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MINIREVIEW

D-Loops and R-Loops: Alternative Mechanisms for the Initiation of Chromosome Replication in *Escherichia coli*

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The process of chromosome replication entails copying of both strands of a duplex. Thus, duplex melting is the first step in the initiation of replication. For linear chromosomes, ends can be a site for opening. For example, adenovirus and *Bacillus subtilis* phage φ29 initiate replication from the ends of the chromosomes with a terminal protein (for a review, see reference 26). For circular duplex molecules, opening can be effected by nicking a strand and peeling it off of the other strand, leading to rolling circle replication (e.g., *B. subtilis* plasmid pT181). For the majority of circular and linear duplex chromosomes, however, initial opening is achieved by formation of a bubble within the duplex. DNA helicase introduced into the initial bubble then enlarges it for assembly of replication proteins. In *Escherichia coli*, a specific interaction between DnaA protein (initiator protein) and *oriC* (the origin of replication) leads to an initial opening followed by enlargement by the action of DnaB helicase. Recent studies of two DNA replication systems which are normally repressed strongly suggest that under certain circumstances, *E. coli* manages to achieve duplex opening by formation of a D-loop or an R-loop (Fig. 1).

Initiation of chromosome replication at *oriC* requires concomitant protein synthesis (41). Under certain specific conditions, however, *E. coli* is known to undergo two modes of DNA replication which do not require protein synthesis. One mode, inducible stable DNA replication (iSDR), occurs in cells induced for the SOS response (23, 24), and the other mode, constitutive stable DNA replication (cSDR), is activated in *rnhA* mutants which lack RNase HI activity (18). The initiation mechanisms for iSDR and cSDR drastically differ not only from the *oriC* system but also from each other. Whereas iSDR depends on homologous recombination, cSDR is strictly dependent on transcription. The salient features of the three initiation systems are summarized in Table 1.

THREE MECHANISMS WHICH OPEN DNA DUPLEXES

Duplex opening by origin recognition proteins. Replication origins usually consist of a core component, which is required for replication initiation under all conditions, and one or more auxiliary components, which facilitate the *ori* core activity (7, 10). The *ori* core contains at least an origin recognition element (ORE) and a DNA-unwinding element (DUE). Auxiliary components are usually transcriptional elements.

The ORE of the 245-bp minimal *oriC* of the *E. coli* chromosome contains four or five DnaA protein-binding sites (DnaA boxes) (41). The *oriC* ORE winds around an aggregate of ca. 20 DnaA molecules in the presence of histone-like protein HU, forming an initial complex (26). The initial complex, with concomitant ATP hydrolysis, is then converted to an open complex where DNA melting is induced in the thermodynamically unstable, three 13-mer sequences (DUE) (27) (Fig. 1). DnaA protein then guides DnaB helicase to the melted region, forming a prepriming complex. The origin of bidirectional replication (OBR), however, has been mapped in the ORE rather than in the melted region. It is suggested that DnaB helicase might be delivered from the initial binding site to minibulges that are induced near the OBR by DnaA protein binding (56). Transcription in the vicinity of *oriC* facilitates DNA melting (51).

Duplex opening by formation of D-loops. In SOS-induced *E. coli* cells, the entire genome can be replicated semiconservatively for many hours in a manner independent of DnaA protein, transcription, and translation (23, 32, 33) (this is iSDR) (Table 1). The origin activity (*oriM1A* and *oriM1B*) for iSDR has been mapped in two adjacent regions within the minimal *oriC* (2). Interestingly, these two regions correspond well with those that have been shown to bind with high affinity to the outer membrane (28). The significance, if any, of this coincidence remains to be investigated. Another major origin (*oriM2*) activity for iSDR is found in the *terC* region (32). The precise position of *oriM2* has not been determined.

