\tau$-DnaB interaction or a requirement to mark the origin region and the site of initiation of leading strand synthesis immediately upon localized denaturation, an event that would only be dependent on the DnaG-DnaB interaction. To address this question, we examined the mode of DNA replication in the presence of pol III HEs reconstituted both with and without the $\tau$ subunit and mutant primases that had altered interactions with DnaB.

pol III HE reconstituted without the $\tau$ subunit could support oriC DNA replication in vitro. On its face, the observation that uncoupled replication forks could support DNA replication seems surprising. However, there is precedent in the monoo- and dipolymerase systems for SV40 DNA replication in vitro (28). HE reconstituted in the absence of $\tau$ presumably synthesizes leading strands in a distributive manner, just as DNA polymerase $\alpha$ does in the monopolymerase system. However, it is probable that this observation is a manifestation of reconstitution of bidirectional DNA replication that was dependent on the DnaG-DnaB interaction. This conclusion is also supported by the observation that mutant primases, having as their only added interactions with DnaB.

An identical switch from asymmetric, randomly initiated replication to bidirectional replication in inverse relationship to the primase concentration was observed when oriC DNA replication was performed using HE reconstituted in either the presence or absence of $\tau$, demonstrating that the lack of a DnaB-pol III HE interaction had no effect on oriC-specific initiation of bidirectional DNA replication that was dependent on the DnaB-DnaG interaction. This conclusion is also supported by the observation that mutant primases, having as their only defect an altered interaction with DnaB, behaved identically to the wild type with the only difference being a shift to the right in the concentration dependence of the switch in replication mode. This also argues against the possibility that it is an interaction between DnaA and DnaG that locates the primase to the origin region. If this were the case, the shift in concentration of primase required to alter the mode of replication when the mutant enzymes were used would not have been observed.

The dominance of the DnaB-DnaG interaction in directing the mode of replication from oriC suggests that it is the location of the first primers synthesized that is the governing feature. These are the primers for leading strand synthesis. How this results in essentially assuring that bidirectional initiation takes place is not clear. It could be that, even if only one replication fork initiates, the second leading strand primer serves as a roadblock to conversion to rolling-circle DNA synthesis. Another intriguing, although highly speculative, possibility is that this reflects a requirement for the formation of a dimeric replisome at the origin, resulting in the coupling of the two replication forks, as has been proposed for initiation at the SV40 origin (29).