Duplex opening for initiation of iSDR is thought to be achieved through formation of an intermediate of homologous recombination, the D-loop (Fig. 1). (i) Initiation of iSDR requires the recombinase activity of RecA (5). (ii) Mutations in the *recB* and *recC* genes, which block the RecBCD pathway of homologous recombination, also block iSDR induction (33). *recD* mutants are proficient in iSDR induction as well as homologous recombination (5). Thus, iSDR requires the DNA helicase activity but not the deoxyribonuclease activities of the RecBC(D) enzyme. (iii) iSDR can be induced in *recBC sbcA* mutants in which the RecE pathway of homologous recombination is active (5). The inducibility is abolished by a mutation in the *recE* gene, which encodes exonuclease VIII (ExoVIII; 5′→3′ exonuclease, with a high degree of preference for double-stranded DNA). (iv) *recBC sbcBC* mutants are also inducible for iSDR, suggesting that the RecF pathway can be used for iSDR initiation (3). (v) Mutations in the *ruvA*, *ruvB*, *ruvC*, and *recG* genes, which are involved in late stages of homologous recombination, stimulate iSDR (5). Thus, homol-

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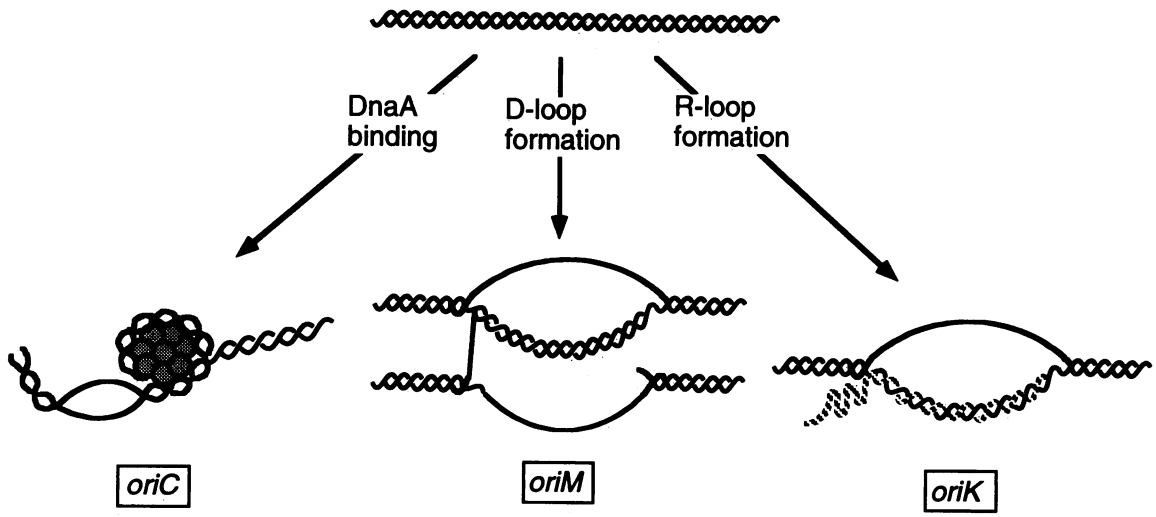

FIG. 1. Three mechanisms which open DNA duplexes. Initial melting of a DNA duplex for initiation of DNA replication by DnaA protein binding, D-loop formation, and R-loop formation is schematically shown. The term D-loop (for displacement loop) was originally coined to represent a replication intermediate of mouse mitochondrial DNA that contained a 7S ssDNA fragment hybridizing to one strand and thus displacing the complementary strand (14). In this article, we use D-loop and R-loop to indicate the loops containing a DNA and an RNA strand, respectively, although both are displacement loops. Solid and striped lines represent DNA and RNA strands, respectively. Shaded circles represent DnaA protein.

ogous recombination and iSDR appear to share D-loops as a common intermediate. On the basis of these observations, it has been hypothesized that, upon induction of the SOS response, a double-strand break (DSB) is introduced into a covalently closed circular DNA molecule within or near *oriM*. RecBC(D) helicase or ExoVIII then generates single-stranded DNA (ssDNA) with a 3′ end from the break. This ssDNA is assimilated into an uncut homolog by the action of RecA recombinase (5). The reaction, yielding a D-loop, accomplishes initial duplex opening for the subsequent steps of replication initiation (see below).

The D-loop model suggests that formation of DSBs is sufficient to induce homologous recombination-dependent DNA replication. This was tested by introducing an artificial DSB into a lambda *cos* site placed on plasmid DNA. When *recBC sbcA* mutant cells were used for host cells, the copy number of *cos*-carrying plasmids increased, without SOS-inducing treatment, in a manner that is dependent on RecA, ExoVIII, and lambda terminase, which cleaves *cos* (4). This mode of initiation, like iSDR, does not lead to rolling circle replication; the major product is covalently closed circular monomers. The activation of RecA protein has previously been shown to be an essential event for induction of iSDR (33). The dispensability of SOS-inducing treatment for the replication of a plasmid with artificially introduced DSBs raises the possibility that activated RecA is involved in activation of the hypothetical endonuclease prior to the onset of iSDR.

Bacteriophage T4 has been shown, both in vivo (42) and in vitro (11), to also employ D-loop formation for duplex opening (for a review, see reference 27a). When replication forks begun from origins reach the ends of the T4 linear genome, the 3′ ends of parental DNA remain unreplicated. The resulting ssDNA end invades a homologous region of another or the same T4 DNA molecule by the action of UvsX (T4 recombinase) and primes the leading-strand synthesis. RecA cannot substitute for UvsX, suggesting that UvsX functions in this type of replication as a component of a specific "protein machine." Although it is not known whether RecA protein interacts with replication proteins, activated RecA could, directly or indirectly, modify replisomes assembled at D-loops in SOS-induced cells. iSDR is known to be considerably resistant to UV irradiation (24) and appears to be mutagenic (29).

Duplex opening by R-loop formation. The *rnhA* gene encodes RNase HI, which is the major ribonuclease H activity in *E. coli*. *rnhA* mutants are capable of chromosome replication in the complete absence of DnaA protein or the *oriC* site and therefore can dispense with the *dnaA* gene and *oriC* for viability (18) (this is cSDR) (Table 1). Chromosome replication in *rnhA* mutants in the absence of *oriC* is initiated at several sites on the chromosome (collectively termed *oriK*s), which are inactive in *rnhA*$^+$ cells (18). The initiation can occur in the absence of protein synthesis and yet is inhibited by rifampin, a transcription inhibitor (55). Thus, the lack of RNase HI activity activates normally repressed origins of DNA replication, and the initiation involves transcription. These

TABLE 1. Comparison of three *E. coli* initiation systems

Initiation system	Type of cells	Mode of duplex opening	Origin used	Requirement for:				
				DnaA	RecA	RecBC	Translation	Transcription
DnaA/*oriC*	Normal	Initiator-*ori* interaction	*oriC*	+	−	−	+	+
iSDR	SOS induced	D-loop formation	*oriM*s	−	+	+	−	−
cSDR	*rnhA* mutant	R-loop formation	*oriK*s	−	+	−	−	+

characteristics of cSDR have led to the R-loop model, which hypothesizes that the transcript of selected transcription units hybridizes to the template strand and displaces the complementary DNA strand, forming an R-loop (55). Normally, RNase HI recognizes the R-loop and removes it. In the absence of the enzyme, however, the R-loop is stabilized and develops into an initiation bubble (Fig. 1).

An actively transcribing RNA polymerase complex contains only a short stretch of transient DNA-RNA hybrid, of perhaps up to 12 bp (57), but possibly much shorter (48). Nonetheless, several lines of evidence point to the existence of persisting DNA-RNA hybrids. (i) The formation of R-loops of significant lengths has been demonstrated in the region of the ColE1-type plasmid replication origin (for a review, see reference 34). In particular, the initiation of replication in the absence of RNase HI and DNA polymerase I (Pol I) requires a minimum of about 40 bp in the hybrid form (an R-loop) (40). The hybrid can grow to about 3 kb in vitro (46) and up to several hundred bp in vivo (13). (ii) *rnhA* mutants chronically express the SOS response, perhaps due either to an increased amount of ssDNA associated with persisting R-loops or to inhibition of DNA replication caused by R-loops (22). (iii) The chronic expression of SOS is exacerbated during growth in rich medium (22), in which certain types of transcription are stimulated. (iv) The response is also enhanced by mutations in *polA*, *recB*, and *recD*. Furthermore, *recA*$^+$ is essential for viability of *rnhA recD* double mutants. These results suggest that removal of persisting R-loops requires Exo V, DNA Pol I, and recombinational repair functions (22). (v) Nascent RNA transcripts synthesized in vitro by *E. coli* RNA polymerase on highly supercoiled circular DNA can hybridize to the template strand, up to 600 bp in length, if RNA polymerase is removed from the transcription complex (49). Since such a long hybrid could not be detected before the removal of polymerase, it has been suggested that RNA polymerase has a site that dissociates nascent RNA from the DNA as the enzyme moves along the template (49). Consistent with this proposal, *rpoB* mutant alleles encoding altered β subunits of RNA polymerase that either enhance or diminish the chronic SOS expression in *rnhA* mutants have been isolated (19). This suggests that the determinant of this property resides at least in part in the β subunit of the polymerase. The *rpoB rnhA* double mutants expressing increased SOS are highly sensitive to rich medium (19).

THE ROLES OF RecA PROTEIN

Both iSDR and cSDR require the recombinase activity of RecA protein (5, 21). Clearly, a role of RecA recombinase in iSDR is to facilitate assimilation of an ssDNA into the homologous region of a duplex to form a D-loop (see above). In contrast, with the exception of *recA*, mutations in other *rec* genes, including *recB*, *recD*, *recF*, *recJ*, *ruvA*, and *ruvC*, neither inhibit nor stimulate cSDR (21). Thus, it is extremely unlikely that cSDR involves homologous recombination. This conclusion is supported by the isolation of a mutation (*rin*) that suppresses the defect of *recA* mutations in cSDR without restoring homologous recombination proficiency (54). Since RecA protein functions at the step of initiation of cSDR (18), it is likely that a nascent transcript which is displaced from the template invades the duplex to form a DNA-RNA hybrid at *oriK* and that the RecA recombinase catalyzes this strand assimilation process. RecA protein has recently been shown to effectively promote DNA-RNA hybrid formation in vitro (15, 16). Presumably, the *rin* mutation activates a recombinase-like activity which promotes assimilation of the RNA strand but not of the DNA strand.

The requirement for RecA can also be suppressed by introduction of the *lexA*(Def) mutation. Thus, derepression of one or more LexA regulon genes activates a bypass pathway which functions in the absence of RecA (9). Interestingly, the bypass requires the nick translation activity of DNA Pol I (8). It has been postulated that instead of a large R-loop which could be generated in the presence of RecA, the bypass would generate segmented hybrids (mini-R-loops) which are interrupted by regions of DNA duplex. The nick translation activity of DNA Pol I would converge the mini-R-loops into a large loop (now a D-loop) by simultaneous digestion of RNA and synthesis of DNA (8).

HELICASE LOADING AND SUBSEQUENT PRIMING

The crucial step following duplex melting is the loading of the replication fork helicase, i.e., a DnaB hexamer, onto ssDNA in the melted region (26). DnaB, while unwinding the duplex in the 5′→3′ direction with respect to the bound ssDNA, interacts with the DnaG primase, which lays down RNA primers for the DNA Pol III holoenzyme. Analyses of the initiation mechanisms of *E. coli* plasmid and phage DNA replication have revealed two systems which deliver DnaB onto ssDNA. Recent studies of iSDR and cSDR suggest the presence of other systems.

PriA-*pas* system (φX-type primosome). In vitro SS-to-RF conversion, i.e., conversion of the ssDNA of phage φX174 to the duplex replicative form (RF), depends on the PriA-*pas* system (for reviews, see references 26 and 34; 39). PriA specifically recognizes and binds to the ca. 70-nt, hairpin structure called *pas* (primosome assembly site) or n′-*pas* on SSB (ssDNA-binding protein)-coated φX174 ssDNA. The binding activates the ATPase activity of PriA, but ATP hydrolysis is not required for the following steps. PriB, through its ssDNA-binding activity, binds to the PriA-DNA complex (1). A single DnaB helicase is then delivered from a DnaB-DnaC complex to the PriA-PriB-DNA complex by the action of DnaT. PriC is also necessary for this pathway, probably to promote the *pas*-directed priming over nonspecific priming (1). DnaG then interacts with the DnaB and synthesizes the first RNA primer.

This system is also implicated in the lagging-strand synthesis of pBR322, both in vivo and in vitro (reviewed in reference 34). RNase HI cleaves the RNA II transcript at the origin, and DNA Pol I begins the leading-strand synthesis from the end of the cleaved RNA II. The synthesis displaces the lagging-strand template (D-loop formation) and exposes a *pas* located on the template about 150 nt downstream of the origin. This allows the assembly of the φX-type primosome at the *pas*. In the absence of RNase HI, formation of an R-loop, instead of a D-loop, can expose the *pas* (34).

This φX-type primosome is not absolutely essential for *E. coli* chromosome replication because *priA*::*kan*(Null) mutants are viable. However, they show reduced viability (31) and chronic SOS induction (44). It has been suggested that, while replication fork assembly at *oriC* is independent of the φX-type primosome, completion of chromosome replication may become dependent on it in the event that the replication complex stalls or dissociates (44).

DnaA protein-DnaA box system (*oriC*-type or ABC primosome). ssDNA from the *ori*γ region of plasmid R6K was found to contain a signal for assembly of an efficient priming system for SS-to-RF conversion of phage ssDNA (38). This system is very different from the φX-type primosome and involves DnaA protein-DnaA box interaction. DnaA binds to a DnaA box present in the stem region of a hairpin structure on an

SSB-coated ssDNA (A site) and delivers DnaB from a DnaB-DnaC complex to the ssDNA. Similarly, at *oriC*, DnaA protein bound to DnaA boxes has been shown, in vitro, to deliver DnaB helicase from a DnaB-DnaC complex onto each strand in the unwound 13-mer region (41). It is not clear whether the loading of DnaB at *oriC* is also mediated by an A site-like structure, because ssDNA containing the entire minimal *oriC* sequence does not support priming for SS-to-RF conversion (43).

In vitro synthesis of the lagging strand of pBR322 can also use a DnaA-DnaA box system, albeit inefficiently (47, 50). DnaA protein binds to the DnaA box located about 100 bp downstream from *ori* and perhaps mediates delivery of DnaB to the ssDNA region. Evidence indicates, however, that this system is distinct from the ABC priming system described above (see reference 38 for further discussion).

PriA-mediated priming at D-loops and R-loops. DnaB, DnaC, and DnaG are necessary for both iSDR (23, 36) and cSDR initiation (18, 20), indicating that a priming mechanism(s) similar to the φX-type primosome or ABC primosome is employed at D-loops and R-loops. The fact that iSDR can be induced in *dnaA*::Tn*10* mutants (32) rules out the involvement of the ABC primosome in iSDR initiation. *priA*::*kan* mutations, on the other hand, completely block iSDR induction (37), suggesting that the φX-type primosome is specifically involved in iSDR initiation. The requirement of DnaT for iSDR initiation supports this idea (36). However, DNA fragments derived from the *oriM1* (*oriC*) region stimulate neither φX174 ssDNA to RF replication (52) nor the ATPase activity of PriA (35). This observation indicates the absence of *pas* sites in the vicinity (within about 2.5 kb) of *oriM1*. Therefore, loading of DnaB at *oriM1* through the φX-type primosome does not seem to require the canonical *pas*. It is probable that PriA can recognize more than one specific sequence or structure to start the assembly of the φX-type primosome.

priA::*kan* mutants are sensitive to rich medium (6) and fail to support replication of pBR322 (6, 31). We recently isolated a revertant, *spa-47*, from *priA*::*kan* mutants that shows resistance to rich medium (6). The revertant shows improved viability and can sustain pBR322 replication. Possibly, requirement of the PriA-*pas* system for chromosome and pBR322 replication is alleviated by an alternative, PriA-independent priming system which is activated by the suppressor mutation. Interestingly, the defect in iSDR induction in *priA*::*kan* mutants is not suppressed by the mutation. The requirement of PriA for iSDR initiation appears to be very strict.

It is envisioned that formation of recombination intermediates (D-loops) displaces one strand of the *oriM* duplex and activates an unknown signal for PriA binding (37). This leads to the loading of a single DnaB helicase on the strand, probably through the assembly of φX-type primosome. Unidirectional DNA synthesis promoted by the helicase would expose a PriA-binding site (*pas* or an unknown signal), leading to bidirectional replication.

The *priA*::*kan* mutation also blocks cSDR initiation completely (37), whereas cSDR can occur in the complete absence of DnaA protein (18). It is likely that the DnaB helicase loading and subsequent priming proceeds at R-loops in a manner similar to that for iSDR. In contrast to iSDR, however, the *spa-47* mutation can partially suppress the defect of *priA*::*kan* mutants in cSDR initiation (6), suggesting that the PriA-independent priming system activated by the *spa-47* mutation can partially substitute for the priming system at R-loops. Thus, the process may not be identical at D-loops and R-loops.

CONCLUSION

The DSB effectively triggers homologous recombination in *E. coli* (17, 53a). The observation that the DSB also initiates DNA replication (i.e., iSDR) poses an intriguing possibility that the homologous recombination process, as well as DSB repair, may involve extensive DNA replication. Consistent with this notion, *priA*::*kan* mutants have been found to be defective in P1-mediated transduction and Hfr-mediated conjugation (25). The ends of linear DNA pieces introduced by P1 phage or by conjugation would trigger DNA replication, yielding recombinants. In *Saccharomyces cerevisiae*, meiotic recombination is initiated by transient DSBs. The ends are processed to yield extensive 3′-overhanging ssDNA, which is most likely to be assimilated to form D-loops (53). It would be extremely interesting to see whether iSDR-like DNA replication can occur in *S. cerevisiae*.

iSDR is a stress-inducible function which could play an important role in cell survival and the creation of genetic diversity within DNA-damaged cell populations (24, 29). Recently, a cSDR-like activity has been detected in *E. coli mhA*$^+$ cells growing in rich medium under certain conditions (12). Thus, the replication may not be a genetic artifact that is seen only in mutants devoid of RNase HI. cSDR could also be, like iSDR, a stress-related or growth-regulated DNA replication activity. To our best knowledge, no DNA replication activity similar to cSDR has been found in eukaryotes. However, transcription activity is known to be closely associated with initiation of DNA replication in eukaryotic systems (10). In particular, the regulatory elements of developmentally regulated amplification of the chorion gene in *Drosophila melanogaster* and the rDNA gene in protozoa, for example, contain promoter- and/or enhancer-like activities (30, 45). Since developmentally regulated gene amplification is designed to increase the copy number of the template for transcription, it would not be very surprising if replication for amplification were triggered by R-loop formation.

DNA replication, recombination, and transcription are each a complex process. Traditionally, these processes have been studied, for the most part, separately. It is increasingly clear, as demonstrated by the studies of iSDR and cSDR summarized here, that these processes are intimately related to and dependent on each other. The two replication systems will continue to provide unique opportunities to explore the interdependency of these vital cellular functions.

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